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Genetic analysis of the M2/ANXA5 haplotype as adverse pregnancy outcomes risk factor- Evaluation in a Portuguese population group

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Genetic analysis of the M2/ANXA5 haplotype as adverse pregnancy outcomes risk factor- Evaluation in a Portuguese population group

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ABBREVIATIONS

APO - Adverse pregnancy outcome

BMI - Body mass index

CI - Confidence interval

FVL - Factor V Leiden

GH - Gestational hypertension

HELLP - Hemolysis, elevated liver enzymes and low platelets

HWE - Hardy-Weinberg equilibrium

IUFD - Intrauterine fetal death

IUGR - Intrauterine growth restriction

MCMC - Monte Carlo Markov Chain

OR - Odds ratio

PB - Premature birth

PCR - Polymerase chain reaction

PE - Pre-eclampsia

RPL - Recurrent pregnancy loss

RR - Relative risk

SGA - Small for gestational age

SNP - Single nucleotide polymorphism

TE - Thromboembolism

VTE - Venous thromboembolism

WG - Week of gestation

RESUMO

Introdução: A gravidez induz um estado de hipercoaguabilidade fisiológica que pode predispor a complicações na gravidez, nomeadamente aborto recorrente (AR), hipertensão gestacional, pré-eclâmpsia (PE), restrição de crescimento intrauterino (RCIU), recém-nascido pequeno para a idade gestacional e tromboembolismo venoso. Os fatores de risco hereditários para trombofilia têm sido identificados como possíveis causas e extensamente investigados. Recentemente, foi identificado um novo fator de risco hereditário para estas complicações, uma variação na sequência da região promotora do gene que codifica a Anexina A5 (*ANXA5*), denominado haplótipo M2. Assim, este estudo pretende avaliar a frequência do haplótipo M2 numa amostra populacional de mulheres portuguesas e averiguar a sua associação com o risco de ocorrência de complicações na gravidez.

Metodologia: Realizou-se um estudo retrospetivo caso-controlo com 102 casos de mulheres portuguesas com antecedentes de complicações na gravidez, incluindo AR, aborto precoce, morte fetal *in* útero, RCIU, PE, eclâmpsia e síndrome HELLP *(hemolysis, elevated liver enzymes and low platelets).* Este grupo foi comparado com um grupo controlo de mulheres portuguesas sem história de complicações na gravidez (n=84), e com dois grupos controlo independentes, previamente usados em estudos semelhantes: os *Münster controls* (n=500) e os *PopGen controls* (n=533). A região promotora do gene da *ANXA5* foi genotipada para a presença dos haplótipos M1 e M2 por PCR/sequenciação de Sanger. Foram estimadas as frequências alélicas, avaliada a conformidade com o equilíbrio Hardy-Weinberg e testada a associação entre os haplótipos da *ANXA5* e a ocorrência de complicações na gravidez utilizando o teste exato de Fisher, cálculo do OR (*odds ratio*), intervalos de confiança (IC) de 95% e valores de p, recorrendo ao *software* Graphpad.

Resultados: O haplótipo M2 estava presente em 31,4% das mulheres com complicações na gravidez (frequência alélica 0,167) e em 15,5% dos controlos portugueses (frequência alélica

0,083). As portadoras do haplótipo M2 apresentavam um risco relativo de ocorrência de complicações na gravidez 1,32 vezes superior ao das não portadoras (OR 2,15, 95% CI 1,13-4,30; p=0,0280), quando comparadas com os controlos portugueses, um risco relativo 1,74 vezes superior quando comparadas com os *PopGen controls* (OR 2,04, 95% CI 1,32-3,10; p<0,0015), e um risco relativo 2,36 vezes superior em comparação com os *Münster controls* (OR 3,27, 95% CI 2,06-5,15; p<0,0001). O haplótipo M1 não estava significativamente associado ao risco de ter complicações na gravidez.

Conclusão: Os resultados obtidos sugerem que o haplótipo M2 pode ser um fator de risco relevante para o desenvolvimento de complicações na gravidez nesta amostra populacional de mulheres portuguesas. Assim, a genotipagem do gene da *ANXA5* para a presença do haplótipo M2, parece ser um procedimento prognóstico adequado em mulheres com complicações na gravidez de causa desconhecida, podendo auxiliar a estabelecer medidas terapêuticas preventivas.

Palavras-chave: Anexina A5, Haplótipo M2, Gravidez, Complicações na gravidez, Fator de risco.

ABSTRACT

Background: Pregnancy alters the hemostatic system into a physiological state of hypercoagulability, which can predispose to adverse pregnancy outcomes (APO), namely recurrent pregnancy loss (RPL), gestational hypertension (GH), pre-eclampsia (PE), intrauterine growth restriction (IUGR), small-for gestational age and venous thromboembolism. Inherited risk factors for thrombophilia have been largely explored as a cause of these APO. Recently a new hereditary risk factor for APO was identified, a sequence variation in the promoter region of the gene encoding annexin A5 (*ANXA5*), called the M2 haplotype. Therefore, we intend to evaluate the frequency of the M2 haplotype in a Portuguese women population sample with a previous history of APO and to assess the association between the M2 haplotype and the risk of APO.

