Reproductive BioMedicine Online (2014) xxx, xxx–xxx

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ARTICLE

Multicentre study of the clinical relevance of screening IVF patients for carrier status of the annexin A5 M2 haplotype

Simon Fishel [a,*], Rashmi Patel [b], Alison Lytollis [b], Jeanette Robinson [c], Mary Smedley [e], Paula Smith [d], Craig Cameron [a], Simon Thornton [a], Ken Dowell [a], Glenn Atkinson [b], Adel Shaker [d], Philip Lowe [e], Rahnuma Kazem [c], Sandra Brett [f], Anna Fox [f]

[a] *CARE Fertility Group, John Webster House, 6 Lawrence Drive, Nottingham Business Park, Nottingham NG8 6PZ, United Kingdom;* [b] *CARE Manchester, 108–112 Daisy Bank Road, Victoria Park, Manchester M14 5QH, United Kingdom;* [c] *CARE Northampton, 67 The Avenue, Cliftonville, Northampton NN1 5BT, United Kingdom;* [d] *CARE Sheffield, 24–26 Glen Road, Sheffield S7 1RA, United Kingdom;* [e] *CARE Nottingham, John Webster House, 6 Lawrence Drive, Nottingham Business Park, Nottingham NG8 6PZ, United Kingdom;* [f] *CARE Dublin, Beacon CARE Fertility, Beacon Court, Sandyford, Dublin 18, Ireland*

[*] Corresponding author. *E-mail address:* simon.fishel@carefertility.com (S Fishel).

Simon Fishel is CEO of CARE Fertility Group. He commenced research at Cambridge University with Bob Edwards in 1975. In 1980, he joined Patrick Steptoe and Bob at the start of Bourn Hall and was also awarded the prestigious Beit Memorial Fellowship and Research Fellowship at Churchill College. He has published more than 200 papers and three books and has received many international awards. Founder of the world's first degree course in IVF in 1992, he was awarded to *an Hominem* Personal Professional Chair in 1997 and 2009 received Liverpool John Moores University highest honour of University Fellow for 'outstanding contribution to science and to humanity'.

Abstract Thrombophilia and impaired placental vasculature are a major cause of adverse pregnancy outcome. In 2007, a new hereditary factor for obstetric complications and recurrent pregnancy loss (RPL) was identified as a sequence variation in the core promoter of the annexin A5 gene, *ANXA5*, called the M2 haplotype. M2 carriership has been demonstrated in couples with recurrent miscarriage and its origin is embryonic rather than specifically maternal, confirmed by subsequent papers. The M2 haplotype is the first report of a hereditary factor related to pregnancy pathology caused by embryonic-induced anticoagulation. It has been demonstrated that couples with RPL had equal and significantly increased M2 carriership and that maternal and paternal carriership confers equal risk. Given its importance for patients with RPL, and potentially implantation failure, this study assessed the incidence of carrier status for the M2 ANXA5 haplotype in both the male and female of couples attending five CARE IVF centres. In 314 patients (157 couples), 44% of couples (one or both partners), 24% of females, 26% of males and 37% of couples with unexplained infertility were M2 carriers. This high incidence has provoked further urgent studies on specific patient populations and on the value of post embryo-transfer therapy.

KEYWORDS: ANXA5, infertility, miscarriage, recurrent pregnancy loss, thrombophilia

http://dx.doi.org/10.1016/j.rbmo.2014.03.019
1472-6483/

Please cite this article in press as: Fishel, S et al. Multicentre study of the clinical relevance of screening IVF patients for carrier status of the annexin A5 M2 haplotype. Reproductive BioMedicine Online (2014), http://dx.doi.org/10.1016/j.rbmo.2014.03.019

Introduction

Thrombophilias are a major cause of adverse pregnancy outcome (Markoff et al., 2011) and there is increasing evidence to suggest that impairment of placental vasculature increases the risk of recurrent pregnancy loss (RPL), intrauterine fetal death, gestational hypertension, pre-eclampsia, venous thromboembolism, fetal growth restriction and small-for-gestational-age (SGA) newborns (Chinni et al., 2009; Grandone and Margaglione, 2003; Grandone et al., 2010; Tiscia et al., 2009, 2012; Younis and Samueloff, 2003).

