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GENETICS

Genetic analysis of the *M2/ANXA5* haplotype as recurrent pregnancy loss predisposition in the Malay population

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Abstract

Purpose The aim of this study was to evaluate a new predisposition factor, *M2/ANXA5* (*RPRGL3*), in recurrent pregnancy loss (RPL) patients of Malay origin, since it was previously known that the prevalence of this condition is relatively high among the Malay population of Malaysia, where conventional hereditary thrombophilia factors have been generally ruled out.

Methods A total of 232 women who had experienced ≥2 unexplained RPL and 141 available male partners were recruited, with 360 healthy Malay and 166 parous female controls. Prevalence of *M2* carriage and RPL odds ratios were calculated in (a) control and patient groups; (b) clinically defined subgroups in categories of pregnancy loss, primary, secondary, and tertiary; and (c) timing of pregnancy loss in early, ≤15th gestation week and "late" fetal losses, and >15th gestation week subgroups.

Results Both male and female subjects had similar *M2/ANXA5* allele frequencies. The carrier rate of *M2/ANXA5* for the general Malay population was 42.2 and 34.9% for parous controls. These carrier rates compared to Malay RPL subjects (52% *M2* carriers) resulted in elevated odds ratios (95% confidence interval) of 1.53 (1.1 to 2.1) and 1.97 (1.3 to 3.1) accordingly for early fetal losses. Moreover, exceeding copy numbers of *M2/ANXA5* alleles seemed to afflict a greater chance of RPL in couples, especially when both partners were *M2* carriers.

Conclusion This study confirmed the proposed role of *M2/ANXA5* as embryonic, genetically associated thrombophilia predisposition factor for early RPL among ethnic Malay of Malaysia.

Keywords Annexin A5 · *M2/ANXA5* · Recurrent pregnancy loss (RPL) · Miscarriage

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Introduction

"Recurrent pregnancy loss" or "repeated pregnancy loss" (RPL), also named "recurrent spontaneous abortions" (RSA), has an adverse psychosocial impact in women and their families, resulting in depression or anxiety for the next pregnancy [1]. RPL is a complex and multifactorial obstetric problem with polygenic background and it involves a variety of genetic, physiological, and environment factors [2]. About 30–40% of RPL cases remain unexplained, categorized as "idiopathic."

In the last decade, evidence has linked hereditary thrombophilia to the etiology of RPL by a mechanism of impeded placental perfusion, ultimately resulting in adverse pregnancy outcome [3–7]. Although factor V Leiden (FVL) and prothrombin G20210A (PTm) variants are accounted for 70% of inherited thrombophilia patients [7], both of these factors are rare in Asian population [8, 9].

Malaysia, located in South East Asia, has a multi-ethnic society. Due to different cultural and socioeconomic backgrounds, the Malay ethnic group (55% of the population) has a higher prevalence of spontaneous abortion and RPL compared to other ethnics [10]. A low incidence of FVL and PTm (1 and 0.3%) in Malay RPL women has been previously reported [11, 12]. There are no official data regarding RPL incidence among Malay to date. Based on the statistics available from the Malaysian Population and Family Survey (MPSF), 14% of early pregnancy losses are recorded from total pregnancies [13]. However, this reported abortion rate could be an underestimate. Notably, a high proportion of pregnancy losses at the Sultan Abdul Halim Hospital from 2013 to 2015, 2296/2575 (89.2%), occurred in Malay women. The spontaneous abortion rate in Malay women at this hospital was 20.3% from a total of 11,306 admissions, although no records were available on RPL. Higher spontaneous abortion rates were recorded among Malay with advancing age, growing number, and shorter intervals of subsequent pregnancies [10]. A conservative average estimate would be between 20 and 25% considering maternal age group 30–40 with multiple consecutive pregnancies. Assuming an equal to the worldwide RPL proportion of 20 to 30%, these would result in a deduced recurrent miscarriage rate of 5 to 8% judging from correlative distribution.

