



Research Paper

Precision Medicine in Assisted Conception: A Multicenter Observational Treatment Cohort Study of the Annexin A5 M2 Haplotype as a Biomarker for Antithrombotic Treatment to Improve Pregnancy Outcome



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ABSTRACT

Background: Pregnancy failure and placenta mediated pregnancy complications affect >25% of pregnancies. Although there is biological plausibility for a procoagulant mechanism underlying some of these events, antithrombotic intervention trials demonstrate limited benefit, possibly through lack of stratification in heterogeneous patient groups. The ANXA5 M2 haplotype is a possible procoagulant biomarker and was tested pragmatically to determine whether this screening and LMWH treatment normalized the outcome for ANXA5 M2 positive couples.

This was a pragmatic study that aimed to measure the effectiveness of a testing (for the M2 haplotype) and treatment (LMWH) pathway in routine clinical practice where there is variation between patients. Such a study in couples with fertility problems can inform choices between treatments; it is then the management protocol which is the subject of the investigation, not the individual treatments.

Methods: Couples (N = 77) with one or both partners ANXA5 M2 positive demonstrated association of this haplotype with adverse IVF outcome. A pragmatic, multicenter, prospective cohort study of ANXA5 M2 haplotype screening, and LMWH treatment following embryo transfer (ET) in 103 IVF couples positive for ANXA5 M2 was performed. They were compared with a group of 1000 contemporaneous randomly selected unscreened and untreated couples undergoing assisted conception, from which 103 matched control couples were derived. The primary outcome measure was live birth incidence. Secondary outcomes were results following embryo transfer (ET) and live birth outcome by gender and M2 carriage, and allelic dose influence.

Findings: The tested and treated cohort of ANXA5 M2 carriers achieved a similar live birth rate (37.9%) per ET cycle compared to both the more fertile comparison group (38.5%), and to the 103 matched controls (33.0%). Significantly more treated male carrier only couples had a live birth versus female M2 only (47.7% vs. 25.0% p = 0.045).

Interpretation: Pragmatic ANXA5 M2 screening and treatment with LMWH in couples undergoing IVF is associated with similar outcome to couples with more favorable prognostic factors. The difference in live birth outcome for treated male only carrier couples may be consistent with an additional maternal thrombophilic factor that may adversely affect pregnancy, although other mechanisms are possible. This study suggests that LMWH treatment should be started prior to clinical pregnancy.

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1. Introduction

Pregnancy failure and placenta mediated pregnancy complications (PMPC), miscarriage, fetal growth restriction (FGR), pre-eclampsia (PE) and abruption, collectively affect over 25% of pregnancies. Fifteen

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per cent of clinically recognized pregnancies end in miscarriage, with 5% of women experiencing ≥ 2 losses, and 1–2% ≥ 3 losses (Rai and Regan, 2006). With assisted conception, 76.3% of all cycles (that include such PMPC) do not result in a live birth (Macaldowie et al., 2014). Diagnosis of PMPC is largely based on clinical outcome rather than cause, despite heterogeneous disease processes generating the same clinical pregnancy outcome (Greer et al., 2014). The largest single cause of first trimester miscarriage is due to embryo aneuploidy. The remainder are unexplained, reflecting limited knowledge of implantation and placentation. The current focus on precision medicine (Collins and Varmus, 2015) emphasizes the need to stratify PMPC by mechanism, without which potential therapies may be rejected as ineffective when assessed in a heterogeneous patient group (Greer et al., 2014). For PMPC there is biological plausibility for coagulation activation being an underlying mechanism in a proportion of these events. The pathological features include deficient implantation, placental infarction, and microvascular thrombosis (Greer et al., 2014). The biological plausibility is enhanced by our knowledge of antiphospholipid syndrome, which is associated with thrombotic placental damage, and increased thrombin generation. Antithrombotic therapy guided by a biomarker, antiphospholipid antibodies, is associated with improved pregnancy outcome (Greer et al., 2014; Empson et al., 2005). In contrast, trials of antithrombotic intervention in unselected women at risk of PMPC, based on outcome of previous pregnancies, has been found to be ineffective (Greer et al., 2014; Kaandorp et al., 2010; Clark et al., 2010; Martinelli et al., 2012; Pasquier et al., 2015; Schleussner et al., 2015). However, an approach based on stratification for a specific thrombotic process underlying these PMPC, would be appropriate before discarding such treatment. This requires a biomarker. One possible biomarker is Annexin-A5 (ANXA5), an anticoagulant protein highly expressed on the apical surfaces of syncytiotrophoblasts, ANXA5 prevents coagulation processes by forming a two dimensional lattice which can be disrupted by antiphospholipid antibodies (Van Genderen et al., 2008), and has been reported to promote cell membrane repair in the syncytiotrophoblast layer (Bouter et al., 2015). The annexin A5 M2 haplotype (ANXA5 M2) is associated with reduced expression of ANXA5 in placentas from M2 haplotype carriers presenting with PE and FGR (Ota et al., 2013; Chinni et al., 2009; Markoff et al., 2010). Further, the M2 haplotype is associated with an increased risk of PMPCs: recurrent pregnancy loss (RPL) (Bogdanova et al., 2007; Tiscia et al., 2009; Miyamura et al., 2011; Rogenhofer et al., 2012; Tüttelmann et al., 2013; Hock et al., 2015; Demetriou et al., 2015), FGR (Tiscia et al., 2009) small for gestational age infants (Tiscia et al., 2012), and gestational hypertension (Tiscia et al., 2009). The haplotype is transmitted equally by males and females (Ota et al., 2013; Chinni et al., 2009; Markoff et al., 2010; Rogenhofer et al., 2012).