Materials and Methods: A retrospective case-control study was conducted with a case group of 102 Portuguese women with history of unexplained APO including RPL, early miscarriage, IUFD, IUGR, PE, eclampsia and hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome. This group was compared with a control group of Portuguese women without history of APO (n=84), and two independent control groups, previously recruited in similar studies: the Münster controls (n=500) and the PopGen controls (n=533). The *ANXA5* gene promoter region was genotyped by PCR/Sanger sequencing for the presence of the M1 and M2 haplotypes. GraphPad software was used to determine the allelic frequencies, concordance with Hardy-Weinberg equilibrium and the association between the *ANXA5* haplotypes and APO through the Fisher's exact test, estimating OR with 95% confidence intervals (95% CI) and p-values.

Results: The M2 haplotype was found in 31.4% of women with APO (allele frequency 0.167) and in 15.5% of the Portuguese controls (allele frequency 0.083). Carriers of the M2 haplotype were found to exhibit a relative risk of APO 1.32 higher than non-carriers (OR

2.15, 95% CI 1.13-4.30; p=0.0280), when compared to the Portuguese controls, a 1.74 higher risk when compared to the PopGen controls (OR 2.04, 95% CI 1.32-3.10; p<0.0015) and a 2.36 higher risk in comparison to the Münster controls (OR 3.27, 95% CI 2.06-5.15; p<0.0001). The M1 haplotype was not significantly associated with the risk of APO.

Conclusion: The obtained results suggest that the M2 haplotype may be a relevant risk factor for APO in this Portuguese women population sample. Thus, genotyping *ANXA5* gene for the M2 haplotype status seems to be a well oriented approach in the prognosis of women with unexplained APO and may guide adequate preventive therapeutic measures.

Key-words: Annexin A5, M2 haplotype, Pregnancy, Pregnancy complications, Risk factor.

BACKGROUND

It is well known that pregnancy is a physiological state of hypercoagulability characterized by a progressive increase in the procoagulant activity, which is maximal around term, while the anticoagulant and fibrinolytic activities are impaired, in order to protect the mother against the bleeding challenges associated with miscarriage and childbirth.[1] On the other hand, these hemostatic changes predispose both the mother and fetus, to adverse pregnancy outcomes (APO), such as recurrent pregnancy loss (RPL), premature birth (PB), intrauterine growth restriction (IUGR), small for gestational age (SGA), intrauterine fetal death (IUFD), gestational hypertension (GH), pre-eclampsia (PE), placental abruption and venous thromboembolism (VTE).[2] These APO collectively complicate up to 15% of pregnancies and are the leading causes of maternal and fetal morbimortality in developed countries,[3] which is in accordance with a Portuguese report, where coagulopathy, hemorrhagic disorders and gestational hypertension were the major maternal death causes (49.6%).[4]

These risks are higher in women with acquired or inherited hypercoagulation disorders that promote venous thrombosis. Amongst the non-inherited predisposing factors, some are considered strong/moderate [odds ratio (OR) >2] provoking factors, such as, major trauma, immobilization, auto-immune diseases, pregnancy or puerperium, hormone replacement therapy and oral contraception.[5] Regarding the inherited risk factors, they have a strong heritability, estimated around 50%.[6] Amongst these, there are classical factors, such as, rare deficiencies in natural coagulation inhibitors (antithrombin, protein C and protein S), Factor V Leiden (*FVL*) and prothrombin G20120A variants.[6] The association between these variants and APO have been widely studied.[7-9] However, while case-control studies suggested the existence of this association, large cohort studies failed to demonstrate it, or when established, it was weak and translated into a small absolute increased risk.[2,8,10,11]

Annexin A5, a member of the annexin family of calcium-dependent phospholipid binding proteins, functions as an inhibitor of coagulation due to its ability to bind to anionic phospholipids, and most avidly to phosphatidylserine, exposed on the surface of platelets,[12] thereby inhibiting aggregation and/or down-regulating the cell surface presentation of tissue factor.[13] Annexin A5, originally named "placental anticoagulant protein", is abundantly found on the apical surface of placental syncytiotrophoblast,[14] the interface between maternal and fetal circulation. Therefore, it has been proposed that Annexin A5 builds an antithrombotic shield, covering the exposed phosphatidylserine content, and thus contributing to the maintenance of blood fluidity in the placenta.[15,16] Most recently, it was found that Annexin A5 functions as an endogenous membrane repair protein essential for the placental integrity.[17] Bogdanova *et al.* identified the presence of two variant haplotypes in the promoter region of the *ANXA5* gene, in addition to the wild-type, termed M1 and M2 haplotypes. The M2 haplotype is a group of single nucleotide polymorphisms (SNP), which comprises of four consecutive nucleotide substitutions (-19G/A, 1A/C, 27T/C and 76G/A), while in the M1 haplotype only two of the four nucleotide substitutions were present (1A/C and 27T/C).[18] Reporter gene assays have demonstrated a reduction in the activity of the *ANXA5* promoter region when the M2 haplotype is present, which was 37-42% of the wild-type promoter.[18,19] The decrease was less pronounced for M1 (57-62% activity).[18] Since the syncytiotrophoblast, which carries the Annexin A5 protein at its surface, is of fetal origin and has both a half-maternal and half-paternal genome, it has been proved that reduced *ANXA5* expression was independent of M2 allele parental origin, making the M2 haplotype the first case of a hereditary factor causing pregnancy pathology by affecting embryonic-induced anticoagulation.[19,20] Moreover, it was shown that there is no genetic compensation through the wild-type allele in heterozygous placentas.[19] It was thus reasonable to speculate that carriage of the M2 haplotype leads to a reduced Annexin A5 cover of the exposed phosphatidylserine