In 2007, a new hereditary predisposition factor associated with thrombophilia-related RPL, termed *M2*, was reported, a haplotype in the proximal core promoter region of the annexin A5 (*ANXA5*) gene, defined as a constellation of four single nucleotide substitutions (SNPs), rs112782763, rs28717001, rs28651243, and rs113588187, respectively [14]. This genetic variant has been acknowledged as a third gene for RPL predisposition, *RPRGL3*, OMIM entry 614391.

Annexin A5 is a placental anticoagulant protein, which binds to the apical surface of placental syncytiotrophoblasts as a putative protective shield [15]. Available evidence demonstrated that *M2* haplotype significantly lowers the expression of *ANXA5* in a reporter gene assay [14, 16]. The reduction of *M2/ANXA5* mRNA [17, 18] and its protein levels were further confirmed in thrombophilia-associated placental complication cases [19].

The *M2* haplotype has been reported in the German and Bulgarian populations with odds ratios for *M2* carriers ranging between 1.3 and 2, as compared to random population controls. The estimated odds ratios in *M2* carriers were somewhat elevated, 1.8 to 3.0, when compared to healthy controls with at least one live birth and with negative history of pregnancy losses in German [5, 14], Italian [4], Bulgarian [5], and Japanese [20] cohorts. However, the criteria for RPL subjects varied slightly between the studies such as the definition of RPL and categories of embryonic development. In addition to available evidence supporting reduced expression of *ANXA5* in chorionic placenta of RPL women, who were *M2/ANXA5* carriers [18, 19], the male *M2/ANXA5* carriers in RPL couples also showed a rather similar risk that would corroborate impeded embryonic anticoagulant function [5, 21–23].

The assessment of thrombophilia-associated obstetric complications should not be a trivial consideration among the Malay ethnic group based solely on the rare incidence of FVL and PTm factors reported previously. Therefore, the aim of the present study was to obtain more information on the prevalence of *M2/ANXA5* as RPL predisposition factor, noted in a preceding pilot investigation, and answer the relevance of the haplotype concerning timing of miscarriage and allelic dependence.

Patients and methods

Study populations

The present genetic association study was approved by the Human Ethics Research Committee of the Universiti Sains Malaysia (USMKK/PPP/JEPeM [245.3.(2)]) and from the National Institutes of Health, Ministry of Health, Malaysia (NMRR-11-1044-9519). The study was carried out in accordance with The Code of Ethics of the World Health Organization (Declaration of Helsinki), and the criteria of strengthening the reporting of genetic association studies were observed as far as applicable. The volunteer subjects who agreed to participate have signed an informed consent before collection of peripheral blood samples.

All cases and controls were of Malay origin verified across three generations that did not have intermarriage. Subjects who had experienced RPL ($n = 237$) were recruited from the Department of Obstetrics and Gynaecology at Hospital Sultan Abdul Halim, Sungai Petani; Hospital Tuanku Jaafar, Seremban; Hospital Tengku Ampuan Afzan, Kuantan; and Hospital Sultanah Bahiyah, Alor Setar, between January 2013 and May 2015. Male partners of 146 of these women agreed to

participate in the study. RPL subjects were pre-screened for potential causes of their repeated pregnancy loss as described previously [21, 24]. Uterine anomalies and endocrine dysfunctions (polycystic ovary syndrome according to the Rotterdam criteria [25] and thyroidal dysfunctions, if anamnestic) were not included. Four abortions in cases of fetal chromosomal abnormalities (mostly numerical aberrations) were excluded from this study through morphological high-resolution ultrasonic examination [26]. Inherited thrombophilia (FVL, PTm) and deficiencies in anti-thrombotic factors (protein C, protein S, factor XII, antithrombin III) were ruled out. Stillbirths were not included in this study. After completion of this diagnostic protocol for RPL patients, 232 subjects with unexplained fetal losses remained; of these, 141 had presented with male partners.

RPL was defined as the occurrence of ≥2 pregnancy loss before the 20th gestational week (GW). Primary RPL comprises ≥2 consecutive RPL before the 20th GW, with no history of live birth; secondary RPL have ≥2 consecutive abortions before the 20th GW, after a live birth; and tertiary RPL represent ≥2 non-consecutive miscarriages that occurred before the 20th GW but are interspersed with live births [27, 28]. RPL was further subdivided into two subgroups based on timing of pregnancy losses: subgroup 1, "early" fetal losses, GW ≤15, and subgroup 2, "late" fetal losses, GW >15 [4].