In a single study using a murine knockout model, significant reductions both in litter size and fetal weight in ANXA5-null mice (ANXA5-KO) was reported (Ueki et al., 2012), if accepted this suggests that maternal expression of ANXA5 in the circulation may be crucial for maintaining normal pregnancy; further administration of heparin on pregnancy days 12, 14, 16 and 18 to ANXA5-KO mice significantly increased litter size so contributing to the biological plausibility for the use for heparin for an intervention.

This study investigated, for the first time, the pragmatic use of the maternal and paternal ANXA5 M2 haplotype as a biomarker to stratify couples at risk of pregnancy failure undergoing assisted conception, for antithrombotic intervention with low molecular weight heparin (LMWH).

2. Methods

2.1. Study Population

Three patient cohorts and a retrospective contemporaneous group of unscreened patients undergoing assisted conception treatment, at

five fertility clinics within in the CARE Fertility Group (CFG) were studied as follows.

- To evaluate the association of the ANXA5 haplotype in a retrospectively tested group of 171 females with adverse outcome and 154 of their partners whose sperm were used. Seventy-seven of these couples had one or both partners who were ANXA5 M2 positive. These formed the “Yardstick” reference cohort (N = 77)
- A Randomly selected unscreened retrospective contemporaneous patient couple group (N = 1000)
- A Prospective, Tested and Treated couple cohort not taken from the retrospective contemporaneous group (N = 103)
- A matched retrospective control couple cohort (N = 103) selected from the 1000 retrospective contemporaneous group

Patients were followed until either clinical miscarriage or in live birth or other pregnancy outcome.

2.2. Yardstick Reference Group

This “Yardstick” reference group permitted observation of the timing of pregnancy losses in untreated M2 carrier couples. ANXA5 M2 screening was performed in 171 female patients and their partners (17 using sperm donors were not tested), with at least one failed previous IVF cycle (mean 1.10). None had achieved a live birth previously, 26% had a previous miscarriage, and their mean duration of infertility was 4.17 years. There were 77 couples (45%) where one or both partners were carriers of ANXA5 M2 (two females were egg recipients and three couples had sperm donation, the donors were not screened). This group had a similar age to the tested and treated cohort (median 35 range 25–44). The timing of losses in this group were compared to those of the unscreened, untreated, retrospective contemporaneous group.

2.3. Retrospective Unscreened Untreated Contemporaneous Group

Of the 1000 couples, 110 were egg recipients, 38 were egg share donors. The patients' ages, infertility status and ethnicity were as described by Fishel et al. (2014).

2.4. Tested and Treated Cohort

696 patients (369 couples) elected prospectively to be screened for carriage of the ANXA5 M2 haplotype. The first 103 patient couples found to be ANXA5 M2 positive were enlisted to the cohort and treated with a prophylactic dose of LMWH on achieving embryo transfer following informed consent, between March 2012 and October 2014. Patient enlistment was based on their own and their clinician's decision to test and treat considering their clinical history, which included duration of infertility, numbers of previous failed cycles and miscarriages. This was conducted as a clinical practice study with patient information documents to guide informed consent. Independent counselling was offered to all patients.

2.5. Matched Controls

The Tested and Treated cohort was matched with a cohort of 103 retrospective untested and untreated controls drawn from the randomly selected group of 1000 unscreened patient couples achieving embryo transfer with no LMWH treatment. The matching characteristics are shown in Table 1. None of the treated or control patients had achieved a live birth previously.

Reporting of this study conforms to the STROBE (The Strengthening the Reporting of Observational Studies in Epidemiology) statement.

Table 1
Matching variables for Treated and Paired Control patient cohorts.

