Annexin A5 M2 Haplotype as a biomarker for antithrombotic treatment to improve pregnancy outcome

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Ovarian Club
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Human placental anticoagulant protein: isolation and characterization.

Funakoshi T1, Heimark RL, Hendrickson LE, McMullen BA, Fujikawa K

Abstract
An anticoagulant protein was purified from the soluble fraction of human placenta by ammonium sulfate precipitation and column chromatography on DEAE-Sepharose, Sephadex G-75, and Mono S (Pharmacia). The yield of the purified protein was approximately 20 mg from one placenta. The purified protein gave a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight of 36,500. This protein prolonged the clotting time of normal plasma when clotting was induced either by brain thromboplastin or by kaolin in the presence of cephalin and Ca2+. It also prolonged the factor Xa induced clotting time of platelet-rich plasma but did not affect thrombin-induced conversion of fibrinogen to fibrin. The purified placental protein completely inhibited the prothrombin activation by reconstituted prothrombinase, a complex of factor Xa-factor Va-phospholipid-Ca2+. The placenta inhibitor had no effect on prothrombin activation when phospholipid was omitted from the above reaction. Also, it neither inhibited the amidolytic activity of factor Xa, nor did it bind to factor Xa. The placenta inhibitor, however, did bind specifically to phospholipid vesicles (20% phosphatidylserine and 80% phosphatidylcholine) in the presence of calcium ions. These results indicate that the placental anticoagulant protein (PAP) inhibits coagulation by binding to phospholipid vesicles. The amino acid sequences of three cyanogen bromide fragments of PAP aligned with those of two distinct regions of lipocortin I and II with a high degree of homology, showing that PAP is a member of the lipocortin family.

PMID: 2960376
Early History

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<tr>
<th>Approved Symbol</th>
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<th>Previous Symbols</th>
<th>Synonyms</th>
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CONCLUSIONS: Antiphospholipid antibodies reduce the levels of annexin V and accelerate the coagulation of plasma on cultured trophoblasts and endothelial cells. The reduction of annexin V levels on vascular cells may be an important mechanism of thrombosis and pregnancy loss in the antiphospholipid-antibody syndrome. Levels of annexin V, a phospholipid-binding protein with potent anticoagulant activity, are markedly reduced on placental villi from women with this syndrome. Hypercoagulability in such women may therefore be due to the reduction of surface-bound annexin V by antiphospholipid antibodies.
Annexins are a family of proteins which share the properties of binding calcium and phospholipids.

The phospholipid-binding property is associated with **anticoagulant** activities which are a consequence of the inhibition of critical phospholipid-dependent coagulation reactions.
ANXA5 essential for repair and maintenance of the syncytiotrophoblast (ST) apical membrane

Without this efficient repair mechanism coagulation occurs which adversely effects the embryo/foetus

Precise remodelling of uterine spiral arteries during the earlier stages of pregnancy necessary for healthy outcome

Defective placentation, impaired trophoblast invasion, and failure of sufficient remodelling of spiral arteries are common features of adverse pregnancy outcomes including: early pregnancy loss, intrauterine growth restriction, and pre-eclampsia

PMPC – Placenta-Mediated Pregnancy Complications
Importance of Syncytiotrophoblast Function

- STs are a continuous, specialized layer of epithelial cells, covering entire surface of villous trees and in direct contact with maternal blood.
- invade maternal myometrial spiral arteries during the first trimester (around 8–10 weeks and completes by 20-22 weeks of gestation in normal pregnancy)
- Surface area of STs is ~5m² at 28 weeks’ Gn & up to 11–12m² at term
- Placental trophoblasts provide:
  - structural and biochemical barriers between the maternal and foetal compartments during pregnancy
  - serve as an important endocrine organ that produces numerous growth factors and hormones that support and regulate placental and foetal development and growth
  - generate growth factors that vascularise the placenta during 2nd & 3rd trimester
SPECTRUM of pathologies associated with ANXA 5