Normal pregnancy is an acquired hypercoagulable state and therefore women with a genetic predisposition to thrombophilia may develop clinical signs of coagulation defects *de novo* during pregnancy or during the post-partum period (Chunilal and Bates, 2009; Rey et al., 2003). The predisposing role of hereditary thrombophilic factors has been reported in several clinical studies (Rodger et al., 2010), and historically, in the majority of patients, the hereditary factor has been Factor V Leiden or prothrombin (Bick, 2000). However, in 2007 a new hereditary factor for RPL and additional thrombophilia-related obstetric complications was identified (Bogdanova et al., 2007; Chinni et al., 2010). This defect, termed the M2 haplotype, is a sequence variation in the core promoter of the annexin A5 gene, *ANXA5*. It consists of four consecutive nucleotide substitutions in the core promoter and results in reduced expression of *ANXA5* in placentas from M2 haplotype carriers when compared with noncarriers.

Annexin A5 is a member of the annexin protein family which share the properties of binding calcium and phospholipids. It is distributed abundantly and ubiquitously, mostly in the kidney, liver and placenta (Morgan et al., 1998). It is most abundant on the apical membranes of placental syncytiotrophoblasts, the interface between maternal and fetal circulation. ANXA5 was originally named 'placental anticoagulant protein'. It has been extensively studied both *in vivo* and *in vitro* (Romisch et al., 1991; Thiagarajan and Tait, 1990). It has potent anticoagulant properties associated with its phospholipid-binding activity and is one of the few annexins to be found extracellularly (Gerke et al., 2005). The ability of ANXA5 to form two-dimensional aggregates on cell membranes has led to the development of the ANXA5 'protective shield' model that postulates that ANXA5 shields phospholipids at this site from availability for coagulation reactions and thus contributes to the maintenance of blood fluidity in the placenta. Annexin 5 is deficient in placentas of patients with antiphospholipid syndrome (APS), and antiphospholipid antibody-mediated reduction of annexin 5 on vascular endothelium may also contribute to systemic thrombosis (Rand, 1999). Bogdanova et al. (2012) revisited the annexin A5 protective shield model and reported that preliminary genotyping analysis of a cohort of 30 lupus anticoagulant-positive patients with obstetric APS revealed that 11 out of the 30 were M2 carriers and this would correspond to a 3-fold relative risk to develop obstetric antiphospholipid antibodies.

Markoff et al. (2010) reported not only that decreased *ANXA5* expression in M2 ANXA5 placentas (including those from women with fetal growth restriction and or pre-eclampsia) is the result of carriage of the M2 haplotype, but that this occurred regardless of parental origin, with obvious consequences for embryonic- rather than wholly maternal-induced risk. They observed that the normal *ANXA5* allele does not compensate for observed M2 allele-specific decreased mRNA concentrations and made the significant finding that, unlike Factor V Leiden and prothrombin where paternal thrombophilic genes are not associated with RPL (Toth et al., 2008), the M2 ANXA5 allele acts via the embryo.

The work of Markoff et al. (2010) led to a pilot study of 30 RPL couples where all other causes of RPL had been excluded (including inherited thrombophilias and APS; Rogenhofer et al., 2012). The study confirmed that male and females in these RPL couples had equal and significantly increased M2 carriership when compared with control populations. The authors concluded that paternal and maternal carriage of the M2 ANXA5 haplotype associate with RPL and confer equal risks. They further reported that M2 ANXA5 is the first instance of a hereditary factor causing pregnancy pathology by affecting embryonic anticoagulation (Rogenhofer et al., 2012).

Tüttelmann et al. (2012) undertook a risk stratification study of an IVF cohort of 695 German women compared with 500 fertile female controls and 533 population controls. The carriers of the M2 haplotype had a higher relative risk (1.4) of belonging to the IVF group in comparison with fertile female controls and a higher relative risk (1.2) compared with population controls. This overall risk was due to a subgroup of women with previous pregnancy losses and for this group the relative risks were 3.8 and 2.3, respectively. The authors reported that there was no association with biochemical pregnancy loss, implantation rate, ovarian reserve, hormone status, number and quality of egg cells and general embryonic development. However, there was no male partner genotyping data available.