surfaces, allowing coagulation factors to compete for phospholipid binding, and consequently leading to a prothrombotic placental environment, which in turn may induce the development of APO.[15] In fact, the M2 haplotype frequency was found to be significantly higher in German patients who had experienced RPL than among controls, and women with de M2 haplotype had about a twofold higher risk of RPL than non-carriers.[18] Subsequent studies confirmed the M2/*ANXA5* association with RPL among Italian, German, Bulgarian, UK white European, Japanese and Malay populations.[21-26] The M2 haplotype has also been associated with other APO such as GH and PE,[20,21,27,28] as well as a risk factor for IUGR[29] and SGA,[21,27,28,30] PB[28] and VTE.[31] However, no significant association was noted between the M1 haplotype and APO when compared with different control groups.[18,21-23,25,26,28,30]

The aims of the present study were i) to determine the frequency of the M2 haplotype in a Portuguese women population sample and ii) to assess the association between the M2 haplotype and the risk of APO, in order to address the unclear causes of APO and predictive value of the *ANXA5* genotyping in pregnancy.

MATERIALS AND METHODS

Sample Selection

The case group included 102 women (mean age of 33.5 years, range 17-41) referred to the Pregnancy and Thrombophilia Consultation Center incorporated by clinicians of both departments - Bissaya Barreto Maternity Hospital and Clinical Hematology department, from Centro Hospitalar e Universitário de Coimbra - in Portugal, between January 2012 and December 2015, with at least one previous episode of unexplained APO including RPL, early miscarriage, IUFD, IUGR, PE, eclampsia and hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome.

RPL was defined as the occurrence of three or more consecutive pregnancy losses before the 22nd week of gestation (WG), early miscarriage was defined as those occurred before the 12th WG,[32] while IUFD was defined as having a fetal loss after completing the 24th WG.[21,33] IUGR was defined as an estimated fetal weight below the 10th percentile for gestational age.[32,34] PE was defined as the occurrence of GH (blood pressure ≥140/90 mmHg in at least two different measurements, more than 6 hours apart, in a previously normotensive woman) and proteinuria (≥300 mg/24 h urine specimen), after the 20th WG.[32] Eclampsia was defined as the presence of new-onset seizures in a woman with PE, and HELLP syndrome was defined by a laboratory documentation of hemolysis with increased lactate dehydrogenase (>600 U/L), elevated liver enzymes (aspartate aminotransferase>70 U/L) and low platelet counts (<100000/μL).[32]

Detailed previous medical history was reviewed, including obstetric and reproductive history, as well as information on medical conditions known to increase the risk for thromboembolism (TE) (i.e. smoking habits, obesity, dyslipidemia, hypertension and diabetes *mellitus*) was obtained.

In order to maximize our ability to detect an association, we excluded APO cases with known risk factors such as: antiphospholipid syndrome, auto-immune diseases, deficiency of antithrombin, protein C or protein S, *FVL* or prothrombin G20120A variants, uterine anomalies and polycystic ovaries. These data were collected retrospectively from the patients' hospital records. Clinically relevant features of the cases are provided in Table 1.

We recruited a total of 84 Portuguese control women (mean age of 42 years, range 26-60) from our Center with at least one successful pregnancy, no documented history of infertility or APO and without TE risk factors (Portuguese controls). We also used as a control population two independent control groups previously recruited in other studies:[18,22,23,28,35] a cohort of fertile women (n=500) from the registry of the Institute of Human Genetics, University Clinic Münster *(*Münster controls),[18] and a female control sample (n=533) drafted from PopGen biobank at University Hospital Schesleswig-Holstein, Kiel (PopGen controls).[36] The Münster fertile control sample had a northern German origin and was selected along the same criteria as the fertile controls sample. The PopGen population controls were from northwest Germany and were unselected healthy subjects identified through official population registers.

Ethical Statement

Written Informed Consent for genetic studies was obtained prior to the participation in this study from each individual, in accordance with the Declaration of Helsinki. The Ethics Committee of the Faculty of Medicine of the University of Coimbra (Coimbra, Portugal) approved all research methodology.

DNA Extraction

Peripheral blood samples were collected from all participants and genomic DNA was extracted from EDTA whole blood by automatic isolation on iPrepTM instrument, using a gDNA Blood Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR), Sequencing and Genotyping

The promoter region, exon 1 and intronic boundaries of the *ANXA5*, which comprises of four consecutive nucleotide substitutions (-19G/A, 1A/C, 27T/C and 76G/A) (Figure 1-A), were amplified by PCR, using primers as described previously: P+ex1.F -CCG AGCCCTGGACAGCTCCCCA; P+ex1.R -GCCCCGCGACCACGCTCTCC TCT.[18] PCR reactions were done in a final volume of 25 µL, and the reaction mix contained the following: 2,5 µL of 10x Taq DNA Polymerase PCR buffer (20 mM Tris HCl (pH 8), 40 mM NaCl, 2 mM Na_2HPO_4, 0,1 mM EDTA, 1 mM DTT), 2 µL of dNTP's (2,5 mM) (Invitrogen, Ca, USA), 3.5 µL of forward and reverse primers (50 ng/µL) (Tib Molbiol, Berlin, Germany), 0,75 µL of $MgCl_2$ (50 mM), 1U of Platinium® Taq (Invitrogen, Ca, USA), 1,25 µL of 5% DMSO, 5 µL of betaine (1M), 1,5 µL of DNA (100 ng/µL), 9,05 µL of double-distilled H_2O. PCR amplifications were performed using a Tpersonal Thermal Cycler (Biometra, Göttingen, Germany). The PCR cycling conditions were 3 min at 94ºC (initial denaturation), followed by 33 cycles of 20 seconds at 94ºC (denaturation), 30 seconds at 61ºC (annealing) and 1 min at 72ºC (extension). In the last cycle, we performed a final extension at 72ºC for 5 min. PCR products were run on a 2% agarose gel electrophoresis. The DNA fragments were stained with SYBR® Safe DNA Gel Stain (Invitrogen, CA, USA), exposed to UV light and visualized using a transilluminator (Vilber Lourmat, Frane) (Figure 1- B).