		Tested and Treated (N = 103)		Matched controls (N = 103)	
Egg recipients	Egg recipient	8	7.8%	8	7.8%
	Standard	95	92.2%	95	92.2%
Number of embryos transferred	1	38	36.9%	38	36.9%
	2	64	62.1%	64	62.1%
	3	1	1.0%	1	1.0%
Embryo type	Cells 1 to 8+	45	43.7%	45	43.7%
	Morula	3	2.9%	3	2.9%
	Blastocysts/compacting	55	53.4%	55	53.4%
No aged <≥35 years	<35	34	33.0%	34	33.0%
	≥35	69	67.0%	69	67.0%
Age of patient (years)	<35: median; (range)	32	(26–34)	32	(22–34)
	≥35: median; (range)	38	(35–47)	38	(35–45)
	Median; (range)	36	(26–47)	36	(22–45)
Previous failed IVF cycles	0	17	16.5%	26	25.2%
	1+	86	83.5%	77	74.8%
Previous miscarriages	0	53	51.5%	56	54.4%
	1+	50	48.5%	47	45.6%

2.6. Ethical Statement and Informed Consent

Clinic practice and procedures are regulated and routinely inspected by the Human Fertilisation and Embryology Authority (HFEA), thus the Internal Review Board (IRB)/external ethical committee are used for clinical trials and embryo research only. This was a clinical observational study for a test and treatment regime for recurrent pregnancy loss that had limited published evidence and as such all CARE medical directors agreed, following their individual clinical judgement to offer the test to patients who had full counselling, written detailed information and signed informed consent based upon patient clinical history. These forms are available for scrutiny.

2.7. Study Design

The study was a pragmatic prospective cohort study of LWMH treatment in IVF couples offered screening and found positive for the ANXA5 M2 haplotype, compared to retrospective matched unscreened and untreated, couples (controls). No exclusions were made. The patient characteristics are shown in Table 1. Ovulation induction and embryology protocols and procedures have been published previously (Fishel et al., 2011). In the tested and treated group, if either or both of the couple were carriers of the M2 haplotype, the female received LMWH (enoxaparin: Clexane; Sanofi Winthrop, France) subcutaneously in a dose of 40 mg daily beginning on the day of oocyte retrieval for fresh embryo transfer or, on the day of frozen embryo transfer, and for a minimum of 12 weeks, with the recommendation to continue until term.

Women were assessed for TH1/TH2 intracellular cytokine ratio, Natural Killer cells and HLADQ at the discretion of the treating clinician. Where the TH1/TH2 intracellular cytokine ratio was disturbed (n = 50), Intralipid® 20% (Fresenius Kabi, New Zealand) was administered (Kwak–Kim et al., 2013).

2.8. Outcome Measures

The primary outcome measure was the incidence of live birth. Secondary outcomes were: incidence of implantation, biochemical pregnancy loss rate, clinical pregnancy rate, clinical miscarriage rate, live birth rate outcome by gender and M2 carriage, and allelic dose influence. Preclinical miscarriage was defined as failure of pregnancy after a positive biochemical pregnancy test and no fetal heart at first ultrasound examination at 5–6 weeks from day of embryo transfer; and clinical miscarriage was defined as pregnancy loss after detection of fetal heart activity.

2.9. Genotyping and Quality Control

DNA collection and analysis together with genotyping was performed, by Sanger sequencing as previously described (Fishel et al., 2014). Purified amplicons were sequenced using standard conditions and electrophoresis on an ABI 3730xl DNA analyser.

2.10. Statistical Analysis

Statistical analysis was independently conducted by Qi Statistics Ltd., Reading, UK. The analyses included: Mantel-Haenzel chi-squared tests for trend to assess the statistical significance of the association between i) treatment group and ii) each pregnancy outcome and each of the ordinal baseline characteristics. Pearson chi-squared tests for general association were used to assess the statistical significance of the association between treatment cohort and of each of the binary and nominal baseline characteristics, and Fisher's Exact Test was used where the expected counts were very low. All tests were 2-sided and statistical significance was viewed as accepted if the p-value was <0.05.

2.11. Statistical Software

All analyses were performed in the statistical software package SAS v9.3 (SAS Institute Inc., 2002–2010). The SAS procedure 'PROC FREQ' (Base SAS 9.3 Procedures Guide: Statistical Procedures) was used to perform all tests of association (Agresti, 2007). Paired Controls were randomly selected from each matched subgroup by generating random numbers using the CALL RANUNI subroutine with SAS.

2.12. Independent Variables

In addition to the study cohort (treatment/control), data on the following independent variables were evaluated: patient age (years), ANXA5 M2 genotyping results (female and male), number of embryos transferred, stage of embryo development at transfer (1–8 cells morula, blastocyst), type of incubator (Embryoscope™/standard), duration of infertility (years), number of previous IVF cycles, number of previous miscarriages, use of intralipid, donor egg use (egg recipient or egg share donor).

All data was obtained from the Clinical Information system (CIS) After 12 weeks clinical miscarriages live births and all other pregnancy outcomes were reported by the patient in compliance with the Human Fertility and Embryo Authority (HFEA) requirements.

2.13. Paired Analyses

The matched cohort of 103 paired Controls was drawn from the 1000 retrospective unscreened, untreated contemporaneous couple group by finding the number of Tested and Treated and Control patients that fell into each of the cross-classifications of baseline characteristics namely; age, number of embryos transferred, type of embryo transferred, previous failed miscarriages, previous failed IVF cycles and egg recipients (a total of 144 possible groups). As this was a pragmatic study of screening and treatment versus non screening, it is important to note that a proportion of the controls will be undetected ANXA5 M2 positive. It has previously been estimated that 44% of couples would be expected to be M2 positive in this IVF population (Fishel et al., 2014). Thus 1000 patient couples could be expected to include 440 M2 carrier couples (one or both partners' carriers).