Ueki et al. (2012) in their knockout murine model found significant reductions both in litter size and fetal weight in *ANXA5*-null mice (*ANXA5*-KO) and thus demonstrated that the maternal supply of ANXA5 to the circulation was crucial for maintaining normal pregnancy. They further observed that cross-breeding of *ANXA5*-KO and wild-type mice showed that only litters bred using *ANXA5*-KO females had reduced numbers of pups. They also demonstrated that administration of heparin on pregnancy days 12, 14 and 16 to *ANXA5*-KO mice significantly increased litter size.

Evidence to date suggests that maternal and paternal carriage of the M2 ANXA5 haplotype confers equal risks and acts via the embryo, causing pregnancy pathology by affecting embryonic coagulation unlike the other well-characterized thrombophilias. Additionally there is a high incidence of carrier status in both control and subfertile populations, including patients with RPL. In the context of the IVF population, it is essential to understand potential endometrial and/or blood-borne factors responsible for IVF failures. Thus, this work performed a multicentre study of the incidence of carrier status of the M2 ANXA5 haplotype in both partners attending IVF clinics and to ascertain the potential relevance to pretreatment screening.

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Materials and methods

Study population

Patients were recruited between March 2012 and February 2013 from patients attending five CARE fertility clinics. Informed consent was obtained from all patients. During this period, 314 patients (157 couples) presented with at least one previously failed IVF cycle (mean 1.9 IVF and 0.2 intrauterine insemination). A detailed clinical history was obtained, and the genotyping for presence or absence of carriage of the M2 ANXA5 haplotype formed part of the diagnostic investigations for infertility.

The mean (range) age of women was 36.3 years (23–49 years) and that of their partners 38.6 years (23–64 years). The mean body mass index of the women was 25.5 kg/m^2 (19–40.5 kg/m^2) and that of their partners was 33.7 kg/m^2 (21–36 kg/m^2). The selection of patients for screening was based on their prior history and the patients' willingness to be tested, following the detailed nature of the study being provided to them at consultation. Women were screened for antiphospholipid antibodies.

With regard to their infertility status, the majority of the male population had oligospermia (48%), astheno/oligoasthenospermia (27%) or azoospermia (13%). These varied according to carrier status with an incidence in the noncarriers of 41%, 26% and 11%, respectively, and for the carriers 35%, 12%, and 12%, respectively. With regard to women, the most prevalent causes of infertility were unexplained (27%), poor ovarian reserve (17%), polycystic ovary syndrome (PCOS; 11%) and endometriosis (6%); according to carrier status, incidence in the noncarriers was 30%, 16%, 16% and 3%, respectively, and for the carriers 26%, 9%, 18%, and 8%, respectively.

The majority of patients were white British (77% men and 75% women) and Indian/Pakistani (8%) the remainder being of diverse ethnicity. As a whole, this cohort is representative of the demography of the UK and Eire. DNA was collected from couples either by a blood sample (the first cohort) or buccal cell analysis on specific collection paper (the remaining cohort). Extensive laboratory tests were undertaken to ensure the transfer to buccal cell collection caused no deterioration in the quality of the DNA. DNA was extracted from white blood cells using QIAmp DNA Blood Mini kit (Qiagen, Hilden, Germany) or from elution from the collecting paper. PCR reactions were carried out on 100 ng genomic DNA isolated from blood samples using the QIAmp Blood Mini kit or from purified collecting paper punches. Amplification was carried out using Biotaq Polymerase (Bioline Reagents, London, UK) in a volume of 25 μl containing 10× NH_4 reaction buffer: 160 mmol/l $(NH_4)_2SO_4$, 670 mmol/l Tris–HCl (pH 8.8), 50 mmol/l $MgCl_2$ (final concentration 1.5 mmol/l), 50 pmol/l forward and reverse primers, 200 mmol/l dNTP, PolyMate Additive (Bioline) and 2.5 U Biotaq polymerase. The cycling conditions were 94°C for 45 s, 30 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 1 min and a final extension step of 7 min. Amplification products were purified using standard column purification methods (Zymo ZR-96DNA Clean and Concentrator kit; Zymo Research, Irvine, CA, USA). Purified amplicons were sequenced using ABI BigDye Terminator chemistry version 3.1 using standard conditions and electrophoresis on an ABI 3730xl DNA analyser and traces were analysed and genotyped using ABI Seqscape version 2.5. (Applied Biosystems, Foster City, CA, USA). The presence of the M2 haplotype (a set of four consecutive nucleotide substitutions in the *ANXA5* promoter: 19G>A (rs112782763), +1A>C (rs28717001), 27T>C (rs28651243) and 76G>A (rs113588187)) was investigated. When only two of the four variants (+1A>C, 27T>C) were present, the haplotype was defined as M1.