Thrombophilia Caused by Defective Production of AnxA5 Manifests as a Spectrum of Pathologies

- *RPL* - Recurrent Pregnancy Loss
- PE - Pre-eclampsia
- GH - Gestational Hypertension
- FGR - Foetal Growth Restriction
- VTE - Maternal venous thromboembolism
- PCOS - Polycystic Ovarian Disease
- aPL - antiphospholipid antibodies
- SGA - Small-for-Gestational-Age

Gestational week

12 weeks
- Development of maternal blood supply to placenta complete
- General ultrasound scans

20 weeks
- PE symptoms start to appear
- In depth ultrasound scans

34 weeks
- Delivery due to early-onset PE
- Ultrasound scans. Final

40 weeks: Delivery

RPL majority at weeks 5 to 12 (range 5 to 23 weeks)

FGR

SGA

GH / PE from week 20

VTE from c.37% pre-partum

63% post-partum
6 weeks
ANXA 5 M2-H haplotype is 4 base pair mutation in the ANXA5 gene causing a reduction in Annexin 5 - present in the syncytiotrophoblasts
ANXA5 M2-Haplootype

- The annexin A5 gene variants

- Initially found through systematic sequence analysis of the ANXA5 gene in 70 patients of Northern German origin with RPL (defined as more than two fetal losses), non-carriers of PTm and/or FVL mutations. In the meantime, since the original publication, more than 4500 patients of different ethnicities and various obstetric complications have been genotyped, where the associated risk in C4/M2 carriers is between 2.4 and 4 for the various thrombophilia related phenotypes.
Studies showing ANXA5 M2 haplotype linked to significant reduction in Annexin

- Reporter gene assay in M2 women w RPL showed in vitro reduction of the ANXA5 promoter of 37-42% 
  (Bogdanova et al 2007)

- Expression of ANXA5 mRNA in placentas from M2 carriers with PE and FGR significantly (3x↓) than normal non carriers (Chinni et al 2009)

- M2 resulted in 42% ↓ placental mRNA levels in heterozygous carriers compared to normal non carriers. (Markoff et al 2010)
Summary of Case and Control Genotyping Studies to date:

Total cases - 4,592; Total controls - 2,577

1) Germany
(Van Genderen et al., 2008; Markoff et al., 2010)
Affected patients: 2,750/ Controls: 1,660
(includes German, French, Bulgarian, Dutch Italian, Spanish, Malay and Mahgreb - African ethnicities)

2) Italian Case control Studies
(Chinni et al 09; Tiscia et al 09; Grandone et al 2010; Tiscia et al 2012)
Affected patients: 307/ Controls: 195

3) UK (Imperial College)

4) Japan
(Miyamura et al 2011; Ota et al 2013)
Affected patients: 996/ Controls: 482

Affected patients: 344/ Controls: 240
Carriage Rates of the ANXA5 M2 Haplotype

- European general population 15%
  - European RPL cohorts 24 to 34%
- Japanese general population 5%
  - Japanese RPL cohorts 11%
- Malay general population 42.6%
  - Malay RPL cohorts 52.6%
Annexin 5 KO Murine Model Demonstrated Plausibility for LMWH Treatment in M2 Patients

- Found significant reductions in litter size and foetal weight in ANXA5 null mice.
- LMWH given to ANXA5-KO mice significantly reduced pregnancy loss.
- Maternal ANXA5 crucial for maintaining intact placental circulation.
- Maternal ANXA5 minimises the risk of thrombosis in the placental circulation and reduces foetal loss.

Pathology of M2 Placentas in PE and Control Patients

- Ota et al 2013 compared pathology of pre-eclamptic and control pregnancies.

- Found the placentas carrying the M2 haplotype showed significantly more severe perivillous fibrin deposition (PVFD) P= 0.004)

- Suggests low levels of ANXA5 at the feto-maternal interface in the chorionic villi of M2 placentas contribute to onset of pre-eclampsia
Previous LMWH studies in RPL and PMPC

- Groups based on clinical outcome, not based on underlying disease mechanisms, so not homogeneous.