Random Malay population subjects were recruited at the Universiti Sains Malaysia, Penang campus from January 2011 to May 2013 with appropriate informed consent. The control group consisted of 360 participants, with 188 (52.2%) men and 172 (47.8%) women. All control participants had healthy status according to the medical register of the university's campus occupational safety and health administration. A history of previous pregnancies and pregnancy losses was not recorded. Another 166 anonymized female control samples with at least one successful pregnancy and no previous gestational pathology (parous controls) were recruited from the Department of Obstetrics and Gynaecology at Hospital Sultan Abdul Halim, Sungai Petani, and Hospital Tuanku Jaafar, Seremban. Clinically relevant features of patients and control groups are summarized in Table 1.

Genotyping and statistical analysis

Genotyping of DNA extracted from peripheral blood was performed for the *M2* haplotype of the *ANXA5* promoter region (*RPRGL3*) for all patient and control subjects of this study. The 360 Malay controls, 77 RPL patients, and 41 male partners thereof were genotyped by amplicon sequencing as previously described [23]. Subsequently, a direct genotyping protocol was developed that utilized allele-specific PCR (AS-PCR) reactions for the *M2* and "normal" haplotypes. This protocol was verified blinded on 100 sequenced samples and the rest of 166 parous controls, 155 RPL women, and 100 available male partners were genotyped with AS-PCR.

AS-PCR for the *M2/ANXA5* haplotype was designed as two nested PCR reactions, the first using common primers for the *ANXA5* 5′ UTR and the second with allele-specific primers sets. The first PCR reactions contained 1× PCR reaction buffer (Biotools, Spain), 1 mM $MgCl_2$ (Biotools, Spain), 5% dimethyl sulfoxide (DMSO), 0.2 μM of each (forward and reverse) common primer, 200 μM dNTPs, approximately 100 ng genomic DNA, and 1 U Taq polymerase (Biotools, Spain). The common primers sequences were as reported previously [14]. Cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 25 cycles of amplification, 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s. The final extension was performed at 72°C for 5 min.

Next, two parallel allele-specific reactions ("normal" and *M2/ANXA5* haplotype) were carried out by using "normal" primers (F: 5′-TGGCGCGGCCGGCCTGCGGTTGG-3′; R: 5′-GAGATGCAGACGCTGAAGGATC-3′) and *M2/ANXA5* primers (F: 5′-TGGCGCGGCCGGCCTGCGGTTGA-3′; R: 5′- GAGATGCAGACGCTGAAGGATCT -3′) in separate PCR mixes. The template was 1 μl of 5× diluted first PCR reaction product. The cycling conditions and PCR mixture were similar to the first PCR reaction, but the next amplification round was performed with 0.5 mM $MgCl_2$, 0.2 μM of each primer, and 1.25 U Taq polymerase. The PCR product thereof with amplicon size of 139 bp was analyzed on 1.5% ethidium bromide-stained agarose gels.

Table 1 Clinical features of cases and control groups

	RPL ($n = 232$)	Parous controls ($n = 166$)	Population controls ($n = 360$)
Age, median (range)	32 (21–45)	30 (20–47)	32 (18–55)
Gravidity, median (range)	5 (2–11)	2 (1–9)	/
Parity, median (range)	7 (0–7)	2 (1–9)	/
No. of fetal losses, median (range)	5 (2–7)	/	/
Weeks of early fetal losses, median (range)	8 (6–14)	/	/
Weeks of late fetal losses, median (range)	16 (16–20)	/	/
GDM	20	6	/
GHT	5	0	/

RPL recurrent pregnancy loss, *GDM* gestational diabetes mellitus, *GHT* gestational hypertension

Odds ratios (OR), 95% confidence intervals (CI), and adjusted OR and 95% CI were estimated with a minimal power of 70% in comparisons with population controls and reached 80% when comparing with parous controls by using multiple logistic regression models that controlled for potential confounding variables, such as age and gravidity (SPSS version 22.0, Chicago, USA). Differences in (a) clinical subgroups, (b) pregnancy loss subgroups, (c) number of alleles, and (d) number of carriers were evaluated using the Pearson chi-square test ($n > 30$) or the two-tailed Fisher's exact test ($n \leq 30$). Deviations from the Hardy-Weinberg equilibrium (HWE) were calculated using a Monte Carlo Markov chain (MCMC) implementation of an exact test, part of the Genepop package (http://genepop.curtin.edu.au/) [29]. Mean and standard deviations of age and number of miscarriages were evaluated with Microsoft Excel 2013 (Microsoft Redmond Campus, Redmond, WA, USA). Statistical significance was defined as $p \leq 0.05$.