Quality control

All genotype calls were made using Seqscape software (Applied Biosystems) with a 25% mixed-base calling threshold. Seqscape was programmed to analyse nucleotide variations at four specific bases, as described in the literature. Results were generated in the form of a mutations report that detailed mutations across the region of interest. Report production was carried out by means of an in-house laboratory information management system, which was programmed to only allow certain combinations of mutation. Any sample that gave an unexpected result was flagged by the system and checked by an operator before repeating the test on a fresh sample.

Genotyping and statistical analysis

Patients who were heterozygous or homozygous carriers of the M2 ANXA5 haplotype were recorded as affected heterozygous or homozygous carriers. Tests for deviations from Hardy–Weinberg equilibrium (HWE) were performed using the method of Guo and Thompson, 1992 (also used by Bogdanova et al., 2007 and Rogenhofer et al., 2012). This test was performed within the male and female groups and overall.

This work also tested all individuals not classified as white British or white Irish to see whether this affected the results. To check whether the significant deviation from HWE observed in the female subgroup could be attributed to chance, 155 individuals were subsampled at random from the entire set (men and women combined) and the *P*-value for deviation from HWE was estimated using the same method. This procedure was performed 1000 times, and of these, only three *P*-values were more extreme than those observed for the all-female group, thus suggesting that the deviation from HWE in women was real and not attributable to chance.

The controls used for comparison were those used by Rogenhofer et al. (2012) from a population control sample drafted from the PopGen biobank at University Clinic Schleswig–Holstein Kiel (n = 533). PopGen population controls were from northwest Germany and were healthy subjects identified through official population registers (Krawczak et al., 2006). The sample used in this study comprised approximately equal numbers of men and women distributed among three age groups (18–30, 30–50 and 50–80 years). The cohort of Muenster fertile controls were anonymized individuals from the institute's registry (Rogenhofer et al., 2012), all with successful pregnancies and no documented history of RPL.

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Results

Six patients were not genotyped: four men (two azoospermia, one oligospermia and one aged 65) and two women (one early menopause and one menopause). Of the remaining 314 patients (157 couples), the overall M2 carriage rate was 25% ($n = 78$) and was of similar incidence in women (24%, $n = 37$) and men (27%, $n = 41$). However, in couples, there was a high incidence of M2 carriage (defined as one or both partners being M2 carriers or homozygotes; 44%, $n = 69$). None of these patients tested positive for APS.

Among these carrier couples were small subsets of couples in which one partner was a noncarrier and one was homozygous (4%, $n = 7$), both partners were carriers (4%, $n = 6$), or one partner was a carrier and one was homozygous (2%, $n = 3$). There were nine homozygotic women and one homozygotic man. The genotype frequencies of *ANXA5* promoter haplotypes observed in this study and expected under HWE in men and women are presented in Table 1. There was no significant deviation from HWE in men, but there was significant deviation from HWE in women ($P = 0.005$). Restricting the analysis to only those individuals classified as white British or white Irish gave similar results (data not shown).

The genotype frequencies of *ANXA5* promoter haplotypes in the current study are compared with two control groups in Table 1. The abundance of the M2 haplotype was enriched in both men and women compared with both the Muenster controls (women) and the PopGen controls (men and women).

The IVF female patients were not in HWE ($P = 0.0052$) owing to the excess of M2 heterozygotes but particularly M2 homozygotes (9 observed versus 3.4 expected). To check whether the significant deviation from HWE observed in women could be attributed to chance, this work subsampled 155 individuals at random from the entire set (men and women combined) and estimated the *P*-value for deviation from HWE using the same method and recorded the *P*-value. We performed this procedure 1000 times, and of these only three *P*-values recorded were more extreme than those observed for the all-female group, thus suggesting that the deviation from HWE in women was real and not attributable to chance.

