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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.

Conceptual definition

In their study arriving at the negative association results for *M2/ANXA5* in selected women who had experienced RSA and fertile controls of Estonian and Danish extraction, Nagirnaja et al. (2015) reach an ambitious conclusion about risk absence in Northern Europe. In an earlier association study by Fishel et al. (2014; citing Nelis et al., 2009), four European areas with differing population structures that could affect the significance of disease-gene associations were outlined: Central and Western Europe; the Baltic countries, Poland and Western Russia; Finland; and Italy. For three of these regions, the incidence in controls from Germany, Southern Italy and Bulgaria have all shown consistency in the M2 haplotype frequency, confirmed by the recent study by Demetriou et al. (2015) on the UK White European population. Subsequent to Nelis et al. (2009), a further study of Northern European populations has revealed genetic diversity in the Northern European population, Estonians having a relatively high proportion of Finnish ancestry (Khrunin et al., 2013). Also, there seems to be significant genetic diversity in North Eastern Europe. As previous and current M2 association studies did not include individuals of Finnish ancestry, the question of specific population structuring that can be raised from work of Nagirnaja et al. (2015) remains open. Most recently a study on Danish population structure reported strong genetic influence from neighbouring Nordic (Sweden and Norway) and Germanic (Germany and Holland) countries and negligible influence from Finland, France and Portugal (Athanasiadis et al., 2015). So the grouping of Estonian and Danish participants together in a disease association study as representatives of "Northern Europe" seems questionable, as the Finns are well known to be genetically different. Therefore, the indicated Estonian incidence of the M2 haplotype in fertile controls and women who have experienced RSA may well differ from previously reported European populations.

Nagirnaja et al. (2015) claim, that there is a "high worldwide prevalence" of the M2 haplotype in population-based samples and "decreased or similar occurrence" among women who have experienced RSA, is just not supported by the actual studies, the only exceptions being their own study and a Chinese study for East China, where genotyping information seems to be missing or incomplete. Along these lines, *M2/ANXA5* seems to be possibly protective and at best neutral to RSA in Estonian and Danish participants; however, considering the reduced prevalence in patient cohorts compared with the fertile controls, and contrary to all previous and current association studies except one, it would be tempting to conclude there might be an evolutionary advantage for M2 carriers. This is suggested for the offspring of M2 carrying couples in the recent work by Demetriou et al. (2015). Although this may be a valid conclusion, another plausible explanation could be provided by a recent model developed for complex diseases that shows that causative inherited genetic lesions may well be more frequent in the unaffected (control) than in the affected (patient) portion of a population, thereby appearing "protective", rather than "predisposing" in an epidemiological sense (Siegert et al., 2015). This finding might seem to be counterintuitive, but the phenomenon results from confounding of the disease association of one genetic lesion by the absence of other lesions necessary for the former to exert its effect in a given individual. In other words, for a given mutation to become phenotypically expressed as a disease phenotype, a second genetic alteration would be necessary that is normally present in a model population as "background". In a different populace however, because of population structuring (confounding and admixing effects), this particular "background" mutation could be missing from the original group of source dwellers but then come in generations later through the influence of another ethnic group. The model is empirically supported by observations of negative correlations between odds ratios and disease prevalence in published genome-wide association studies. Such negative correlations of causative genetic defects would potentially arise as a result of the heritage tree structure of the human gene genealogy. Therefore, population structuring could be a relevant issue for the study by Nagirnaja et al. (2015), in which a negative correlation may be demonstrated by the above mechanism of confounding.

A negative M2 haplotype association with early RSA, seems to be caused by the high prevalence of chromosomal abnormalities as discussed above. As the proportion of such chromosomal lesions inherited from the parents is tiny (<1%), a possible prospective study eliminating this issue would use rapid genotyping of post-conceptuses using microarrays or quantitative fluorescence polymerase chain reaction for negative selection of aneuploidies and submicroscopic aberrations among patient cohorts. Therefore, it is worth mentioning that almost all recent association studies on the M2 haplotype (Rogenhofer et al., 2012; Tiscia et al., 2012; Tüttelmann et al., 2013; Demetriou et al., 2015; Thean Hock et al., 2015) have excluded both fetal and parental chromosomal aberrations (for most studies numerical) and come to the same M2 incidence in their early RSA samples.

Genetic association studies, with properly selected patient and control groups, are a valid approach to deliver evidence on a risk factor; however, the ultimate proof of such risk, possibly conferred by M2 carriage, would be data derived from a randomized observational trial comparing live birth and spontaneous abortion rates in untreated M2 carriers with untreated non-carriers of the haplotype. Until such data are available, no definite proof can be generated on the proposed risk role of *M2/ANXA5* for RSA and thrombophilia-related obstetric complications.

Minor issues

Use of restriction fragment length polymorphism analysis for genotyping

It is somewhat puzzling that Nagirnaja et al. (2015) opted to use restriction fragment length polymorphism (RFLP), described as the genotyping option in Figure 1c by Bogdanova et al. (2007), on the Danish sample set of their participants involving 227 patients and 115 controls. A comparison of valid genotyping methods is definitely not in favour of RFLP as it is prone to errors, and therefore of limited reliability (Bianchi et al., 2010). Although this seems a minor issue assuming that the genotyping deviation through RFLP would not exceed 5%, valid comparisons to previously published work seem problematic because this analysis accounts for the M2 haplotype only, thus rendering concomitant promoter genotypes

reconstruction and genetic equilibrium calculations impossible, which in turn could verify the accuracy of genotyping.

Re-sequencing of the *ANXA5* proximal promoter region in Estonian patients who have experienced RSA and fertile controls

As stated in their results section, the Nagirnaja et al. (2015) confirm the four SNPs building the M2 haplotype in a single linkage disequilibrium block. They also added a fifth upstream SNP to their analysis: 180 C/T (rs62319820), which according to a statement in the second paragraph of their results section and to the legend of, gives identical linkage disequilibrium results when phasing together with the M2 haplotype. This statement is, however, not supported by Fig. 1c (Nagirnaja et al., 2015), showing clearly different r^2 scores for rs62319820, which seems to associate at best and only partially with the M1 haplotype.

Comparative genotyping datasets of worldwide cohorts

Nagirnaja et al. (2015) presented a summary on comparative genotyping data of their Estonian and Danish cohorts in. Although the genotype fractions from their own cohorts do not add up to 100%, whicn can be interpreted as rounding error, this error increases from 0.4% to 9.4% in the worldwide datasets, raising the possibility of missing data. In addition, the cited study by Hayashi et al. (2013) that used merged controls of different regions in Japan is incorrectly referred to as representative for the population of Central Japan. As previously discussed, this study delivered inflated M2 prevalence values, compared with the study by Miyamura et al. (2011) for Central Japan. Altogether, it seems that Nagirnaja et al. (2015) used accurate software for polymorphism identification, which was followed by manual checking; however, the results of their procedure summarized in Table 4 (Nagirnaja et al., 2015) leave reasons for doubt.

Conclusion

It is not a mutually exclusive option that a North–South European population distribution gradient may exist for the M2 haplotype, similar to other common genetic lesions (Nelis et al., 2009). Therefore, it might be difficult to replicate previous association findings in a specific population, because of genealogy-actuated negative correlation between causative mutations in complex disease (Siegert et al., 2015). In conclusion, it is our opinion that a risk role attributable to the M2 haplotype of the *ANXA5* gene for idiopathic RSA cannot be excluded or disproved from the data presented by Nagirnaja et al. (2015).

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