Results

Genotyping by AS-PCR

The genotyping method developed accurately discriminated the *M2* and "normal" haplotypes as a sum of two independent reactions (Supplementary Fig. S1) with 100% reliability as verified blinded on 100 previously sequenced samples. As already documented in numerous studies and lately confirmed in the pilot investigation [23], the *M1* haplotype was not a predisposing factor for RPL. Therefore, the screening of *M1* was not included in further analysis.

Overall RPL predisposition

The RPL dependence of *M2* carrier status was first assessed by comparison to the random Malay population (Table 2). As previously shown [23], the control group of random Malay subjects ($n = 360$) fulfilled HWE for *ANXA5* promoter haplotypes with $P = 0.6622$. The *M2* carrier rate of the random control group was 42.2%. The second control group comprised of parous female controls ($n = 166$) without gestational pathology had an *M2* carrier rate of 34.9% (Table 2) and was not in HWE for *M2/ANXA5* ($P = 0.036$), due to a lack of *M2* heterozygotes (respective excess of *M2* homozygotes). This control group was specifically used to assess *M2* predisposition in RPL patients and not in their male partners, because the post hoc statistical analyses involved the confounders age and gravidity/parity specifically applying to the repeated miscarriages phenotype in women.

A total of 182 out of 373 subjects (RPL women and partners thereof, median age of 33, range 21 to 64) experiencing RPL (in ≤20th GW) were *M2/ANXA5* carriers (48.8%), which resulted in adjusted odds ratio of 1.32 (95% CI 0.99 to 1.78, $p = 0.062$) compared to random population controls.

RPL subjects were further divided into three clinically relevant subgroups and carrier rates of the *M2* haplotype were reassessed. As shown in Fig. 1a, the primary (1°) RPL, $n = 139$, and secondary (2°) RPL, $n = 147$, subgroups had *M2* carrier rates of 49.6 and 54.4% accordingly, with an overall combined prevalence of 52.0% (Table 2). The estimated *M2* carrier rate for the tertiary (3°) RPL subgroup, $n = 87$, was 37.9%, somewhat lower than the random population prevalence of 42.2%, and it showed a statistically significant difference when compared to the first two subgroups ($p = 0.02$). Therefore, the consequence of *M2* carriage was reassessed for the 1° and 2° RPL subgroups ($n = 286$) and further compared to the random Malay population. The comparison yielded an adjusted overall odds ratio of 1.52 (95% CI 1.1 to 2.1, $p = 0.01$) for *M2* carriers of the first two RPL subgroups (Fig. 2a). For subsequent analyses, the focus was on the clinically relevant 1° and 2° RPL subjects, where simple logistic regression was used for further assessment in subgroups.

RPL predispositions of women and their male partners as carriers of the *M2* haplotype

To determine whether the *M2/ANXA5* haplotype would be an RPL predisposing factor of importance in both sexes, 1° and 2° RPL subjects were subdivided into female patients and

Table 2 Genotype distributions of female and male partners in the RPL cohort, according to clinically relevant pregnancy loss categories