The patients' previous IVF, intrauterine insemination and pregnancy histories are shown in Table 2. The numbers of previous failed IVF cycles were highest in couples who had one homozygotic partner and one noncarrier (mean 3.1 previous IVF) and in couples where the male partner was a carrier (mean 2.1 previous IVF).

Previous live births were very low in all carrier/homozygous groups (range 0–4) and a slightly higher incidence was observed in noncarrier couples ($n = 13$). The patients' most recently reported miscarriage in carrier couples occurred at a mean of 10.1 weeks (range 5–23 weeks) in the 17 miscarriages where date of loss was reported. In noncarrier couples, miscarriage ($n = 53$) occurred at a mean of 9 weeks (range 5–26) in 25/53 miscarriages.

Male infertility and M2 frequency

Overall, 63 of 157 men (40%) had associated infertility factors. Carriage incidence in this group was 27% ($n = 17$). Overall, oligospermia was the most frequent finding (40%, 25 infertile men) followed by oligoasthenoteratozoospermia (13%, eight infertile men). However there is unlikely to be any relationship or causal linkage between the existence of the M2 haplotype and male infertility.

Of 157 women, 93 (59%) had a diagnosis of infertility other than unexplained or male factor. Additionally, 25 of the 93 women with a diagnosis (27%) were also found to be M2 carriers. Unexplained, poor ovarian reserve/ovulation failure often linked to age plus PCOS were the most frequently cited causes of infertility in both groups. However, male infertility was cited as the primary cause of infertility

Table 1 Observed and HWE expected genotype distribution in men and women in the current study with results from test of departure from HWE and in two control groups.

Genotype	*This study*				*Muenster controls*[a]		*PopGen controls*[a]	
	Men (n = 153)		*Women (n = 155)*		*Women (n = 500)*		*Men and women (n = 533)*	
	Observed	*Expected*	*Observed*	*Expected*	*Observed*	*Expected*	*Observed*	*Expected*
N/N	88 (57.5)	91.8	97 (62.6)	94.5	356 (71.2)	343.6	415 (77.9)	413.3
N/M1	24 (15.7)	20.9	21 (13.5)	17.2	87 (17.4)	99.5	35 (6.6)	47.8
M1/M1	0 (0)	1.2	0 (0)	0.8	16 (3.2)	7.2	1 (0.2)	1.5
N/M2 or M1/M2	40 (26.1)	36.2	28 (18.1)	39.2	31 (6.2)	48.4	77 (14.4)	69.0
M2/M2	1 (0.7)	2.9	9 (5.8)	3.4	10 (2.0)	1.4	5 (0.9)	1.4
Total haplotypes	306		310					
Estimated *P*-value	NS		0.00517					
P-value standard error	0.0001		<0.0001					

Expected values correspond to those expected under HWE; test of departure from HWE computed via Markov Chain Monte Carlo.
Haplotypes in the *ANZXA5* promoter: N = normal/wild type; M1 = 1A>C and 27T>C (six heterozygotes); M2 = 19G>A, 1A>C, 27T>C and 76G>A (16 heterozygotes).
HWE = Hardy–Weinberg equilibrium; NS = not significant.
[a]Previously recruited control groups of healthy population controls, data adapted from Rogenhofer et al. (2012): a cohort of fertile women from the registry of the Institute of Human Genetics University Clinic Muenster selected along the same criteria as the Munich fertile control sample and population controls from northwest Germany in the PopGen biobank at University Clinic Schleswig–Holstein Kiel.

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Table 2 Couples' previous IVF and intrauterine insemination cycles and pregnancy history.

	Carrier couples (n = 69)						*Noncarrier couples (n = 88)*
	Total	*Both carriers (n = 6)*	*Male carrier only (n = 31)*	*Female carrier only (n = 22)*	*One homozygote, one carrier (n = 3)*	*One homozygote only (six women, one man) (n = 7)*	
IVF cycles	191 (2.0)	17 (1.9)	66 (2.1)	36 (1.6)	5 (1.7)	22 (3.1)	153 (1.9)
IUI	23	5	3	12 (all same couple)	0	3	12 (0.2)
Pregnancies	63 (0.9)	5 (0.6)	33 (1.1)	17 (0.8)	3	9 (1.3) (one woman had four)	83 (0.9)
Total miscarriages	50 (0.7)	3	26 (0.8)	15 (0.7)	3	6 (one woman had four, one woman had two)	53 (0.6)
Live births	4	1	1	2	0	0	13
Time of last miscarriage (gestational weeks)[a]	10.1 (5–23) (*n* = 17)	6 (7–15)	9.6 (7–22) (*n* = 11)	23	7, 15, very early	5, 9	9 (5–26) (*n* = 25)

Values are *n* (mean), *n*, mean (range) or means.
[a] *n* = number of women for which data were available.

in 21% of noncarrier couples but noted in only one of 37 women who carried the M2 haplotype. Six out of 17 PCOS cases (35%) were also carriers.