3. Results

The yardstick reference group M2 positive couples had poor pregnancy outcome with high rates of pregnancy loss at various stages and no live births. Table 2 shows where in gestation each pregnancy was lost at each stage up to and including clinical miscarriage, so enabling

Table 2

Pregnancy failures showing where losses occur in untreated groups after embryo transfer.

	Unscreened untreated group	Unscreened untreated group	Yardsticks	p-Value
	Egg donors and egg recipients	Standard	Yardstick standard	Unscreened untreated Std vs. Yardstick Std
	(N = 75)	(N = 534)	(N = 75)	
Number of patients with failed live birth after embryo transfer	75	534	75	
Number of embryos transferred	106	774	115	
Number of embryos with fetal heart activity detected	9	31	12	
Implantation incidence (fetal hearts/embryos transferred)	8.5%	4.0%	10.4%	0.0027
Positive pregnancy test	30	88	28	
Biochemical pregnancy rate (per patient)	40.0%	16.5%	37.3%	<0.0001
Biochemical loss/preclinical miscarriage	23	58	16	
Biochemical loss rate	76.7%	65.9%	57.1%	0.4005
Clinical pregnancy	7	30	12	
Clinical pregnancy rate	9.3%	5.6%	16.0%	0.0009
Clinical miscarriage	5	30	12	
Clinical miscarriage rate	85.7%	100.0%	100.0%	

Note: one control egg recipient and one egg donor were lost to follow up after clinical pregnancy and outcome unknown so assumed to have maintained pregnancy.

Note that only patients with an embryo transfer that failed to result in a live birth have been included.

9 control patients with an ectopic pregnancy or termination were excluded.

comparisons of the timing of pregnancy loss for the Yardstick and the retrospective, unscreened, untreated contemporaneous 1000 couple group.

Excluding egg recipients, the untreated ANXA5 M2 carrier Yardstick group had a statistically higher incidence of implantation, biochemical and clinical pregnancy than Controls. Their clinical miscarriages all occurred by 12 weeks gestation: two at 6 weeks one at 7 weeks, four at 8 weeks, four at 9 weeks and two at 12 weeks gestation. The incidence of implantation and clinical pregnancy was significantly higher than either controls or egg recipients, but patients then lost their clinical pregnancies. Thus the untreated M2 carriers have a different pattern of timing of loss both to the unscreened, untreated standard controls and to unscreened, untreated egg recipients.

The Tested and Treated cohort, with a potential for more adverse outcome due to a higher mean maternal age, a greater number of

miscarriages, a longer duration of infertility, and more unsuccessful assisted conception cycles, achieved a live birth rate of 37.9%, compared to the 1000 retrospective unscreened and untreated group of 38.5% (Table 3). Statistical analysis showed no significant impact of the use of Embryoscope™ time-lapse incubators and Intralipid (data not shown but provided in Supplementary Tables). Significantly more favorable outcomes were observed for younger patients, egg recipients and for those who had blastocysts or two earlier cleavage stage embryos transferred (data not shown but provided in Supplementary Tables).

The Tested and Treated cohort had a similar outcome to the Paired Control cohort See Table 4.

Of the four clinical miscarriages in the Tested and Treatment cohort, one was due to chorionamnionitis at 15 weeks gestation; one was a twin pregnancy with Dandy Walker variant delivered at 23 weeks, and two at 12–13 weeks with no diagnosis made.

Table 3

Pregnancy outcome for patients with an embryo transfer.

Pregnancy outcomes	Study group		Odds ratio (95% CI) (Treated cohort/Unscreened untreated)	p-Value (Treated vs. Unscreened untreated)
	Tested and Treated cohort	Contemporaneous retrospective unscreened and untreated group		
Patients with an embryo transfer	103	1000		
Number of embryos transferred	169	1454		
Number of embryos transferred with fetal heart activity subsequently detected	56	494		
Implantation incidence (fetal hearts/embryos transferred)	33.1%	34.0%	0.96 (0.69, 1.35)	0.83
Biochemical pregnancy rate (per patient)	53.4% (N = 55)	50.9% (N = 509)	1.11 (0.74, 1.66)	0.63
Biochemical loss rate (per biochemical pregnancy)	21.8% (N = 12)	15.9% (N = 81)	1.47 (0.75, 2.92)	0.26
Clinical pregnancy rate (per patient)	41.7% (N = 43)	42.8% (N = 428)	0.96 (0.63, 1.44)	0.84
Patients with clinical miscarriages (miscarriages after fetal hearts detected)	4	36		
Patients with ectopic or terminated clinical pregnancy	0	9		
Clinical miscarriage rate (per clinical pregnancy) ^a	9.3%	8.6%	1.09 (0.37, 3.23)	0.87
Patients with live births	39	373		
Patients lost to follow up/live birth data unavailable	0	9		
Live birth rate (per patient) ^{a,b,c}	37.9%	38.5%	0.97 (0.64, 1.48)	0.89

^a Excludes eight controls with a termination of pregnancy after patients electing to have amniocentesis or chorionvillous biopsy and one control with an ectopic pregnancy.