Index	Population controls	Parous controls	All RPL		Primary and secondary RPL		Tertiary RPL	
	n (%)	*n* (%)	Women *n* (%)	Men *n* (%)	Women *n* (%)	Men *n* (%)	Women *n* (%)	Men *n* (%)
Genotypes	$n = 360$	$n = 166$	$n = 232$	$n = 141$	$n = 179$	$n = 107$	$n = 53$	$n = 34$
N/N	208 (57.8)	108 (65.1)	119 (51.3)	72 (51.1)	88 (49.2)	49 (45.8)	31 (58.5)	23 (67.6)
N/*M2*	134 (37.2)	46 (27.7)	105 (45.3)	65 (46.1)	83 (46.4)	55 (51.4)	22 (41.5)	10 (29.4)
M2/*M2*	18 (5.0)	12 (7.2)	8 (3.4)	4 (2.8)	8 (4.5)	3 (2.8)	0 (0.0)	1 (2.9)
M2 AF	0.236	0.211	0.261	0.259	0.277	0.285	0.208	0.176
M2 carriage	152 (42.2)	58 (34.9)	113 (48.7)	69 (48.9)	91 (50.9)	58 (54.2)	22 (41.5)	11 (32.3)

RPL recurrent pregnancy loss, *N* "normal" allele including M1 heterozygous and homozygous combinations, *AF* allele frequency

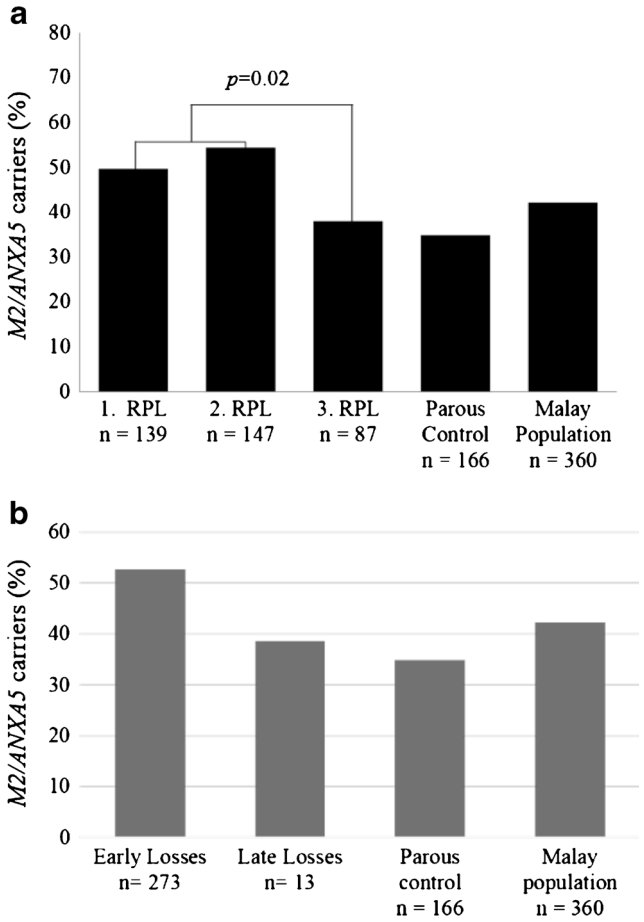

Fig. 1 Distribution of *M2* carriers (%) in relevant clinical subgroups of Malay patients and male partners who had experienced RPL according to repeated pregnancy loss categories (**a**). Embryonic categories, gestational weeks 6 to 15 and >15 (**b**)

their male partners to further assess the independent contributions to estimated *M2* carrier rates. The 1° and 2° RPL subgroups comprised of 179 women and 107 male partners accordingly. When compared to random Malay population, the subgroup of RPL women yielded an odds ratio of 1.42 (95% CI 1.0 to 2.0, $p = 0.05$) for *M2* carriers. The comparison to parous controls without gestational complications resulted in an odds ratio of 1.93 (95% CI 1.25 to 2.97, $p < 0.01$). When male partners carrying *M2* were compared to the population controls, the odds ratio was 1.62 (95% CI 1.1 to 2.5, $p = 0.03$) that is comparable to the general population predisposition in RPL females. Male partners with 54.2% *M2* carrier rate (Table 2) had a carrier status only three percentage points higher (95% CI 0.7 to 1.9, $p = 0.74$) than RPL women from this study.

RPL predispositions of *M2* carriers in patient subgroups according to timing of miscarriage

The miscarriage proportions in the early ($n = 273$) and late ($n = 13$) fetal losses subgroups were 95.5 and 4.5% accordingly. Table 3 shows genotype distributions in each subgroup of fetal losses related to the predisposition haplotype *M2*. *M2* carrier rate in RPL subjects was higher in early, compared to late fetal losses, with 52.7 vs. 38.5% (Fig. 1b).