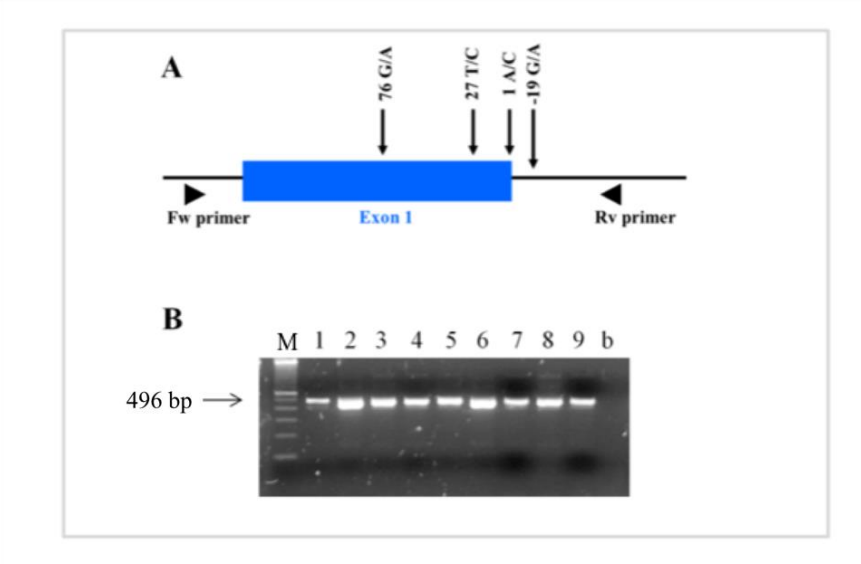

Figure 1. Location of the SNPs within the sequenced region of the *ANXA5* promoter and PCR analysis. (A) The SNPs positions were given according to the first *ANXA5* haplotypes detection study.[18] The exon 1 is indicated by a blue box; vertical arrows indicate the SNPs location; triangles indicate the position recognized by primers used to amplify the promoter region. (B) The photo shows an agarose gel electrophoresis of the 496 bp PCR products (lines 1-9). Line M indicates the 100 bp DNA size marker; line b indicates the negative control without DNA.

After the amplification, the Sanger sequencing was performed according to the manufacturer's instructions: products were purified using a cleanup reagent ExoSAP-IT™, the purified amplicons were then sequenced using BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA), and the sequencing products were run on a 3130 Genetic Analyzer (Applied Biosystems, CA, USA) through capillary electrophoresis. Traces were examined and compared using Seqscape® software v2.5. (Applied Biosystems, CA, USA) to available DNA sequences in the GenBank database[37] (Figure 2).

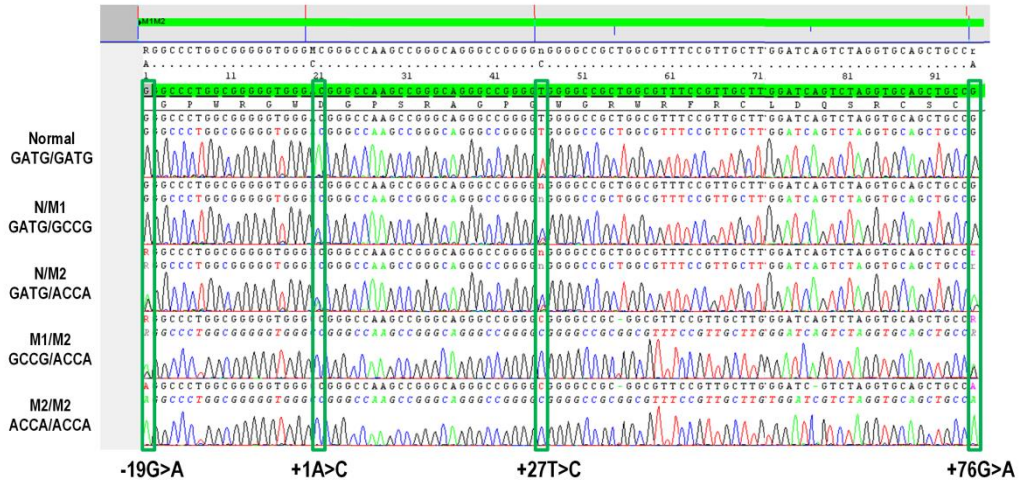

Figure 2. Electropherogram from Sanger sequencing. The figure shows the four different SNP's which comprises the *ANXA5* haplotypes (M1 and M2) and all the possible genotypes.