^b Note that for the purposes of calculating the Live Birth Rate per patient, nine Control patients with Clinical Pregnancy who were lost to follow up were assumed to have had a live birth.

^c Includes one live birth (499 g) to a Control patient that was neonatal death.

Table 4
Pregnancy outcome for matched Treated and Paired Control cohorts.

Pregnancy outcomes	Study group		Odds ratio (95% CI) (Treated/Control)	p-Value (Treated vs. Control)
	Tested and Treated	Paired Controls		
Paired patients with an embryo transfer	103	103		
Number of embryos transferred	169	169		
Number of embryos transferred with fetal hearts detected	56	51		
Implantation incidence (fetal hearts/embryos transferred)	33.1%	30.2%	1.15 (0.72, 1.81)	0.56
Biochemical pregnancy rate (per patient)	53.4% (N = 55)	46.6% (N = 48)	1.31 (0.76, 2.27)	0.33
Biochemical loss rate (per biochemical pregnancy)	21.8% (N = 12)	14.6% (N = 7)	1.63 (0.59, 4.56)	0.35
Clinical pregnancy rate (per patient)	41.7% (N = 43)	39.8% (N = 41)	1.08 (0.62, 1.89)	0.78
Clinical miscarriage rate (per clinical pregnancy)	9.3% (N = 4)	17.1% (N = 7)	0.49 (0.13, 1.85)	0.29
Patients with live births	39	34		
Patients lost to follow up/live birth data unavailable ^a	0	1		
Live birth rate (per patient)	37.9%	33.0%	1.24 (0.70, 2.19)	0.47

^a 1 patient was lost to follow up in the control cohort after clinical pregnancy at 12 weeks and has been included as a live birth.

The seven clinical miscarriages in the matched control cohort occurred at 8, 9, 10, 11, 12, 13 (twins) and 19 weeks respectively with no diagnosis made. Three of these controls were egg recipients.

Comparing couples with only males who demonstrated carrier or homozygosity, to couples where only the female had carrier/homozygous status, the live birth outcome is statistically significantly different at the 5% level ($p = 0.0452$) with 58.3% of male ANXA5 M2 only treated couples having a live birth versus 25.0% of female ANXA5 M2 only treated couples (Table 5). This result is close to the statistically significant cut-off so needs to be interpreted in the context of the size of the difference and the population.

There is no statistically significant evidence of an allelic dose effect on live birth outcome.

4. Discussion

The Yardstick reference group allowed observation of the gestational stage at which pregnancy losses occur following ET, and demonstrate the known association (Ota et al., 2013; Markoff et al., 2010; Tiscia et al., 2009; Rogenhofer et al., 2012) between ANXA5 M2 parental status and adverse outcome, with higher risk of early clinical pregnancy loss.

The timing of clinical pregnancy loss is consistent with the previously reported time of loss in natural pregnancies with ANXA5 M2 carriers with RPL (Bogdanova et al., 2007; Tiscia et al., 2009; Miyamura et al., 2011; Tüttelmann et al., 2013; Hock et al., 2015; Demetriou et al., 2015).

The association between parental M2 status and adverse clinical pregnancy outcomes, coupled with the biological plausibility of the M2 haplotype operating through procoagulant mechanisms leads to the hypothesis that the M2 haplotype acts as a biomarker for adverse pregnancy outcome, which is amenable to intervention with LMWH.

Stratification of assisted conception patients by parental ANXA5 M2 status would offer targeted intervention with LMWH, delivering a precision medicine approach.

The lack of such stratification may underlie the inconsistent data from trials of LMWH for PMPC (Martinelli et al., 2012; Rodger et al., 2014) due to inclusion of heterogeneous disease mechanisms rather than a focus on those associated with a procoagulant mechanism. These findings also suggest that for LMWH treatment to be effective it should be introduced before a clinical pregnancy is confirmed, as later initiation appears too late to improve live birth. The timing of LMWH administration may also have been a factor in the outcome of trials of LMWH for recurrent pregnancy loss, where treatment was often started following diagnosis of clinical pregnancy and LMWH was without benefit (Kaandorp et al., 2010; Clark et al., 2010; Pasquier et al., 2015; Schleussner et al., 2015). The ANXA5 M2 haplotype may also be a relevant biomarker to stratify treatment in a proportion of unexplained infertility and recurrent miscarriage cases and should be explored.