- Not stratified by **both** parents characteristics
  No male partners included.

- Treatment w. LMWH normally at clinical pregnancy detection i.e. from 5 weeks +
  - too late as M2 clinical pregnancy losses start at 5 weeks.
Heparin has potentially beneficial effects on trophoblast that may facilitate implantation. Benefits may be limited to particular phenotypes or genotypes.

- Women with three or more recurrent miscarriages, who may represent a more homogeneous group.
- Specific thrombophilias and their interaction with disease.
- Thrombotic damage such as placental infarction.

Need for biomarkers or phenotypes to guide treatment.

Current treatment is imprecise, generalized by disease outcome and not stratified by maternal (or paternal) characteristics or the disease mechanism.

While awaiting randomised trials we must avoid medicine based on hope rather than evidence as we await precision medicine approaches for placental mediated pregnancy complications.
As a potential biomarker for PMPC it’s presence may be deleterious to the maintenance of a healthy pregnancy for mother and child.
CARE published studies on the incidence in our IVF population

- Male 26%
- Female 24%
- Both partners carriers ~10%
- Couples 44%
- Couples w. unexplained infertility 37%
- Co-exists w. male and female infertility patients (27%)
- Co exists w PCOS (35%)
Key differences Between the ANXA5 M2 Haplotype and FVL and PT Thrombophilia

The **incidence** is much higher than for: FVL & PT

<table>
<thead>
<tr>
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<th>Incidence General pop</th>
<th>RPL/obstetric</th>
<th>IVF incidence Females</th>
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<tbody>
<tr>
<td>FVL</td>
<td>3-7%</td>
<td></td>
<td></td>
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<tr>
<td>PT</td>
<td>2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANXA5 M2-H</td>
<td>15%</td>
<td>22- 38%</td>
<td>24%</td>
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</table>
## Known Hereditary Thrombophilic Risk Factors – All Maternally Transmitted

<table>
<thead>
<tr>
<th>Thrombophilic defect</th>
<th>Normal population</th>
<th>Incident VTEa</th>
<th>Recurrent VTE</th>
<th>Relative Thrombotic Risk</th>
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<tbody>
<tr>
<td>Antithrombin deficiency</td>
<td>0.02-0.04</td>
<td>1-2</td>
<td>2-5</td>
<td>5-10</td>
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<td>Protein C deficiency</td>
<td>0.2-0.5</td>
<td>2-5</td>
<td>5-10</td>
<td>6-10</td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>0.1-1</td>
<td>1-3</td>
<td>5-10</td>
<td>2-10</td>
</tr>
<tr>
<td>Factor V Leiden</td>
<td>3-7</td>
<td>20</td>
<td>40-50</td>
<td>3-7 (heterozygotes) 50-100 (homozygotes)</td>
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<tr>
<td>Prothrombin G20210A</td>
<td>1-3</td>
<td>3-8</td>
<td>15-20</td>
<td>2-8 (heterozygotes)</td>
</tr>
<tr>
<td>C4 / M2</td>
<td>15</td>
<td></td>
<td></td>
<td>3 times and additive to other thrombophilias</td>
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</table>
**CARE Observational Study**

**Tested and treated patient couples cohort N=103**
696 patients (369 couples) screened prospectively
All M2 positive couples female treated w LMWH

**Female given LMWH (40 mg daily) from day of oocyte retrieval for fresh embryo transfer and on day of embryo transfer if frozen embryo transfer for minimum of 12 weeks but advised to delivery**

**Pregnancy outcomes analysed**

**Untreated couples (Yardstick cohort, N-77) screened retrospectively after failure (screened 171 female/154 male partners) for M2 after IVF failure**

**Not treated previously w LMWH**

**Pregnancy outcomes analysed**

**Retrospective contemporary control group N=1000**

**103 couples** selected randomly from this group as paired untreated control group

**Unscreened and untreated w LMWH**

**Pregnancy outcomes analysed**
Tested and Treated and Control Profiles

- Control Group Median age 35. Median DOI 3 years

- Treated group (n=103) with one or both partners an M2 carrier Median DOI 5 years, Median age 36
  - Compared to a cohort of 1000 unscreened contemporaneous controls untreated w LMWH, randomly selected over the same time period and in the same clinic ratio as the treateds.