Statistical Analyses

The allele frequencies were estimated by simple counting and deviations from the Hardy-Weinberg equilibrium (HWE) were performed using a Monte Carlo Markov chain (MCMC) implementation of an exact test.[38] The distribution of the *ANXA5* haplotypes between the studied groups was analyzed using Fisher's exact test. OR with 95% confidence intervals (CI) were calculated. Tests were assumed significant whenever $p<0.05$. All these statistical analyses were performed using GraphPad Prism 6.02 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

RESULTS

Population characteristics

The case group included 102 women with previous history of unexplained APO. 101 women were Caucasian and one was African. They had a mean age of 33.5 years (17-41), a median number of pregnancies of 2 (0–7) and a median parity of 1 (0–2). Mean body mass index (BMI) was 24.62±3.98 Kg/m^2 (mean ±SD). Acquired risk factors for TE were distributed as following: smoking habit 13.7% (n=14), obesity 13.7% (n=14), dyslipidemia 11.7% (n=12), hypertension 2.9% (n=3) and only 1 had previous history of diabetes (Table 1).

Table 1: Demographic and clinical data of women with APO.

Characteristics	**Cases (n=102)**
Demographic data	
Age, mean (range)	33.5 (17-41)
Number of pregnancies, median (range)	2 (0-7)
Parity, median (range)	1 (0-2)
Acquired TE risk factors, n (%)	
Smoking habit	14 (13.7%)
Obesity	14 (13.7%)
Dyslipidemia	12 (11.7%)
Hypertension	3 (2.9%)
Diabetes *mellitus*	1 (0.9%)

TE – Thromboembolism; n – Number.

The diverse APO summarized in Table 2 evidence that almost half of the pregnant women showed pregnancy-related hypertensive disorders, and 48 had experienced a median of 3 pregnancy losses (range 1-5), with a total of 108 pregnancy losses, 91% in the first trimester. We highlight that some women had more than one subtype of APO.

Table 2: Characterization of APO in the case group.

Subtype of APO	**n *(%)***
Pregnancy-related hypertensive disorders *(PE/HELLP syndrome/eclampsia)*	50 (49.02)
Pregnancy losses *Early miscarriages (<12 WG)* - 37 (77.08%) *RPL (≥3)* - 24 (50%)	48 (47.05)
IUGR	28 (27.45)
IUFD (27-39 WG)	10 (9.8)

PE – Pre-eclampsia; HELLP – Hemolysis, elevated liver enzymes, and low platelets; RLP – Recurrent pregnancy loss; WG – Week of gestation; IUGR – Intrauterine growth restriction; IUFD – Intrauterine fetal death; n – Number.

Association of the *ANXA5* M2 haplotype with APO

When comparing the study case group with the Portuguese control group, the allele frequency of the M2 haplotype was found to be substantially higher in APO cases (0.167) than among Portuguese controls (0.083) (Table 3). Consequently, a relative risk of 1.32 for APO was observed in the M2 carriers *versus* non-carriers (OR 2.15, 95% CI 1.13-4.30; p=0.0280), when compared with the Portuguese controls (Table 4). The Portuguese control group and cases group were in HWE for the *ANXA5* haplotypes (MCMC p=0.797 and p=0.857, respectively). The frequency of the M2 allele was also higher in the APO cases (0.167) in comparison to the Münster controls (0.051) and the PopGen controls (0.082). The M2 haplotype contributes to a relative risk of 2.36 for APO (OR 3.27, 95% CI 2.06-5.15; p<0.0001), when compared with the Münster controls, and contributes to a relative risk of 1.74 (OR 2.04, 95% CI 1.32-3.10; p<0.0015), when compared with the PopGen controls. In the Münster control group, a significant deviation from the HWE was observed (MCMC

p<0.0001), which was mainly owing to the excess of M2 homozygotes (10 observed *versus* 1.4 expected). The PopGen control group was in HWE for the ANXA5 haplotypes (MCMC p=0.409).

No association between carriage status for the M2 haplotype and the subtype of APO was identified. No other differences in age, BMI, TE risk factors were identified when comparing the M2 carriers *versus* non-carriers.

Genotype M1/M1 was absent in the APO cases group and in the Portuguese control group, thus no significant association of the M1 haplotype with APO was noted when comparing with different control groups.

Table 3: Genotype frequencies of *ANXA5* gene promoter haplotypes in APO cases group *versus* three different control groups.

Genotype	**Cases**		**Portuguese controls**		**Münster controls**		**PopGen controls**	
	Observed n (%)	*Expected*	*Observed n (%)*	*Expected*	*Observed n (%)*	*Expected*	*Observed n (%)*	*Expected*
N/N	58 (56.9)	58.1	52 (61.9)	53.4	356 (71.2)	343.6	415 (77.9)	413.3
N/M1	12 (11.8)	12.1	19 (22.6)	16.0	87 (17.4)	99.5	35 (6.6)	47.8
M1/M1	0 (0)	0.6	0 (0)	1.2	16 (3.2)	7.2	1 (0.2)	1.5
N/M2, M1/M2	30 (29.4)	28.4	12 (14.3)	12.8	31 (6.2)	48.4	77 (14.4)	69.0
M2/M2	2 (2.0)	2.8	1 (1.19)	0.6	10 (2)	1.4	5 (0.9)	1.4
Total	102	102	84	84	500	500	533	533
M2 carriage (%)	31.4		15,5		8.2		15.4	
M2 AF	0.167		0.083		0.051		0.082	

Expected – genotype frequency expected at the Hardy–Weinberg equilibrium; AF – allele frequency; n – Number.

