Investigation of the Annexin A5 M2 haplotype in 500 white European recurrent miscarriage couples

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ABSTRACT

Annexin A5 is a placental anti-coagulant protein that contains four nucleotide substitutions (M2 haplotype) in its promoter. Studies have shown that this haplotype is a risk factor for recurrent miscarriage (RM). Our aim was to investigate the influence of the M2 haplotype in the gestational timing of miscarriages, assessing the paternal risk and investigate for relationships with known risk factors. Five hundred white European couples with three or more consecutive miscarriages and two fertile control groups were selected for this study. The allele frequency of M2 was found to be significantly higher among early RM patients than among controls (P=0.002) and there was no difference between controls and patients with late miscarriages. Moreover, there was no difference between RM patients who had a live birth or no live births or between patients who were positive or negative for known risk factors. Male and female partners in each group had similar allele frequencies of M2. In conclusion, the M2 haplotype is a risk factor for early miscarriages, before the 12th week of gestation and confers about the same relative risk to carriers of both sexes. Having one or more M2 allele(s) in combination with other risk factors further increases the RM risk.

Key Words: annexin A5, ANXA5, M2 haplotype, recurrent miscarriage, risk factor.
INTRODUCTION

Recurrent miscarriage (RM) is defined as more than three consecutive pregnancy losses and it affects about 1% of couples trying to conceive (Stirrat, 1990). RM is not only associated with higher rates of morbidity but is also associated with complications later in pregnancy including fetal growth restriction, prematurity and pre-eclampsia (Rai and Regan, 2006). A number of these miscarriages occur due to chromosomal abnormalities in the fetus and the chance of this taking place increases as the maternal age rises (Nybo Andersen et al. 2000).

Risk factors for RM include antiphospholipid syndrome (APLS), which is an autoimmune disease caused by the presence of circulating maternal antiphospholipid antibodies (aPL) such as anticardiolipin antibodies and lupus anticoagulant (LA). These antibodies cause thrombosis of the placental vessels which in turn leads to fetal loss (Salafia et al. 1997; Lim, 2009). Another risk factor includes a point mutation (Arg506→Gln) in the Factor V gene referred to as the Factor V Leiden (FVL) mutation. This mutation causes slower cleavage (10-fold) of Factor V by activated protein C (APC) and therefore an increased level of thrombin and predisposition to clot formation (Kalafatis et al. 1994). Additionally, a mutation in the prothrombin gene (nt 20210 G→A) that is associated with increased prothrombin levels is a risk factor for venous thrombosis (Poort et al. 1996).

Annexin A5 (ANXA5) is a placental anti-coagulant protein that occurs on normal placental villi. Due to its ability to bind to anionic phospholipids that are found on platelets, it impedes aggregation and hence it is thought to function as an inhibitor of coagulation (Thiagarajan and Tait, 1990). ANXA5 is a ubiquitous, but not abundantly expressed protein, manifesting highest levels in liver, kidney and placenta (Morgan et al. 1998). ANXA5 abundance was reduced in the placental trophoblast in the presence of antibodies, characteristic of the APLS (Rand et al. 1994).
The ANXA5 gene is found on human chromosome 4q27 and consists of 13 exons and 12 introns. The gene spans 29 kb and encodes a single transcript of approximately 1.6 kb and a protein product of 320 amino acids with a molecular weight of about 35 kDa. The region genomic locus encompassing the promoter is very GC rich (73% GC) (Cookson et al. 1994).

Bogdanova et al. (2007), reported the presence of two variant ANXA5 promoter haplotypes in addition to the wild-type (WT) in the promoter region of this gene; the M1 and M2 haplotypes, which are common in the normal population. M1 haplotype comprises of two nucleotide substitutions (1A→C and 27T→C) and M2 haplotype comprises of four substitutions (-19G→A, 1A→C, 27T→C and 76G→A) that are in linkage disequilibrium (LD) with each other (Bogdanova et al. 2007).

The frequency of the M2 haplotype was found to be significantly higher in RM patients than among controls (Bogdanova et al. 2007). In fact, subsequent studies have shown that the M2 haplotype is present in 11% of fertile Japanese controls and 21% in RM Japanese patients (Miyamura et al. 2011), and in 15% of European populations and 21-30% in RM patients of European origin (Tüttelmann et al. 2013; Tiscia et al. 2009). No significant association of the M1 haplotype was found with RM. Other studies have also shown an association between the M2 haplotype and pre-eclampsia or gestational hypertension (Tiscia et al. 2009), as well as being a risk factor for fetal growth restriction and small for gestational age newborns (Chinni et al. 2009; Tiscia et al. 2012).