- Treated group were significantly older( p=0.0001) and longer infertile (p=<0.0001) than controls.
  - Increasing patient age is associated with a lower odds of a live birth (OR = 0.908, i.e., -9.2% per year; 95% CI = 0.882, 0.933; p<0.0001).

- 55.3%of treateds were >4years infertile v. 28.8% of 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.

None had achieved a live birth previously.

Matched on: egg recipient or standard, numbers of embryos transferred, embryo type, ♀ age, previous failed IVF cycles, previous miscarriages.
A “yardstick” group of patients who initially had an IVF failure or miscarriage but then elected to be screened for M2 and were positive were included to observe the pregnancy stage their losses occurred following embryo transfer.

Whilst they are not true controls because of selection on previous failure (71/77) they were in all other respects similar to controls except a higher incidence of 2-3 failed cycles (23.4% v 9.2% controls)
Profiles of Yardsticks

- Median age 35
- Median DOI 4 years
- 1 Previous Miscarriage 26%
- 2+ Previous miscarriage 1%
- Previous failed IVF cycles
  - 1 = 31.2%
  - 2 = 18.2%
  - 3+ = 10.4%
Summary  Outcome of Tested and Treated Group

- Most controls were:
  0-4 years infertile, first time IVF and 5% RPL

- Treateds were:
  49% 5-9 years infertile, 46.6% 2-3 failed cycles and 17.5% RPL

- Screening and treating “normalises” this group to achieve the same live birth rates of 38% per embryo cycle as statistically younger first time IVF couples.
- Statistics show their chances of a live birth were NOT affected by years infertile if identified and treated.
- Type of incubator and intralipid had no bearing on pregnancy outcome
C4M2 – treatment results

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

Simon Fishel, Deborah Baker, Janine Elson, Maha Ragunath, Glenn Atkinson, Adel Shaker, Ahmed Omar, Rahnuna Kazem, Ashley Beccles, Ian A. Greer

* CARE Fertility, John Webster House, 6 Lawrence Drive, Nottingham NG8 6PZ, UK.
Table 2
Pregnancy failures showing where losses occur in untreated groups after embryo transfer.

<table>
<thead>
<tr>
<th></th>
<th>Unscreened untreated group</th>
<th>Unscreened untreated group</th>
<th>Yardsticks</th>
<th>p-Value</th>
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<td></td>
<td>(N = 75)</td>
<td>(N = 534)</td>
<td>(N = 75)</td>
<td></td>
</tr>
<tr>
<td>Number of patients with failed live birth after embryo transfer</td>
<td>75</td>
<td>534</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Number of embryos transferred</td>
<td>106</td>
<td>774</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>Number of embryos with fetal heart activity detected</td>
<td>9</td>
<td>31</td>
<td>12</td>
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<tr>
<td>Implantation incidence (fetal hearts/ embryos transferred)</td>
<td>8.5%</td>
<td>4.0%</td>
<td>10.4%</td>
<td>0.0027</td>
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<tr>
<td>Positive pregnancy test</td>
<td>30</td>
<td>88</td>
<td>28</td>
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<tr>
<td>Biochemical pregnancy rate (per patient)</td>
<td>40.0%</td>
<td>16.5%</td>
<td>37.3%</td>
<td>&lt; 0.0001</td>
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<td>Biochemical loss/preclinical miscarriage</td>
<td>23</td>
<td>58</td>
<td>16</td>
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<tr>
<td>Biochemical loss rate</td>
<td>76.7%</td>
<td>65.9%</td>
<td>57.1%</td>
<td>0.4005</td>
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<tr>
<td>Clinical pregnancy</td>
<td>7</td>
<td>30</td>
<td>12</td>
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<tr>
<td>Clinical pregnancy rate</td>
<td>9.3%</td>
<td>5.6%</td>
<td>16.0%</td>
<td>0.0009</td>
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<tr>
<td>Clinical miscarriage</td>
<td>5</td>
<td>30</td>
<td>12</td>
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<tr>
<td>Clinical miscarriage rate</td>
<td>85.7%</td>
<td>100.0%</td>
<td>100.0%</td>
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</table>