Next, the relative prevalence of *M2* carriers in fetal loss categories was estimated by comparing patients and their male partners (combined) of these subgroups against the population controls. Because of the relatively small number of individuals in the late fetal loss subgroup, the comparison performed used Fisher's exact test. Obtained odds ratios were 1.53 (95% CI 1.1 to 2.1, $p = 0.01$) for early fetal losses and 0.86 (95% CI 0.3 to 2.7, $p = 1.0$) for late fetal losses (Fig. 2a). The comparison of *M2*-carrying patients of these subgroups to parous controls yielded odds ratios of 1.97 (95% CI 1.3 to 3.1, $p < 0.01$) and 1.12 (95% CI 0.3 to 4.8, $p = 1.0$) accordingly (Fig. 2b).

Assessment of RPL predisposition in *M2*-carrying couples

In order to better assess the *M2* carrier predisposition for RPL, the comparisons were drawn to couples' ($n = 141$) rather than to individuals' status. *M2/ANXA5* carrier rates among patients were 47.5 and 48.9% for their male partners accordingly (Supplementary Table S1). Statistical comparisons on the

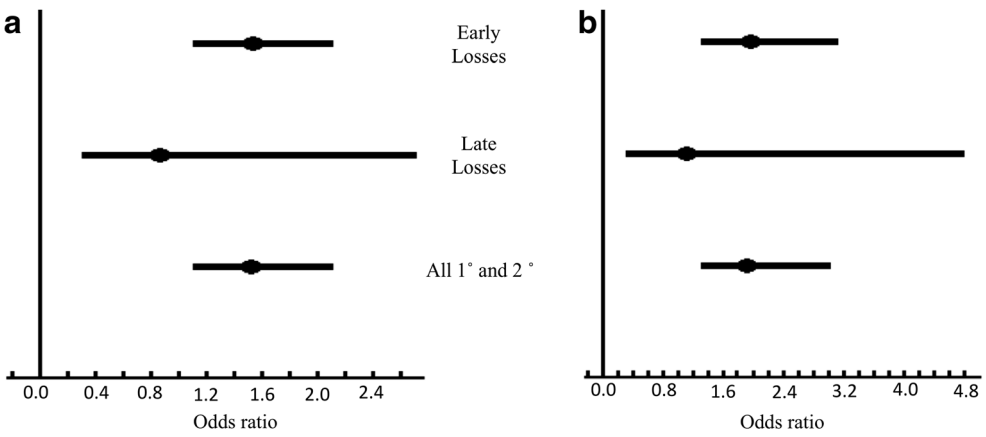

Fig. 2 Forrest plot showing odds ratios for *M2* carriers in RPL subgroups compared to random Malay population (**a**) and to parous controls (**b**)

Table 3 Genotype distributions of female and male partners in the RPL cohort according to fetal losses categories of miscarriage

Genotype	All 1° and 2° RPL		Subgroup 1: early fetal losses, ≤15th GW		Subgroup 2: late fetal losses, >15 GW		Population controls	Parous controls
	Women *n* (%)	Men *n* (%)	Women *n* (%)	Men *n* (%)	Women *n* (%)	Men *n* (%)	*n* (%)	*n* (%)
Genotypes	*n* = 179	*n* = 107	*n* = 171	*n* = 102	*n* = 8	*n* = 5	*n* = 360	*n* = 166
N/N	88 (49.2)	49 (45.8)	83 (48.5)	46 (45.1)	5 (62.5)	3 (0.6)	208	108 (65.1)
N/*M2*	83 (46.4)	55 (51.4)	80 (46.8)	53 (51.9)	3 (37.5)	2 (0.4)	134	46 (27.7)
M2/*M2*	8 (4.5)	3 (2.8)	8 (4.7)	3 (3.0)	0 (0.0)	0 (0.0)	18	12 (7.2)
M2 AF	0.277	0.285	0.281	0.289	0.188	0.200	0.236	0.211
M2 carriage	91 (50.9)	58 (54.2)	88 (51.5)	56 (54.9)	3 (37.5)	2 (33.3)	134 (42.2)	58 (34.9)