This concept of the parental M2 Haplotype, acting as a biomarker to stratify assisted conception patients for LMWH therapy, was explored in the cohort comparison described in this paper. The results of the cohort comparison show that a tested and treated cohort of ANXA5 M2 carriers do not achieve a significantly different live birth rate (37.9%) per embryo transfer cycle both in comparison to a larger, unselected, untreated, more fertile group and to a paired cohort who would both have a lower prevalence of M2. Additionally the live birth rate of 37.9% compares favorably to the live birth rate per embryo cycle of 36.1% achieved from 6572 fresh embryo transfer cycles in CARE patients using their own eggs in the two years 2013 and 2014. It is further of note that whereas in 2014 the HFEA reported that the mean age of women having IVF treatment was 35 and their duration of infertility (DOI) was 4 years, 67% of the tested and treated cohort in this study were aged 35 and over (median age 38) and had an mean DOI of 5.1 years. These data suggest that screening for ANXA5 M2 haplotype and treatment with LMWH can improve prognosis for M2 positive couples, to that expected in a first assisted conception cycle in the general population.

The ANXA5 M2 haplotype has been reported to pose an equal risk whether transmitted paternally or maternally (Ota et al., 2013; Chinni

Table 5
Live births for treated patients by carrier status and allelic dose.

All treated patients with complete genetic testing data		Live births		No live births		Total (N = 96) ^a	p-Value
		(N = 36)	%	(N = 60)	%		
C4M2 carrier status	Female carrier/homozygote	9	25.0%	26	43.3%	35	0.0452 (Male vs. Female only)
	Male carrier/homozygote	21	58.3%	23	38.3%	44	
	Both carrier/homozygote	6	16.7%	11	18.3%	17	
Number of alleles	1	29	39.7%	44	60.3%	73	0.4222
	2 to 3	7	30.4%	16	69.6%	23	

1 includes: (WT/WT)/(WT/M2); (WT/WT)/(M1/M2); (WT/M2)/(WT/M1); (WT/M2)/(M1/M1).

2 includes: (WT/M2)/(WT/M2); (WT/M2)/(M1/M2); (WT/WT)/(M2/M2); (WT/M1)/(M2/M2).

3 includes: (WT/M2)/(M2/M2).

^a Seven couples with incomplete genetic testing data have been excluded (five untested males and two untested females).

et al., 2009; Markoff et al., 2010; Rogenhofer et al., 2012; Demetriou et al., 2015). However, the significant difference in live birth outcome reported in Table 5 between treated male only carrier couples versus treated female only carrier couples ($p = 0.045$) suggests an additional maternal thrombophilic factor that may further adversely affect pregnancy outcome. Ota et al. (Ota et al., 2013) noted that women with recurrent pregnancy loss suffer repeat miscarriages even though the maternal and paternal ANXA5 M2 haplotype will be transmitted to the fetus/placenta with a 50% frequency. The possibility that the ANXA5 M2 haplotype is a thrombophilia is also supported by Grandone et al. (2010a, b) who reported that deep vein thrombosis in men and nonpregnant women (Grandone et al., 2010a), and in pregnant women (Grandone et al., 2010b) is independently associated with M2 status. The less favorable outcome for female only carriers or homozygotes versus males also suggests that the maternal contribution is not confined to the transmission of the M2 haplotype to the fetus and needs further elucidation. There is growing evidence that male factors are important for abnormal placentation and the development of PE (Katsi et al., 2015). The association shown in this study of the paternal ANXA5 M2 haplotype with adverse outcomes adds weight to the hypothesis that male factors play also play a role in PMPC.

In the cohorts studied, duration of infertility, type of incubator and the use of intralipid did not have any bearing on pregnancy outcome. The effect of allelic dose (1 versus 2 or 3 alleles) as reported by Demetriou et al. (2015) is not observed in this study.

This may be because the allelic dose effect has been reduced by LMWH treatment.

4.1. Limitations of the Study

Classically for a confirmatory trial it is required to specify upfront the clinically meaningful difference of interest and then set up the trial to adequately collect the right amount of data. However, conducting randomized controlled trials in the assisted conception population is inherently difficult as advancing maternal age is associated with a decline in fertility and such females are reluctant to be randomized. Trials of this type in naturally-conceived pregnancy require several years to complete, for example the TIPPS trial of antithrombotic intervention for women with thrombophilia in pregnancy (Rodger et al., 2014) took 12 years. There is further difficulty in spontaneous pregnancy if LMWH is to be introduced early in pregnancy. Given the effect of age on fertility, the age of the patient cohort, the impact of age on treatment outcome, and the time required to perform a randomized trial in this population, a pragmatic approach to screening and treatment was preferred with rigorous matching of variables. Clark (2013) has suggested that cohort-controlled are useful in identifying variables associated with treatment outcomes before proceeding to an RCT. Therefore a pragmatic approach as described here with a retrospective case control design is the most practicable first step in assessing the hypothesis that the ANXA5 M2 haplotype is a biomarker for IVF failure, which can be overcome by treatment with LMWH.

Based on the work of Fishel et al. (2014) the highest incidence we may estimate for control IVF couples positive for M2 would be 44%; hence the M2 dilution effect would be at the least a factor of 2.3 (100/44).

Within the known limitations of the study and the population, however, no significant evidence was found for differences between the groups in terms of the primary live birth outcome.