Table 4: Association between APO and the M2 haplotype in comparison with the control groups.

Index category	Cases (n=102)	Cases (n=102)	Cases (n=102)
Reference category	Portuguese controls (n=84)	Münster controls (n=500)	PopGen controls (n=533)
RR	1.32	2.36	1.74
OR	2.15	3.27	2.04
95% CI	1.13-4.30	2.06-5.15	1.32-3.10
p value	0.0280	<0.0001	0.0015

RR – Relative risk; OR – Odds ratio; CI – Confidence interval; N – number.

DISCUSSION AND CONCLUSIONS

In this work we report a study of the M2 *ANXA5* promoter haplotype, previously found associated with APO in several different ethnic populations. Prior studies using controls with a German, Italian, Bulgarian and UK white European origin, with at least one successful pregnancy and no former history of APO, conveyed a M2 carriage rate of 15-17%,[18,21-23] which is in agreement with the frequency of 15.5% found in our control population sample of Portuguese origin. In contrast, a lower frequency of 11% has been observed in Japanese populations,[20,24] whereas in Northern European populations the reported carrier rate was about 23.4%-27%[39] and 35% in Malay populations.[25]

Portuguese women with history of APO had a higher M2 allele frequency compared to women with uncomplicated pregnancies with the same origin (0.167 *versus* 0.083) and also, when compared with two independent control groups previously recruited in other studies, the Münster controls group (0.051) and the PopGen controls (0.082) of German origin.[18,22,23,28,35] Both control groups, the Portuguese controls and the PopGen controls, were in HWE for the M2 haplotype. Thus, our selected control group could be viewed as a representative sample of the Portuguese population. However, a deviation from HWE was observed in the Münster controls like in previous studies, owing to an excess of M2 homozygotes. A possible explanation to this is the positive ascertainment bias resulting from the Münster controls group consisting entirely of women who had no history of APO, whereas APO usually occurs in up to 15% of women worldwide.[3,23] We have shown that carriers of the M2 have a higher APO risk than non-carriers, with an estimated OR close to 2 for M2 carriers when compared to controls, which strengthens the significant association of the M2 haplotype of *ANXA5* with APO, already demonstrated in European and Asian APO cohorts, with OR for the M2 carriers ranging between 1.4 and 3.1.[18,21-25,28,30,31] Although these findings were in disagreement with the Northern European population-based study, where a lower M2 carriage rate was detected

among the APO patients compared to the controls (15%-18% versus 23.4-27%).[39] However, it is important to bear in mind that the differences between studies may arise from different aspects, such as differences in selection criteria for APO subjects and controls, experimental setup or study design.[39]

The M2 haplotype frequency was very similar among early miscarriage, RPL, pregnancy-related hypertensive disorders, IUGR and IUFD, which was in agreement with the previously obtained results for these APO,[21,28] except for IUFD, which has not been significantly associated with the M2 haplotype.[21] In fact, it has been suggested that ANXA5 plays a more important role before de 15th WG,[21-23,25,26] which relates to a time in the course of pregnancy when embryonic implantation[21] and placental vascular remodeling happens[22], in contrast to the *FVL* and prothrombin G20120A variants reported to lead to adverse events after the 10th WG.[8,10,40]

The diagnostic protocol in Bissaya Barreto Maternity Hospital for women with history of unexplained APO, involves the screening of antiphospholipid antibodies and the more common inherited risk factors for thrombophilias (*FVL* and prothrombin G20120A variants), or others if there is a documented family history. However the screening of inherited risk factors for thrombophilias in women with history of unexplained APO, but without personal or family history of VTE, is controversial since large cohort studies failed to demonstrate a significant association between them and APO.[2,8,10,11] In contrast, we found a significant association between the M2 haplotype and APO, besides the fact that the M2 haplotype carriage rate in the Portuguese control sample was equal or about 3 times higher than the frequency of the *FVL* in the European population.[32,41] Thus, we consider that genetic testing for *ANXA5* haplotypes could be considered in the thrombophilic risk assessment algorithm of women who previously suffered from an unexplained APO, mainly if it occurs in early pregnancy stages. The usefulness of screen *ANXA5* haplotypes is even more supported by the

fact that there are biological reasons for supposing that M2 carriers may benefit from low-molecular-weight heparin treatment starting before a new clinical pregnancy is confirmed.[42] Recent studies conducted with couples with history of APO, rather than mothers alone, had demonstrated that paternal M2 carriage confers a similar relative risk for APO as maternal M2 carriage[20,22,23,25,35,43] so on the basis of the findings from these studies, screening for M2 carriage on both partners in APO couples should be considered.

Some limitations of the present study need to be taken into account when interpreting the results obtained. Indeed, the cases group included some women with known acquired risk factors for TE, which may have functioned as confounding factors, distorting the M2/*ANXA5* APO association studied. This was a small population-based study, with a small patient number for each APO. Even though the Hardy-Weinberg analysis did not reveal any evidence of population disequilibrium in the Portuguese controls and cases groups, the diminished total size sample does not allow the generalizability of the study results. The clinical data was retrospectively collected, thus there may be some confounding factors that were not possible to control within the study. To overcome these limitations, further studies prospectively conducted with a larger total sample size and for each APO, are required. Moreover, as already done in studies conducted in other countries, would be also interesting to verify the role of paternal M2 carriage and the relation of M2 carriage to the timing of APO in the Portuguese population, in order develop a thrombophilic risk assessment algorithm for couples with history of unexplained APO.