Reporter gene assays demonstrated a 60% reduction in the ANXA5 promoter activity when the M2 haplotype was present in comparison with the WT promoter (Bogdanova et al. 2007). Therefore, in patients carrying the M2 haplotype, the anti-coagulant properties of ANXA5 are reduced and may lead to a hypercoagulable state in the intervilous space, potentially explaining an increased risk of RM. Moreover, a study on ANXA5 expression in
RM placentas demonstrated that mRNA levels are reduced regardless of the parental origin of the M2 haplotype (Markoff et al. 2010).

To further elucidate the role of the M2 ANXA5 haplotype and its association with RM, we genotyped a large cohort consisting of 500 white European couples with RM. Our aim was to investigate the influence of the ANXA5 M2 haplotype on the timing of miscarriages, to assess the male risk and to investigate any interaction with known risk factors, such as APLS FVL and prothrombin mutation.
MATERIALS AND METHODS

Study populations

Patient blood samples used in this study were collected from patients and their male partners who attend the Recurrent Miscarriage Clinic at St Mary's Hospital, Imperial College London, and who agreed to participate in research with signed, informed consent. A total of 996 white European samples (501 female patients and 495 male partners) were used in this study. Patients with uterine anomalies, polycystic ovaries (Rotterdam, 2004) and fetal and parental chromosomal abnormalities were excluded. APLS was confirmed by the presence of lupus anticoagulant (LA) antibodies and/or anticardiolipin (aCL) and/or β2-Glycoprotein IgG and IgM autoantibodies (Miyakis et al. 2006) following routine testing by the Clinical Biochemistry service. Data for these tests was therefore extracted from each patient’s hospital records. An additional test for the prothrombin variant (G20210A) was conducted as described below.

Patients were broadly divided into different classes according to the gestational period in which the miscarriage occurred. A total of 310 women and 309 male partners with 3 or more early miscarriages (before 12th week of gestation) and no late miscarriages were classified as “early miscarriage patients”. The women in this group had an average age at referral of 34.6 ± 4.9 years (mean ± standard deviation). A total of 191 women and 186 men who had at least 1 late miscarriage (after 12th week of gestation) were classified as “late miscarriage patients”. The women in this group had an average age at referral of 34.0 ± 5.3 years. Still births were not included in this group of patients. The study was approved by the Imperial College Hospital Ethics Committee (REC ref: 12/WA/0196).

A total of 241 White European control trio (mother, father and placenta) samples (Moore controls) with at least one successful pregnancy and no previous history of
miscarriage were used. This control cohort consisted of White European trios recruited at Queen Charlotte and Chelsea Hospital, London (Apostolidou et al. 2007). The average age of the control women was 33.6 ± 4.3 years.

The PopGen control group of 533 randomly selected German individuals (UKSH, Kiel) was also used as a control population (Bogdanova et al. 2007). These samples were previously genotyped by Professor Arseni Markoff’s research group in Germany and were used as an additional Caucasian control group for our study.

**DNA extraction**

DNA was extracted from blood and placenta tissue using the iPrep™ PureLink™ gDNA Blood Kit and the iPrep™ ChargeSwitch® gDNA Tissue Kit, respectively, using the iPrep Purification Instrument based on the manufacturer’s instructions (Invitrogen, UK).

**Polymerase Chain Reaction (PCR), sequencing and genotyping**

Genomic DNA was amplified by PCR before sequencing. Primers (5’ to 3’ sequence) used for sequencing the promoter region of *ANXA5* are FP cegacccggtgagctcc and RP gcaccgaccagctctc. 20 μl reactions were prepared using the HotStarTaq DNA polymerase kit (Qiagen) according to the manufacturer’s instructions. Primers (5’ to 3’ sequence) used for sequencing the 3’UTR of the prothrombin gene for the G20210A mutation (PTm), are FP acaaccgctgatcaaatgg and RP gagctgcccatgaatagactg. 20 μl reactions were prepared using the Taq DNA polymerase kit (Bioline) according to the manufacturer’s instructions.

Sequencing reactions were prepared according to the manufacturer’s instructions (Applied Biosystems) using the ABI Prism Big Dye terminator cycle sequencing ready
reaction kit (BDT v1.1). Sequencing products were run on an ABI Prism 3730 DNA analyzer. Sequences were examined for SNP genotypes using Sequencher™ v4.6 (Gene Codes Corporation) bioinformatics software and called following visual inspection.