RPL recurrent pregnancy loss, *N* "normal" allele including M1 heterozygous and homozygous combinations, *AF* allele frequency, *GW* gestational week

relative distribution of *M2* carriers and RPL associated attributable population predisposition per couple in the pregnancy loss categories (1°, 2°, and 3°) yielded odds ratios that were similar to those obtained for individuals with appropriately higher significance in individuals, due to the increased sample size. Estimated odds ratios of *M2* carriers in early (≤15th GW) and late (>15th GW) couples were 1.53 (95% CI, 1.1 to 2.1, $p < 0.01$) and 0.86 (95% CI, 0.3 to 2.7, $p = 1.0$).

M2/ANXA5 allelic and carrier status dependence of RPL predisposition in couples

To elucidate the effect of increasing *M2* allele numbers in couples, Pearson's chi squared tests were used to estimate the difference in relative abundances. *M2* alleles number ranged from zero (in cases in which both partners were wild type for *ANXA5*) to three copies (in cases in which one partner was a *M2* homozygote and the other was *M2* heterozygous) per couple. There were no homozygous *M2* couples detected in this study. The fractions of 1° and 2° RPL couples with one or more copies of the *M2* allele in relation to non-carriers were compared in a 2 × 2 table with couples of the tertiary RPL subgroup and among the fetal losses categories (Supplementary Tables S1 and S2). Couples who had ≥2 alleles (32/102 compared to 5/34 of 3° couples) were significantly more frequent among 1° and 2° RPL subjects ($p = 0.01$), with a greater share in the early fetal losses subgroup (Supplementary Table S2).

In order to estimate the odds of increasing carrier numbers per couple, RPL couples were categorized into non-*M2* carrier (both partners are wild type for *ANXA5*), one *M2* carrier (one of the partners is heterozygous or *M2* homozygous), and two *M2* carriers (in cases that two partners are heterozygous or in cases that one partner is heterozygous and the other is *M2* homozygous). Thus, the substantial difference between the *M2* copy number and carrier status analyses is that heterozygous and homozygous individuals were both counted as carriers. Relative abundances of two carriers per couple were compared to the occurrences of non-carrier couples among RPL categories. There was higher prevalence of two-carrier *M2* couples (26/102 vs. 5/34) among 1° and 2° RPL subjects ($p = 0.03$), further corroborated with their relative share in the early fetal losses subgroup, ≤15th GW.

Discussion

The *M2* haplotype of the *ANXA5* gene is significantly associated with RPL. As reported in previous studies, *M2* prevalence in population and healthy subject controls of German, Italian, Bulgarian, and UK white European origin are about 15–17% [4, 5, 14]; whereas in Asian populations, the reported carrier rate is 11% with 5.4% *M2* allelic frequency for the Japanese [20]. A recent study finding no association of *M2*/*ANXA5* with RPL in 86 Estonian and 227 Danish subjects reported 27.3% prevalence with 15.2% allelic frequency in Estonian and 23.5% prevalence with 12.6% allelic frequency in Danish parous women [30]. Surprisingly, the *M2* carrier rate estimated for the Malay population of Malaysia is relative high with 42.2%. Although the Japanese and Malay share a common ancestral haplogroup origin in the Austronesian population [31], they show markedly different *M2* prevalences that could be explained with diverse phylogenetics. It is thus tempting to speculate that the relatively high spontaneous abortion and according RPL rates recorded for the Malay [10, 32] are at least in part due to the abundance of the *M2* haplotype, as previously suggested in a pilot study [23].

The diagnostic protocol for RPL workup in Malaysia still involves the "classic" genetic thrombophilia screening, i.e., factor V Leiden (FVL) and prothrombin (PTm) variants. However, these two genetic thrombophilia lesions have very low prevalence in Malay RPL women [11, 12]. The second study reported that despite the extensive screening, 38% of their recurrent miscarriage cases remained idiopathic, which could at least partly involve the risk haplotype *M2* [12].