In conclusion, our results suggest that the M2 haplotype of the *ANXA5* gene may contribute to the risk of APO in Portuguese women carriers of this haplotype. Considering the estimated high general frequency of the M2 haplotype, the data collected emphasizes the usefulness of the incorporation of M2 carrier status screening in risk APO assessment models for women with history of unexplained APO.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest in this paper.

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REFERENCES

1. O'Riordan MN, Higgins JR. Haemostasis in normal and abnormal pregnancy. Best practice & research Clinical obstetrics & gynaecology. 2003;17(3):385-96.

2. Simcox LE, Ormesher L, Tower C, Greer IA. Thrombophilia and Pregnancy Complications. Int J Mol Sci. 2015;16(12):28418-28.

3. Greer IA, Aharon A, Brenner B, Gris JC. Coagulation and placenta-mediated complications. Rambam Maimonides medical journal. 2014;5(4):e0034.

4. Ventura, Maria, Gomes, Manuel. Mortes maternas em Portugal 2001-2007. Lisbon; 2009.

5. Heit JA. Epidemiology of venous thromboembolism. Nature reviews Cardiology. 2015;12(8):464-74.

6. Morange PE, Suchon P, Tregouet DA. Genetics of Venous Thrombosis: update in 2015. Thrombosis and haemostasis. 2015;114(5):910-9.

7. Lykke JA, Bare LA, Olsen J, Lagier R, Arellano AR, Tong C, et al. Thrombophilias and adverse pregnancy outcomes: results from the Danish National Birth Cohort. Journal of thrombosis and haemostasis : JTH. 2012;10(7):1320-5.

8. Rodger MA, Betancourt MT, Clark P, Lindqvist PG, Dizon-Townson D, Said J, et al. The association of factor V leiden and prothrombin gene mutation and placenta-mediated pregnancy complications: a systematic review and meta-analysis of prospective cohort studies. PLoS medicine. 2010;7(6):e1000292.

9. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. Lancet (London, England). 2003;361(9361):901-8.

10. Rodger MA, Walker MC, Smith GN, Wells PS, Ramsay T, Langlois NJ, et al. Is thrombophilia associated with placenta-mediated pregnancy complications? A prospective cohort study. Journal of thrombosis and haemostasis : JTH. 2014;12(4):469-78.

11. Bogdanova N, Markoff A. Hereditary thrombophilic risk factors for recurrent pregnancy loss. Journal of community genetics. 2010;1(2):47-53.

12. Thiagarajan P, Tait JF. Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. The Journal of biological chemistry. 1990;265(29):17420-3.

13. Ravassa S, Bennaghmouch A, Kenis H, Lindhout T, Hackeng T, Narula J, et al. Annexin A5 down-regulates surface expression of tissue factor: a novel mechanism of regulating the membrane receptor repertoir. The Journal of biological chemistry. 2005;280(7):6028-35.

14. Krikun G, Lockwood CJ, Wu XX, Zhou XD, Guller S, Calandri C, et al. The expression of the placental anticoagulant protein, annexin V, by villous trophoblasts: immunolocalization and in vitro regulation. Placenta. 1994;15(6):601-12.

15. Bogdanova N, Baleva M, Kremensky I, Markoff A. The annexin A5 protective shield model revisited: inherited carriage of the M2/*ANXA5* haplotype in placenta as a predisposing factor for the development of obstetric antiphospholipid antibodies. Lupus. 2012;21(7):796-8.

16. Rand JH. The annexinopathies: a new category of diseases. Biochimica et biophysica acta. 2000;1498(2-3):169-73.

17. Carmeille R, Degrelle SA, Plawinski L, Bouvet F, Gounou C, Evain-Brion D, et al. Annexin-A5 promotes membrane resealing in human trophoblasts. Biochim Biophys Acta. 2015;1853(9):2033-44.

18. Bogdanova N, Horst J, Chlystun M, Croucher PJ, Nebel A, Bohring A, et al. A common haplotype of the annexin A5 (*ANXA5*) gene promoter is associated with recurrent pregnancy loss. Human molecular genetics. 2007;16(5):573-8.

19. Markoff A, Gerdes S, Feldner S, Bogdanova N, Gerke V, Grandone E. Reduced allele specific annexin A5 mRNA levels in placentas carrying the M2/*ANXA5* allele. Placenta. 2010;31(10):937-40.

20. Ota S, Miyamura H, Nishizawa H, Inagaki H, Inagaki A, Inuzuka H, et al. Contribution of fetal *ANXA5* gene promoter polymorphisms to the onset of pre-eclampsia. Placenta. 2013;34(12):1202-10.

21. Tiscia G, Colaizzo D, Chinni E, Pisanelli D, Scianname N, Favuzzi G, et al. Haplotype M2 in the annexin A5 (*ANXA5*) gene and the occurrence of obstetric complications. Thromb Haemost. 2009;102(2):309-13.

22. Tuttelmann F, Ivanov P, Dietzel C, Sofroniou A, Tsvyatkovska TM, Komsa-Penkova RS, et al. Further insights into the role of the annexin A5 M2 haplotype as recurrent pregnancy loss factor, assessing timing of miscarriage and partner risk. Fertility and sterility. 2013;100(5):1321-5.