**Statistical analysis**

Odds ratio (OR) with 95% confidence intervals (CI) and chi-squared tests were calculated using online software packages: (http://www.hutchon.net/ConfidOR.htm) and (http://www.socscistatistics.com/tests/chisquare/Default.aspx). Departure from Hardy-Weinberg equilibrium (HWE) was assessed using a Monte-Carlo Markov Chain (MCMC) implementation of an exact test, part of the Genepop package (http://genepop.curtin.edu.au/). Statistical significance was defined as P<0.05.
RESULTS

A possible association between RM and the M2 haplotype was investigated by genotyping RM patient and control cohorts. The M2 allele frequency (AF) of female and male partners of the RM group was 0.119 and 0.128, respectively and that of the Moore control group was 0.098 and 0.089 for females and males, respectively. Chi-squared tests were used to investigate whether the prevalence of the M2 haplotype was similar between women and men either in the control group or in the patients. No significant differences were observed between women and men in the control group or between female and male patients. This was also true when patients were divided into early RM patients or late miscarriage patients (Table 1).

In order to assess the male risk in our study, male patients were compared with male Moore cohort controls. Male carriers of M2 face a 1.4 times higher risk of RM than non-carriers (odds ratio 1.51, 95% CI 1.02 - 2.24, P=0.04), similar to the previously reported female risk (Tüttelmann et al. 2013).

The RM groups were in Hardy-Weinberg equilibrium (HWE) for the ANXA5 haplotypes (Table 2). Although male controls in the Moore cohort were in HWE, female controls were found to deviate (MCMC P=0.03). Taking into consideration the fact that the prevalence of the M2 haplotype was similar between women and men within both the control group and the patient group (Table 1) and that the male risk of M2 carriers was similar to the female risk, it was decided to combine male and female frequencies in each group. In this combined grouping, no deviation from HWE was observed in either the patient or the Moore control cohorts. The association between the M2 haplotype and RM was also tested by comparison to an independent White-European patient cohort comprising of PopGen controls (Table 2).
Overall RM Risk

The AF of the M2 haplotype was found to be higher among all RM patients (0.123) than among Moore controls (0.093) or PopGen controls (0.082). Consequently, carriers of M2 face a 1.3 times higher risk of RM than non-carriers (odds ratio 1.41, 95% CI 1.07 - 1.86) compared with the Moore controls and a 1.5 higher risk (odds ratio 1.63, 95% CI 1.24 - 2.16) in comparison with the PopGen controls (Table 3A). The M1 haplotype was not associated with a higher RM risk.

Risk in patient subgroups in relation to timing of RM

Patients were then divided into two different groups according to the timing of miscarriage; early miscarriage patients (n=619) and late miscarriage patients (n=377). In early miscarriage patients, the AF of M2 was 0.137 that contributes a relative risk of 1.4 (odds ratio 1.61, 95% CI 1.21 - 2.17) as compared with Moore controls and a relative risk of 1.6 (odds ratio 1.87, 95% CI 1.39 - 2.51) when compared with PopGen controls (Table 3B). In late miscarriage patients, the AF of M2 was 0.101 and no significant difference was observed between late miscarriage M2 carriers in either Moore or PopGen controls (Supplementary table 1).

Risk in relation to whether or not patients had a previous live birth

Patients (females plus male partners) were stratified according to those that had no live births (n=495) and those that had at least 1 live birth (n=352). Live birth information was not available for 75 RM couples. Relative risks for M2 carriers that either had or did not have a live birth compared with the Moore controls were 1.5 (odds ratio 1.60, 95% CI 1.15 - 2.24) and 1.4 (odds ratio 1.53, 95% CI 1.12 - 2.09), respectively (Table 3A). The same relative risks were observed for early RM M2 carriers when compared with Moore controls (Table
3B), but no significant difference was observed between late RM M2 carriers and Moore controls (Supplementary table 1). Similar relative risks were observed when patients were compared with the PopGen controls. More importantly, no significant differences were observed between early RM patients who are M2 carriers whether they had a live birth or not.

Risk in relation to whether patients were positive or negative for known risk factors

In order to investigate whether ANXA5 promoter mutations interact with other general thrombophilic factors in terms of RM risk, patients were divided into those who were either positive for known risk factors (APLS, FVL or PTm) (n=276) or those who were negative (n=466). Risk factor information was not available for 127 RM couples. The relative risks compared with the Moore cohort for M2 carriers who were positive for known risk factors was 1.5 (odds ratio 1.61, 95% CI 1.12 - 2.31) and for those who were negative was 1.3 (odds ratio 1.45, 95% CI 1.05 – 1.99), while compared with the PopGen controls was 1.6 (odds ratio 1.87, 95% CI 1.31 - 2.68) and 1.5 (odds ratio 1.68, 95% CI 1.22 - 2.30), respectively (Table 3A).