4.2. Conclusions

In conclusion, this study suggests that the identification and treatment of M2 carrier pregnancies by screening both partners for the M2 haplotype, in assisted reproduction, may identify an adverse prognostic factor for assisted conception, which can be used to stratify couples for treatment with LMWH. Such an intervention results in live birth

outcomes similar to those achieved in assisted conception cohorts with more favorable prognostic factors. This study also suggests that such treatment should be started prior to clinical pregnancy. In view of its association with PMPC particularly in the first trimester of pregnancy, studies on the role of the M2 haplotype are warranted in these conditions.

Conflicts of Interest

Simon Fishel reports he is Founder and President of CARE Fertility Group and a minority share holder and has no conflict of interest.

Ian Greer reports personal fees from Sanofi, outside the submitted work.

Deborah Baker reports personal fees and other from IHG Pharmaco, outside the submitted work; In addition, she has a patent WO 2015/155523 pending.

Janine Elson, Maha Ragunath, Glenn Atkinson, Adel Shaker, Ahmed Omar, Rahnuma Kazem, Ashley Beccles are all employees of CARE Fertility and report no conflicts of interest.

Authors' Contributions

Simon Fishel: co-originator of the study and protocols, collation of data and manuscript author.

Ian Greer: literature search, study design, data interpretation, writing and critical review of drafts and manuscript and STROBE check list editing.

Deborah Baker: conducted literature searches, participated in study design, participated in data collection, liaised with the statisticians for data interpretation, co-wrote the paper and STROBE statement checklist, revised drafts and is the corresponding author.

Janine Elson, Maha Ragunath, Glenn Atkinson, Adel Shaker, Ahmed Omar, Rahnuma Kazem: co ordination of the patients for the study and contribution of the data for the manuscript.

Ashley Beccles: data mining and collation.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2016.06.024>.

References

- Agresti, A., 2007. *An Introduction to Categorical Data Analysis*. John Wiley & Sons, Second Edition, New York.
- Bogdanova, N., Horst, J., Chlystun, M., et al., 2007. A common haplotype of the annexin A5 (ANXA5) gene promoter is associated with recurrent pregnancy loss. *Hum. Mol. Genet.* 16, 573–578.
- Bouter, A., Carmelle, R., Gounou, C., et al., 2015. Review: annexin-A5 and cell membrane repair. *Placenta* 36 (Supplement 1), S43–S49 (Trophoblast Research, Vol. 29).
- Chinni, E., Tiscia, G., Colaizzo, D., Vergura, P., Margaglione, M., Grandone, E., 2009. Annexin V expression in human placenta is influenced by the carriership of the common haplotype M2. *Fertil. Steril.* 91 (3), 940–942.
- Clark, D.A., 2013. Aspirin and heparin to improve live birth rate in IVF for unexplained implantation failure? *Reprod. BioMed. Online* 26, 51.
- Clark, P., Walker, I.D., Langhorne, P., et al., on behalf of the Scottish Pregnancy Intervention Study (SPIN) collaborators, 2010. SPIN (Scottish Pregnancy Intervention) study: a multicenter randomized controlled trial of low-molecular-weight heparin and low-dose aspirin in women with recurrent miscarriage. *Blood* 115 (21), 4162–4167.
- Collins, F.S., Varmus, H., 2015. A new initiative on precision medicine. *N. Engl. J. Med.* 372, 793–795. <http://dx.doi.org/10.1056/NEJMp1500523> (February 26, 2015).
- Demetriou, C., Peskett, E., Abu-amer, S., et al., 2015. Investigation of the annexin A5 M2 haplotype in 500 white European couples who have experienced recurrent spontaneous abortion. *Reprod. BioMed. Online* 31 (5), 681–688.
- Empson, M., Lassere, M., Craig, J., Scott, J., 2005. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant. *Cochrane Database Syst. Rev.* (2) <http://dx.doi.org/10.1002/14651858.CD002859.pub2> (Art. No.: CD002859).