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.
Comparison of Untreated M2 (Yardstick) Losses v Treated M2 Losses

<table>
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<th>Treated M2</th>
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<td>N=103</td>
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<tr>
<td>Implantation incidence</td>
<td>10.4%</td>
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<tr>
<td>Biochemical pregnancy</td>
<td>37.3%</td>
<td>53.4%</td>
</tr>
<tr>
<td>Biochemical loss rate</td>
<td>57.1%</td>
<td>21.8%</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>16.0%</td>
<td>41.7%</td>
</tr>
<tr>
<td>Clinical Miscarriage rate</td>
<td>100.0%</td>
<td>9.3% N=4 (1 genetic, 1 infection)</td>
</tr>
<tr>
<td>Live birth rate</td>
<td>0</td>
<td>37.9%</td>
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</table>
Although this group had high rates of pregnancy loss at various stages their implantation incidence and clinical pregnancy rates were significantly higher than controls.

However all clinical pregnancies were lost early at 5-12 weeks.

Thus they have a different pattern of pregnancy loss to unscreened patients.
Comparing couples with only males who carried M2 versus couples where only the female was an M2 carrier

The live birth outcome was statistically higher in male carrier only couples 58.3% versus 25% of Female M2 only couples (p=0.0452)
Key differences Between the ANXA5 M2 Haplotype and FVL and PT thrombophilia

- ANXA 5 M2-H is a placenta mediated complication of pregnancy (PMCP) transmitted equally by males and females, acting via the embryo and via maternal disturbance in normal annexin 5 function in female carriers.

- The other well established thrombophilias are ONLY maternal.

- They are NOT transmitted by the male

- Their incidence is far less in the general and IVF populations

- They will however exacerbate the ANXA 5 M2-H effects if present in the female
Carriers of ANXA 5 M2-H face a 2 fold higher RPL risk (when defined as 2 or more miscarriages) as compared to the general population.

The relative RPL risk grows to 3 when the number of miscarriages is taken to be 3 and above;

A 4 fold increase in risk of early pregnancy loss compared to fertile controls;

A 2 fold increase in risk of GH, PE and VTE;

A 3 fold increase in risk of restricted fetal growth;

A 2.6 fold increase in risk of SGA;

A 3 fold relative risk to develop aPL antibodies;

Present in >30% of the PCOS population.
Risks if Both Parents are Carriers or for Women who are Homozygous (Demetriou et al 2015)

- Risk appears to be “dose related” *
- Couples with a total of two M2 alleles (i.e. both partners carriers or one a homozygous) significantly risk of RPL than control couples \( (P=0.03) \)
- One or more alleles plus additional thrombophilia (APL, PT) also elevates risk

* Demetriou et al Investigation of the Annexin A5 M2 haplotype in 500 white European couples who have experienced RSA Reproductive Biomedicine Online 2015; 31( 5 ) : 681-688

- The effect of allelic dose (1 versus 2 or 3 alleles) as reported by Demetriou et al (2015) was not observed in this study.
- This may be because the allelic dose effect has been reduced by LMWH treatment
If BOTH male and female are carriers then in embryos the relative risk should be between 4 and 6, since each of the carrier parents has 50% chance of transmitting the M2 allele.