The current extended study involving a total of 232 Malay RPL women and 141 male partners thereof corroborated the risk estimates for *M2* carriers from the initial pilot study [23]. This study also confirmed the increased odds ratios for RPL patients from the primary and secondary pregnancy loss clinical categories as opposed to the subgroup of tertiary pregnancy losses, where a genetic factor would be of lesser importance, due to the relatively increased sporadic share of miscarriages. The clinical consideration of the first and second subgroups is rather important for the diagnostic workup of ethnic Malay, as it is common for a Malay family to have more than three children.

The parous controls had a *M2* carrier rate of 34.9%, comparable to the tertiary RPL couples group with 37.9%, where RPL per definition appears as a condition much more dependent on environmental and physiological than on genetic factors. The observed HWE deviation in *M2* distribution for parous controls is analogous to other selected fertile control groups from previous studies [14, 22]. A possible explanation is the positive ascertainment bias resulting from selection of the Malay parous control group comprised only of women with at least one successful pregnancy and without miscarriages or other gestational pathology, whereas spontaneous abortion generally occurs in about 10% of women worldwide [33]. The observed ascertainment bias is thus indirectly indicative for the proposed role of *M2* as RPL factor, apparently the consequence of adjustment for phenotype.

The odds ratios of *M2*-carrying RPL patients from this study, 1.4 with population controls and 1.9 with parous women, are similar to these of European RPL cohorts, 1.5 to 2.5 with population controls and 3 to 5 with parous controls [5, 21, 22], as well as to the reported odds ratio of 2.4 with parous women from the Japanese study [20]. Attributable predisposition of male *M2* carriers in Malay RPL couples is thus in general agreement with these previous studies, supporting the proposed pathophysiological expression of *M2/ANXA5* in impediment of embryonic anticoagulation.

Therefore, stratifying RPL according to timing of miscarriage (categories of fetal loss) was necessary, in order to specifically determine the role of annexin A5 in pregnancy-relevant pathology. Because of the relatively high conception rate in Malay women, and the systemic influence of *M2/ANXA5* as hereditary factor, the last recorded pregnancy loss was considered as reference index loss. The highest *M2* carrier rates were observed in the early fetal losses subgroup, less than 15 gestational weeks, in primary and secondary RPL patients. This is in agreement with the proposed role of *M2/ANXA5* as "early" RPL factor [4, 5], in contrast to the "classic" thrombophilia factors FVL and PTm, of greater importance for late miscarriages, >20th week [2, 34]. It should also be noted that for this study, uncertainties in the exact timing of conception should allow for ±1 to 2 weeks difference, when recording the timing of pregnancy loss similar to previous studies. Furthermore, even if some more patients with fetal aneuploidies undetected by morphological ultrasound in addition to the four that were already excluded, would have participated in this study, the average age of the RPL patients included was below 35 years, which should considerably reduce the chance of fetal chromosomal aberrations.

Raising the number of predisposing alleles in couples should generally increase the chance of their pathologic expression in the progeny. A higher count of *M2/ANXA5* alleles would elevate the miscarriage risk of RPL couples accordingly. Without exception, the analysis showed that 2 and 3 *M2/ANXA5* alleles are significantly associated with "early" RPL ($p = 0.01$). Likewise, if both partners are *M2/ANXA5* carriers, they would have higher chances to experience RPL ($p = 0.03$), specifically enriched in the subgroup of "early" fetal losses between the 6th and 15th gestational weeks, similar to the UK cohort study [22].

In conclusion, *M2/ANXA5* seems to be a predisposing factor for primary and secondary RPL in Malays with unusually high abundance in the general population, even more so on the background of insignificant occurrences of "classic"' inherited thrombophilia reported [11, 12]. Male and female *M2* carriers are subject to similar RPL predisposition with the highest exposure for early pregnancies, less than 15 weeks of gestation. Considering the relatively high pregnancy loss rates of Malay women and the estimated high general prevalence of the *M2* haplotype, couples who have experienced primary and secondary RPL might be screened for *M2* carrier status as a biomarker of possible successful anticoagulant treatment, as suggested by recent genetic evidence from the EThIGII and the CARE Fertility Group clinical trials [35, 36]. Related to this, Malay RPL couples appear as a very suitable population model for a properly powered therapeutic randomized clinical trial.

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

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