Slightly higher relative risks were observed for early RM M2 carriers when compared with both control groups (Table 3B), while no significant difference was observed between late RM M2 carriers and controls (Supplementary table 1). The relative risks became even higher when patients that were positive only for APLS were compared with controls, as the AF of M2 increased to 0.142 for all patients and to 0.163 for early RM, with an M2 carriage of 30.4%. However, no significant differences were observed between M2 carriers of RM patients that were positive for known risk factors and those that were negative.

Overall RM risk and total number of M2 alleles in couples
Analysis was also carried out after splitting the patients into different subgroups according to couples rather than as individuals. In early miscarriage couples, a relative risk of 1.3 (odds ratio 1.47, 95% CI 1.03 - 2.09) as compared with Moore control couples was observed and no significant difference was noted between late miscarriage couples and Moore couples. Overall, similar results were observed in couples as to those observed in individuals, although in some cases P-values were increased (Supplementary Table 2). This could be because the overall numbers were halved in this analysis.

Estimated risks rates were also stratified according to the total number of M2 alleles present in couples and chi-squared tests were used to highlight differences between couples (Table 4). M2 alleles per couples ranged from zero (in cases where both partners were wildtype for ANXA5) to three (in cases where one partner was a M2 homozygote and the other was M2 heterozygous). No couples in this study were both homozygous for M2. Chi-squared tests were performed and P-values were calculated by comparing all four categories together (0, 1, 2 or 3 M2 alleles) between patients and Moore controls in a 4 x 2 table and also by comparing category “0” (having no M2 allele) with all of the other three categories combined together (having any M2 alleles) in a 2 x 2 table.

Couples that had a total of two M2 alleles were significantly more frequent in early RM couples as compared with control couples (P=0.03), while no differences were observed in the total number of M2 alleles between late miscarriage couples and control couples (Table 4). Interestingly, when early RM couples were divided into those who were positive or negative for known risk factors, significant differences were only observed in the positive for known risk factor group when all four categories were compared in a 4 x 2 table (P=0.02). Looking at each subgroup separately, early RM couples sharing two M2 alleles, reached significance (P=0.01) if they were positive for known risk factors as compared with control
couples, whereas those sharing one M2 allele failed to reach significance. This suggests that an additional M2 allele further influences the risk of RM.

**M2 haplotype in control placentas**

Although not statistically significant, the AF of M2 was found to be higher in control placentas (0.114) as compared with the parents (0.093). This was true for placentas of both female (0.107) and male fetuses (0.121) (Table 5). In fact, out of 47 control couples where one parent was WT and the other was an M2 carrier, M2 was inherited by 30 offspring.
DISCUSSION

Previous studies have shown that polymorphisms in the promoter region of the \textit{ANXA5} gene are significantly associated with RM. Women with the M2 haplotype have a higher risk of fetal loss than non-carriers. This was shown initially in a population of 70 German women with RM (Bogdanova et al. 2007), and reproduced in cohorts of 103 Italian (Tiscia et al. 2009) and 243 Japanese women (Miyamura et al. 2011). Recently these findings have been confirmed in populations of 243 German and 236 Bulgarian patients with RM (Tüttelmann et al. 2013).

In our study, a deviation from HWE was observed in the Moore cohort female controls. This was similar to the effect previously described in the female Muenster control group, investigated by Bogdanova et al. (2007), due to a lack of M2 heterozygotes. A possible explanation is positive ascertainment bias resulting from the Moore control group consisting entirely of women who had no miscarriages, while miscarriage usually occurs in about 10% of women worldwide (Everett, 1997). When women and men were combined together, this deviation was no longer present.

Findings from our study on 501 White-European females and 495 male partners are in agreement with previous studies, as we have shown that carriers of M2 exhibit a higher RM risk than non-carriers. In this study we show that the haplotype confers about the same relative risk to carriers of both sexes, suggesting that paternal M2 carriage confers an equal risk for RM as M2 carriage in mothers and that both maternal and paternal promoter alleles of the \textit{ANXA5} gene contribute equally to the lower expression levels seen in the M2 carriers. This confirms a pilot study that showed that the M2 haplotype confers the same relative risk to carriers of both sexes, based on 30 couples (Rogenhofer et al. 2012) and a more recent
study based on 109 couples (Tüttelmann et al. 2013). Based on the findings from these studies, screening for the M2 haplotype in both partners in RM couples should be considered.