Unexplained infertility and M2 frequency

Overall, 104 patients (33%) presented as having no explanation for infertility. Of these, 38 patients (37%) were identified as M2 carriers: 25 men (24%) and 13 women (13%). There were nine homozygotic women (6% of all women) There was also one homozygotic man aged 49 for whom the couple had no other known diagnosis although his partner had had two IVF cycles which had resulted in miscarriage.

Discussion

Carriership of the M2 ANXA5 haplotype in this cohort of patient couples was 44%, representing a very high incidence. Furthermore it was present in 27% of male infertility patients, 27% of female infertility patients and in 37% of patients with previously unexplained reasons for infertility. Additionally, it was present in 35% of PCOS patients, which has been reported by Rogenhofer et al. (2013) who note that the M2 ANXA5 haplotype is independently associated with RPL in PCOS patients. Of the patients who carried the M2 haplotype in the present study, none tested positive for APS. Bogdanova et al. (2010), in a cohort of 30 lupus anticoagulant-positive patients with obstetric APS, reported 11 as M2 carriers; and Markoff et al. (2011) reported that 'preliminary results (Cherkelova et al., 2010, unpublished observations) suggest about a twofold higher incidence of M2/ANXA5 in SLE and aPL [systemic lupus erythematosus and antiphospholipid antibody] patients with obstetric complications'. It is possible that the observed variance is a result of the infertility cohort in this study being a different group of patients than those with 'obstetric complications'.

The genotype distribution in men and women was similar to that reported by Rogenhofer et al. (2012) where a RPL cohort was compared with three different control groups. Genotype M1/M1 was absent in the RPL cohort and rare in controls. Genotype M1/M2 was not observed in the RPL cohort and seen only in a total of eight from control groups and in only four patients in the current IVF cohort. However, the incidence of M2 homozygotic women was elevated at 6% in this cohort and one M2 homozygotic man was recorded. Female homozygote frequency was 3-times higher than that reported from other control groups and double that of RPL women (Rogenhofer et al., 2012).

The use of the PopGen and Muenster controls is justified as Nelis et al. (2009) concluded that four areas could be identified — namely (i) central and western Europe; (ii) the Baltic countries, Poland and Western Russia; (iii) Finland; and (iv) Italy — which, if not corrected for, the inter-population differences would affect the significance of disease gene associations. The incidence in controls from published studies from Germany, southern Italy and Bulgaria — representatives of three of these regions — have all shown consistency in the M2 haplotype frequency. The majority of the IVF patients were white British (77% men, 75% women), which correspond to the central and western Europe region. This study had no Finnish patients and analysis with and without the subset of Indian/Pakistani and others still showed the significant departure from HWE in women but not in men, mainly due to the abundance of M2 homozygotes.

In terms of ethnicity, this study found M2 carriers in a wide range of ethnicities, including Jewish, Turkish and Middle Eastern in addition to Indian and Pakistani patients. The possible differences in carriage rate and clinical effects in

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these ethnicities warrants further investigation since there may be significant differences in incidence and pathology. The incidence in Caucasian populations of Europe is well established (Markoff et al., 2011) and Miyamura et al. (2011) reported that carriage of the haplotype resulted in risks for RPL in the Japanese population similar to that observed in the populations of central Europe; however, the incidence of RPL was lower in Japan (5.5 versus 15%). Thus further study of different ethnicities other than white Europeans and Japanese is warranted.