- Fishel, S., Craig, A., Lynch, C., et al., 2011. Assessment of 19,803 paired chromosomes and clinical outcome from first 150 cycles using array CGH of the first polar body for embryo selection and transfer. *J. Fertiliz. In Vitro* 1, 101. <http://dx.doi.org/10.4172/jfiv.1000101>.
- Fishel, S., Patel, R., Lytollis, A., et al., 2014. Multicentre study of the clinical relevance of screening IVF patients for carrier status of the annexin A5 M2 haplotype. *Reprod. BioMed. Online* 29, 80–87.
- Grandone, E., Tiscia, G., Colaizzo, D., Vergura, P., Pisanelli, D., Margaglione, M., 2010a. The haplotype M2 within the ANXA5 gene is independently associated with the occurrence of deep venous thrombosis. *Thromb. Haemost.* 103, 1102–1103.
- Grandone, E., Tiscia, G., Colaizzo, D., et al., 2010b. Role of the M2 haplotype within the annexin A5 gene in the occurrence of pregnancy-related venous thromboembolism. *Am. J. Obstet. Gynecol.* 203 (5), 461 (e1–5).
- Greer, I.A., Brenner, B., Gris, J.C., 2014. Antithrombotic treatment for pregnancy complications: which path for the journey to precision medicine? *Br. J. Haematol.* 165, 585–599.
- Hock, T.T., Bogdanova, N., Cheen, A.K., et al., 2015. M2/ANXA5 haplotype as a predisposition factor in Malay women and couples experiencing recurrent spontaneous abortion: a pilot study. *Reprod. BioMed. Online* 30 (4), 434–439.
- Kaandorp, S.P., Goddijn, M., van der Post, J.A., et al., 2010. Aspirin plus heparin or aspirin alone in women with recurrent miscarriage. *N. Engl. J. Med.* 362, 1586–1596 (2010).
- Katsi, V., Felekos, I., Siristatidis, C., et al., 2015. Preeclampsia: what does the father have to do with it? *Curr. Hypertens. Rep.* 17 (8), 60.
- Kwak-Kim, J., Han, A.R., Gilman-Sachs, A., Fishel, S., Leong, M., Shoham, Z., 2013. Current trends of reproductive immunology practices in in vitro fertilization (IVF) – a first world survey using IVF-worldwide.com. *Am. J. Reprod. Immunol.* 69 (1), 12–20 (Jan).
- Macaldowie, A., Wang, Y.A., Chughtai, A.A., Chambers, G.M., 2014. Assisted reproductive technology in Australia and New Zealand 2012. Sydney: National Perinatal Epidemiology and Statistics Unit, the University of New South Wales.
- Markoff, A., Gerdes, S., Feldner, S., Bogdanova, N., Gerke, V., Grandone, E., 2010. Reduced allele specific annexin A5 mRNA levels in placentas carrying the M2/ANXA5 allele. *Placenta* 31 (10), 937–940.
- Martinelli, I., Ruggenenti, P., Cetin, I., et al., 2012. Heparin in pregnant women with previous placenta-mediated pregnancy complications: a prospective, randomized, multicenter, controlled clinical trial. *Blood* 119, 3269–3275.
- Miyamura, H., Nishizawa, H., Ota, S., et al., 2011. Polymorphisms in the annexin A5 gene promoter in Japanese women with recurrent pregnancy loss. *Mol. Hum. Reprod.* 1, 447–452.
- Ota, S., Miyamura, H., Nishizawa, H., et al., 2013. Contribution of fetal ANXA5 gene promoter polymorphisms to the onset of pre-eclampsia. *Placenta* 34, 1202–1210.
- Pasquier, E., De Saint, Martin L., Bohec, C., et al., 2015. Enoxaparin for prevention of unexplained recurrent miscarriage: a multicenter randomized double-blind placebo-controlled trial. *Blood* 125 (14), 2200–2205.
- Rai, R., Regan, L., 2006. Recurrent miscarriage. *Lancet* 368, 601–611.
- Rodger, M.A., Hague, W.M., Kingdom, J., et al., 2014. Antepartum dalteparin versus no antepartum dalteparin for the prevention of pregnancy complications in pregnant women with thrombophilia (TIPPS): a multinational open-label randomised trial. *Lancet* 384 (9955), 1673–1683.
- Rogenhofer, N., Engels, L., Bogdanova, N., Tüttelmann, F., Markoff, A., Thaler, C., 2012. Paternal and maternal carriage of the annexin A5 M2 haplotype are equal risk factors for recurrent pregnancy loss: a pilot study. *Fertil. Steril.* 98 (2), 383–388.
- Schleussner, E., Kamin, G., G., S., et al., for the ETHIG II group, 2015. Low-molecular-weight heparin for women with unexplained recurrent pregnancy loss a multicenter trial with a minimization randomization scheme. *Ann. Intern. Med.* 162, 601–609.
- Tiscia, G., Colaizzo, D.S., Margaglione, M., Grandone, E., 2009. Haplotype M2 in the annexin A5 (ANXA5) gene and the occurrence of obstetric complications. *Thromb. Haemost.* 102 (2), 309–313.
- Tiscia, G., Colaizzo, D., Favuzzi, G., et al., 2012. The M2 haplotype in the ANXA5 gene is an independent risk factor for idiopathic small-for-gestational age newborns. *Mol. Hum. Reprod.* 18 (10), 510–513.
- Tüttelmann, F., Ivanov, P., Dietzel, C., et al., 2013. Further insights into the role of the annexin A5 M2 haplotype as recurrent pregnancy loss factor, assessing timing of miscarriage and partner risk. *Fertil. Steril.* 100, 1321–1325.
- Ueki, H., Mizushina, T., Laoharatchathathanin, T., et al., 2012. Loss of maternal annexin A5 increases the likelihood of placental platelet thrombosis and foetal loss. *Sci. Rep.* 2, 827. <http://dx.doi.org/10.1038/srep00827> (Epub 2012 Nov 9).
- Van Genderen, H.O., Kenis, H., Hofstra, L., Narula, J., Reutelingsperger, C.P.M., 1983. Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. *Biochim. Biophys. Acta* 2008, 953–963.