23. Demetriou C, Abu-Amero S, White S, Peskett E, Markoff A, Stanier P, et al. Investigation of the Annexin A5 M2 haplotype in 500 white European couples who have experienced recurrent spontaneous abortion. Reproductive biomedicine online. 2015;31(5):681-8.

24. Miyamura H, Nishizawa H, Ota S, Suzuki M, Inagaki A, Egusa H, et al. Polymorphisms in the annexin A5 gene promoter in Japanese women with recurrent pregnancy loss. Molecular human reproduction. 2011;17(7):447-52.

25. Ang KC, Kathirgamanathan S, Ch'ng ES, Lee YY, Roslani AL, Naidu B, et al. Genetic analysis of the M2/*ANXA5* haplotype as recurrent pregnancy loss predisposition in the Malay population. Journal of assisted reproduction and genetics. 2017;34(4):517-24.

26. Thean Hock T, Bogdanova N, Kai Cheen A, Kathirgamanathan S, Bin Abdullah R, Mohd Yusoff N, et al. M2/*ANXA5* haplotype as a predisposition factor in Malay women and

couples experiencing recurrent spontaneous abortion: a pilot study. Reproductive biomedicine online. 2015;30(4):434-9.

27. Chinni E, Tiscia GL, Colaizzo D, Vergura P, Margaglione M, Grandone E. Annexin V expression in human placenta is influenced by the carriership of the common haplotype M2. Fertility and sterility. 2009;91(3):940-2.

28. Rogenhofer N, Nienaber LRM, Amshoff LC, Bogdanova N, Petroff D, Wieacker P, et al. Assessment of M2/*ANXA5* haplotype as a risk factor in couples with placenta-mediated pregnancy complications. Journal of assisted reproduction and genetics. 2017.

29. Sifakis S, Soufla G, Koukoura O, Soulitzis N, Koutroulakis D, Maiz N, et al. Decreased annexin A5 mRNA placental expression in pregnancies complicated by fetal growth restriction. Thrombosis research. 2010;125(4):326-31.

30. Tiscia G, Colaizzo D, Favuzzi G, Vergura P, Martinelli P, Margaglione M, et al. The M2 haplotype in the *ANXA5* gene is an independent risk factor for idiopathic small-for-gestational age newborns. Molecular human reproduction. 2012;18(10):510-3.

31. Grandone E, Tiscia G, Colaizzo D, Chinni E, Pisanelli D, Bafunno V, et al. Role of the M2 haplotype within the annexin A5 gene in the occurrence of pregnancy-related venous thromboembolism. American journal of obstetrics and gynecology. 2010;203(5):461.e1-5.

32. Graça L, Araújo C, Bispo R, Borges A, Brazão R, Calhaz-Jorge C, et al. Medicina Materno-Fetal. 4 ed. Lisboa: LIDEL; 2010.

33. Siassakos D, Fox R, Draycott T, Winter, C. Late intrauterine fetal death and stillbirth. Royal College of Obstetricians and Gynaecologists. 2010.

34. Lausman A, Kingdom J. Intrauterine growth restriction: screening, diagnosis, and management. Journal of obstetrics and gynaecology Canada : JOGC = Journal d'obstetrique et gynecologie du Canada : JOGC. 2013;35(8):741-8.

35. Rogenhofer N, Engels L, Bogdanova N, Tuttelmann F, Markoff A, Thaler C. Paternal and maternal carriage of the annexin A5 M2 haplotype are equal risk factors for recurrent pregnancy loss: a pilot study. Fertility and sterility. 2012;98(2):383-8.

36. Krawczak M, Nikolaus S, von Eberstein H, Croucher PJ, El Mokhtari NE, Schreiber S. PopGen: population-based recruitment of patients and controls for the analysis of complex genotype-phenotype relationships. Community genetics. 2006;9(1):55-61.

37. National Center for Biotechnology Information. *ANXA5* annexin A5 [H*omo sapiens* (human)] [Internet] U.S.: National Center for Biotechnology Information; 2018 March [cited 2018 March 12]. Available from: https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=308.

38. Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics. 1992;48(2):361-72.

39. Nagirnaja L, Nommemees D, Rull K, Christiansen OB, Nielsen HS, Laan M. Annexin A5 Promoter Haplotype M2 Is Not a Risk Factor for Recurrent Pregnancy Loss in Northern Europe. PloS one. 2015;10(7):e0131606.

40. Roque H, Paidas MJ, Funai EF, Kuczynski E, Lockwood CJ. Maternal thrombophilias are not associated with early pregnancy loss. Thrombosis and haemostasis. 2004;91(2):290-5.

41. Jadaon MM. Epidemiology of activated protein C resistance and factor v leiden mutation in the mediterranean region. Mediterranean journal of hematology and infectious diseases. 2011;3:e2011037.

42. Rogenhofer N, Markoff A, Wagner A, Klein HG, Petroff D, Schleussner E, et al. Lessons From the EThIGII Trial: Proper Putative Benefit Assessment of Low-Molecular-Weight Heparin Treatment in M2/*ANXA5* Haplotype Carriers. Clinical and applied thrombosis/hemostasis : official journal of the International Academy of Clinical and Applied Thrombosis/Hemostasis. 2017;23(1):27-33.

43. Fishel S, Patel R, Lytollis A, Robinson J, Smedley M, Smith P, et al. Multicentre study of the clinical relevance of screening IVF patients for carrier status of the annexin A5 M2 haplotype. Reprod Biomed Online. 2014;29(1):80-7.