M2 is a hereditary factor that causes various pathologies during pregnancy by adversely affecting embryonic anticoagulation (Markoff et al., 2010; Rogenhofer et al., 2012). A very recent paper on RPL in German and Bulgarian patients by Tüttelmann et al. (2013) provides further evidence that paternal carriage contributes similar risk to that of maternal carriage, as reported by Markoff et al. (2010) who showed nonpreferentially and equally reduced *ANXA5* mRNA expression in chorionic placenta carrying maternal or paternal alleles.

Although Ueki et al. (2012) could only demonstrate a maternal influence on pregnancy viability from their *ANXA5*-KO murine model, the human placental study of Markoff et al. (2010), which has been further confirmed by Rogenhofer et al. (2012), supports earlier work on the embryonic influence on placental function (Rand et al., 1997). Rand et al. (1997) demonstrated that the fetal component has a characteristically evident pattern of *ANXA5* expression on the apical surface of the syncytiotrophoblast layer lining the chorionic villi. Furthermore, as concluded in Malassiné et al. (2003), there should be caution in extrapolating data from experimental models, particularly in studies of the pathophysiology of complications of pregnancy with a placental origin.

Any impairment of embryonic coagulation is of particular importance in IVF practice since the focus is often on managing and providing for healthy gametes and embryos, selecting for optimal embryo viability and ensuring a healthy uterus able to sustain a pregnancy. However, although the largest single cause of miscarriage is believed to be the aneuploid embryo, other factors are clearly of significance, especially in RPL cases, where it can remain an issue even after the transfer of euploid embryos following IVF. The relatively recently discovered genetic factor M2 ANXA5 is alone in influencing placental function via adverse effects on embryonic anticoagulation and, if undetected, could negate the considerable work and cost incurred to establish a healthy pregnancy via IVF. In this study, there were a significant number of patients, equally distributed between men and women, where M2 carriage was either an additional factor to those already determined or it was present in a significant number of patients with no other infertility diagnosis. There is a growing body of evidence of the risks of carriage of the M2 ANXA5 haplotype to maternal health (RPL, venous thromboembolism, pre-eclampsia, gestational hypertension, APS; Bogdanova et al., 2012; Grandone et al., 2010; Tiscia et al., 2009). Bogdanova et al. (2012) postulated that carriage of the M2/ANXA 5 haplotype leads to reduced ANXA5 cover of exposed phosphatidylserine surfaces, and this reduced shielding would allow coagulation factors to compete for phospholipid binding. Secondly, there would be greater exposure of phospholipid antigenic factors, that would then lead to antiphospholipid antibody development, which in turn would further disrupt the ANXA5 shield. Sifakis et al. (2010) demonstrated significant differences in mRNA expression between normal and fetal growth restriction pregnancies but no difference in ANXA5 protein concentration. However, the authors did not genotype their samples for M2 ANXA5.

A significantly higher prevalence of the M2 haplotype in a group of women with a history of idiopathic SGA babies has been reported (Tiscia et al., 2012), demonstrating a 2-fold higher risk of giving birth to a SGA newborn. All the M2 homozygotes in this study (there were no homozygotes in the controls) had a history of a severe SGA (below the 3rd percentile).

Recently, a large cross-sectional study (Henriksson et al., 2013) was determined the incidence of pulmonary and venous thromboembolism in pregnancies after IVF and reported an increased risk of thromboembolism and, importantly, pulmonary embolism. The risk of venous thromboembolism increased during all trimesters, particularly during the first trimester, as did the risk of pulmonary embolism. The study concluded that 'efforts should focus on the identification of women at risk of thromboembolism, with prophylactic anticoagulation considered in women planning to undergo *in vitro* fertilization.'