Most importantly, our data demonstrate that M2 is associated with ‘early’ RM and not with ‘late’ RM which is in agreement with Tiscia et al. (2009). In addition the M2 haplotype appears to be enriched in the subgroup of early RM patients that are positive for known risk factors in comparison with those that are negative, although the observed difference is not statistically significant. Filtering out the FVL and prothrombin positive individuals from this group, results in M2 carriage rate of almost 31% for APLS patients. This is in general agreement with the proposed function of the M2/ANXA5 as genetic predisposition for the development of obstetric APLS (Bogdanova et al. 2012).

The highest relative risk for M2 haplotype (1.7) carriage was observed in patients who had early fetal losses before the 12th week of gestation and who were also positive for APLS (odds ratio 2.07, 95% CI 1.35 - 3.20) when compared with the Moore controls and increased to 2.0 (odds ratio 2.41, 95% CI 1.56 - 3.71) when compared to PopGen controls. This is generally in agreement with Tüttelmann et al. (2013), where the highest relative risk of the M2 haplotype was observed in women that had early fetal losses.

Interestingly, in our study, out of 30 couples where both parents were M2 carriers, 24 had only early miscarriages (average 4.3 miscarriages). Of the remainder, 3 couples had both early and late miscarriages and 3 couples had only late miscarriages. Of the 14 couples where one partner was an M2 homozygote and the other was wildtype, 9 couples had only early miscarriages (average 3.9 miscarriages), 3 couples had both early and late miscarriages and 2 couples had only late miscarriages.

Reduced levels of ANXA5 have been observed in women with APLS, who suffered RM and these may contribute to the placental thrombosis observed in these patients (Rand et
al. 1994). However, even in the absence of aPL, reduced ANXA5 expression would probably lead to a hypercoagulable state in the intervillous placental space. This may be a risk that could be attenuated by anticoagulant therapy. The fact that no association was seen between the M2 haplotype and late miscarriages implies that reduced levels of ANXA5 may be used as a marker only for early miscarriages.

Early but not late miscarriages are usually associated with aneuploidy and the risk of fetal loss increases after the age of 35 (Nybo Andersen et al. 2000). Idiopathic recurrent miscarriages are caused mostly by chromosomal abnormalities and as shown recently, with only a residual miscarriage rate of 7% (Hodes-Wertz et al. 2012). Information on aneuploidy in the pregnancy losses of RM patients in our study was not available, so it is hard to accurately differentiate the effect of the ANXA5 M2 haplotype from an elevated risk of aneuploidy. However the average age of our RM cohort was less than 35 and was similar to the average age of our control population.

In Bogdanova et al. (2007), reporter gene assays have shown that the M2 haplotype decreases the activity of the promoter of this anti-coagulant gene, potentially leading to fetal loss. If the M2 haplotype was completely disadvantageous, one might have expected it to disappear or at least diminish in frequency over generations due to natural selection. However, data from the control placentas in our study may suggest that the M2 haplotype could be advantageous at some point in pregnancy. Similar suggestions of possible survival advantages of a prothrombotic phenotype have previously been described for both FVL and Prothrombin. There remains a high population frequency of the FVL mutation despite the associated disadvantages such as APC resistance, increased risk of venous thrombosis and miscarriage (Bertina et al. 1994; Dahlbäck, 2009). Some sort of evolutionary advantage for carriers has been suggested, such as aversion of sepsis (Kerlin et al. 2003) or protection from bleeding, as APC-resistant women were characterized by less intrapartum blood loss than non
APC-resistant women (Lindqvist et al. 1998). Findings from a more recent study also suggest that the Prothrombin G20210A variant as well as FVL might convey a survival advantage (Lussana et al. 2012).

In conclusion, our study has shown that the $M2/ANXA5$ haplotype is a risk factor for early miscarriages, before the 12th week of gestation. The M2 haplotype confers about the same relative risk to carriers of both sexes, suggesting a role of $ANXA5$ expression levels in the fetus and/or the extraembryonic membranes. In addition, from our RM couple analysis, it appears that the RM risk increases with increasing dosage of the M2 allele. In most cases (24/33), both parents contribute one M2 allele each, further supporting the hypothesis that the M2 haplotype confers the same risk if carried either by males or females. Finally this analysis suggests that having one or more M2 alleles in combination with other risk factors, further increases the RM risk.
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Competing interests

The authors report no conflict of interest.
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