However in 47 control couples with one carrier only M2 was inherited by 30 children = 64% inheritance (Demetriou et al 2015)

For SGA ALL female homozygotes had a history of severe SGA (below the 3rd percentile) (Tiscia et al 2012)
Conclusions

- Identification of M2 carriers in both partners allows precision approach to use of LMWH
  - Include gamete donors.
- Particularly for patients aged 35 plus, those with a previous failed cycle, miscarriage, unexplained fertility
- Achieves an acceptable LB rate similar to controls (38% in current study).
CARE Fertility Group of Clinics

- Dublin
- Bolton
- Manchester
- Derby
- Birmingham
- Leicester
- Milton Keynes
- Sheffield
- Mansfield
- Nottingham
- Boston
- Peterborough
- Northampton
- London
- Tunbridge Wells
Data from control placentas suggests the high population frequency could confer survival advantages at some stage of pregnancy.

- e.g. aversion of sepsis and reduced intrapartum bleeding as reported for FVL and PT

(see Demetriou et al 2015)
Summary of Tested and Treated Outcomes

- **Treated patients live birth rate**
  - Despite their less favourable prognosis (i.e. older, greater number of miscarriages, longer duration of infertility) achieved a **live birth rate of 37.9%** compared to 38.5% in the younger unscreened controls and to the 33% in the matched controls.
  - Increasing patient age is associated with a lower odds of a live birth (OR = 0.908, i.e., -9.2% per year; 95% CI = 0.882, 0.933; p<0.0001).
  - The live birth rate compares well 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 2013 and 2014.
Recap of M2 v FVL+ PT
Incidence of ANXA5 M2-H in PGD Embryos

- 174 embryos from 22 patients
- 14 of these patients were carriers (64%)
- Out of the 174 embryo samples
  - 20 (11.5%) were found to carry the M2 allele
  - 2 (10% - 1.1%) in homozygous condition (M2/M2).
This means either the parents are both tested prior to PGD and if either is a carrier LMWH is given.

OR screening the embryo and selecting those which are not carriers.

BUT if the only viable embryo is a carrier it still means treatment.

Female carriers will also require treatment to address maternal coagulation adverse effects and prevent VTE.
They compare the M2 incidence in 313 patients with unexplained RPL to a fertile women control group of 214 subjects from Estonia and Denmark and arrive at the conclusion that M2/ANXA5 is not a RPL risk factor in Northern Europe.

the exclusion criteria for ‘unexplained RPL’ as defined by the authors did not take into account fetal chromosomal abnormalities, known to be the most common cause of early RPL. According to a fairly recent review on the subject by van den Berg et al, Biochim Biophys Acta 2012;1822:1951-9, chromosomal and submicroscopic genetic abnormalities on average are prevalent in ca. 45% of early recurrent miscarriage samples.

Control groups problem.

KORA controls do not adhere to the definition of random population controls, since they comprise of DNA samples from blood donors, defined as ‘healthy’, recruited in the Augsburg area of Bavaria, South Germany. This presents methodological age, gender and status bias. In contrast, PopGen controls are true random population controls as defined (Krawczak et al., Community Genet 2006;9:55-61).

- Haplotype reconstruction problem. Statistically deriving haplotypes from KORA genotype data usually results in incidence overestimates, even greater when common haplotypes, due to phase reconstruction errors that are inherent to array genotyping (Heidl et al., Gesundheitswesen 2005;67 Suppl 1:S132-6; Lamina et al., PLoS One. 2008;3:e1853).
From all M2/ANXA5 published research that confirms the haplotype as RPL risk factor, 3 original studies use random population controls, according to their strict definition.

the varying incidences reported for the M2 haplotype among world populations but even the very recent Malaysian study that posits 23.6% genetic incidence of M2 with a corresponding 42.2% carriage rate for Malays, agrees on the risk role of the haplotype in idiopathic RPL women and couples.