Nelson and Greer (2008) conducted an extensive review of the similarities of heparin and heparan, the haemostatic changes induced by ovarian stimulation and the risk of thrombosis, the contribution of thrombophilia to pregnancy and infertility outcomes, early embryonic–maternal dialogue and how these various aspects of assisted conception may be modified by heparin. The authors concluded that heparin has the potential to improve pregnancy rates and outcomes. Recently, Seshadri et al. (2012) conducted an extensive meta-analysis of observational and randomized studies on the effect of heparin on the outcome of IVF treatment. The meta-analysis of the observational studies showed a significant increase in clinical pregnancy and live birth rates and the authors concluded that that the role of heparin as an adjuvant therapy during IVF treatment required further evaluation in adequately powered high-quality randomized studies. They further suggested that such studies could either target the general IVF population or a specific subgroup of patients including those with known thrombophilia or recurrent implantation failure. In the absence of such studies and in view of the recent important findings from Henriksson et al. (2013) and the high incidence of the M2 ANXA5 haplotype within the current IVF cohort, this study's fertility centres have taken a pragmatic view to identify and treat patients who are carriers of the M2 ANXA5 haplotype, which is now known to be an inherited thrombophilia adversely affecting embryonic anticoagulation. In 2001, the Royal College of Obstetricians and Gynaecologists reviewed four randomized controlled trials in women with two or more pregnancy losses treated with low-dose aspirin with and without low-molecular-weight heparin (Scientific Impact Paper 26). It noted that these studies failed to demonstrate improvement in live birth outcome. They further noted that these studies were underpowered to be able to confirm or refute effects in women with three or more losses or those with thrombophilia. However, when this opinion was advanced there was no knowledge of

Please cite this article in press as: Fishel, S et al. Multicentre study of the clinical relevance of screening IVF patients for carrier status of the annexin A5 M2 haplotype. Reproductive BioMedicine Online (2014), http://dx.doi.org/10.1016/j.rbmo.2014.03.019

the existence of the M2 ANXA5 haploype in women with RPL. Indeed the authors stated that 'there remain unidentified inherited thrombophilias'. Furthermore the findings that paternal carriage contributes a similar risk to that of maternal carriage and that the defect is conveyed embryonically were also unknown, reflecting the need to understand an appropriate stratification of patients. This study's fertility centres are adopting the approach of offering screening of patients for carriage of the M2 haplotype with a view to identifying women at risk not only of pregnancy loss but for the additional risks conferred by this thrombophilic genetic defect. While appreciating that this is an incidence study only, the current practice advice for women identified at risk (either because she and or her partner are carriers) in this study's fertility centres is that they be treated from implantation to near term with low-molecular-weight heparin. If the woman is a carrier, treatment for 6 weeks post partum is advised to reduce the risk of maternal venous thromboembolism. In terms of risk to the fetus, a recent case–control study (Tiscia et al., 2012) reported that carriage of the M2 ANXA5 haplotype was an independent risk factor for idiopathic SGA newborns and that women carrying the M2 haplotype had a 2-fold higher risk of giving birth to an SGA baby. In addition they reported a 6% incidence of homozygotes which is similar to the 6% incidence in the current cohort. In their study, all M2 homozygotes had a history of a severe SGA (below the 3rd percentile).

It is possible to speculate that M2 homozygotic women may be at greater risk of thrombotic events by virtue of the decrease in their own endogenous ANXA5 during pregnancy; thus identification of this subset of patients before IVF treatment is important since from this study their IVF cycle failure rate is higher than for noncarriers. This study reports a single homozygotic man with no other infertility diagnosis whose partner had had two previous failed IVF cycles. Rogenhofer et al. (2012) interestingly noted no M2 homozygotic men in their cohort of 30 RPL couples. It is already well established (RCOG–SAC Opinion Paper 8, 2007) that the risk of low birthweight for IVF singletons is significantly higher than for naturally conceived singletons (incidence of SGA 12.6% versus in England 7.5%, reported by the London Health Observatory (2002–2004)). Thus identifying and treating women who are themselves M2 carriers or whose partner is a carrier may assist in reducing the incidence of SGA by mitigating the adverse effects on embryonic anticoagulation. There are long-lasting health costs associated with low birthweight in infants and this aspect warrants further study.

In conclusion, since the defect is conveyed embryonically and affects embryonic anticoagulation and also the risk is independent of any specific parental transmission (i.e. it can be induced whether the transmission is maternal or paternal or both), screening of both partners presenting for IVF for carriage of the M2 ANXA5 haplotype ought to be considered as routine and early in the diagnostic work up of couples being treated with their own gametes. The M2 haplotype appears to be an additional independent factor that contributes to the risk of pregnancy failure.

Further work accessing trio genotyping data of paternal, maternal and infant origin together with outcome is required to determine whether there are differences in outcome if both mother and child are carriers of the M2 haplotype. Additionally further consideration should be given to a test-and-treat critical pathway for those receiving donated gametes, embryo donors and surrogate mothers.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 7 June 2013; refereed 26 March 2014; accepted 27 March 2014.