Conceptual definition problem
Genetic association studies with properly selected patient and control groups are a valid approach to deliver evidence on a risk factor, the ultimate proof of such risk, possibly conferred by M2 carriage, would be data from a randomized observational trial comparing live birth / miscarriage rates in untreated M2 carriers vs. untreated non-carriers of the haplotype. Until such data are available, no definite proof can be generated on the proposed risk role of M2/ANXA5 for RPL and thrombophilia related obstetric complications.
Conclusions

- Identification of M2 carriers in both partners allows precision approach to use of LMWH
  - Include gamete donors.
- Particularly for patients aged 35 plus, those with a previous failed cycle, miscarriage, unexplained fertility
- Achieves an acceptable LB rate of 38%.
CARE FERTILITY GROUP OF CLINICS

- Beacon CARE fertility
- Dublin
- Manchester
- Bolton
- Halifax
- Mansfield
- Sheffield
- Nottingham
- Boston
- Peterborough
- Northampton
- Birmingham
- Leicester
- Milton Keynes
- London
- Tunbridge Wells
Both reduce trophoblast apoptosis and platelet aggregation.

Both exert an antithrombotic effect but via different mechanisms –

Annexin A5 provides a membrane repair mechanism for the syncytiotrophoblasts thus preventing coagulation and fibrin deposition (1)

LMWH facilitates an antithrombin anticoagulant effect, enhances endometrial angiogenesis and reduces complement activation (2)

LMWH can also modulate many mechanisms for apposition, adhesion and penetration of the developing embryo

Clinically APS syndrome patients respond well to LMWH treatment =+/- aspirin
Comparison of Untreated M2 (Yardstick) Losses v Treated M2 Losses

<table>
<thead>
<tr>
<th></th>
<th>Untreated M2</th>
<th>Treated M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>N= 75</td>
<td>N=103</td>
</tr>
<tr>
<td>Implantation incidence</td>
<td>10.4%</td>
<td>33.1%</td>
</tr>
<tr>
<td>Biochemical pregnancy rate</td>
<td>37.3%</td>
<td>53.4%</td>
</tr>
<tr>
<td>Biochemical loss rate</td>
<td>57.1%</td>
<td>21.8%</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>16.0%</td>
<td>41.7%</td>
</tr>
<tr>
<td>Clinical Miscarriage rate</td>
<td>100.0%</td>
<td>9.3%</td>
</tr>
<tr>
<td>N=4 (1 genetic, 1 infection)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live birth rate</td>
<td>0</td>
<td>37.9%</td>
</tr>
</tbody>
</table>
Key differences Between the M2 Haplotype and FVL and PT Thrombophilia

- M2 is a placenta mediated complication of pregnancy (PMCP), transmitted equally by males and females, acting via the embryo and via maternal disturbance in normal annexin A5 function in female carriers.
- The other well established thrombophilias are **ONLY** maternal.
- They are NOT transmitted by the male.
- Their incidence is far less in the general and IVF populations.
- They will however exacerbate the M2 effects if present in the female.
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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h Faculty of Medicine & Health, University of Nottingham, Jubilee Campus, Nottingham, NG11 8GZ, UK
i Ashley Beccles, Consultant, Wrexham Maelor Hospital, Wrexham, North Wales, Wrexham, LL13 1QY, UK
What is the biological plausibility for coagulation changes causing placental mediated pregnancy complications (PMPC)?

- Overlap of features of PMPC
  - Deficient implantation
  - Placental infarction.
  - Microvascular thrombosis
  - Women with RPL more likely to have SGA baby (Canda et al, 2012)

- Are these different ‘downstream’ manifestations of common pathological processes triggered by a variety of primary problems?
  - Is coagulation a common pathway for PMPC?
  - Vascular thrombosis or non-thrombotic mechanisms?
  - Link between heritable thrombophilia and PMPC
Pragmatic, multicentre, randomised, controlled trial in women with a history of ≥2 consecutive unexplained losses at ≤24 weeks

- Enoxaparin / LDA vs intensive pregnancy surveillance
- Enoxaparin / low-dose aspirin plus intensive surveillance group (n=147)
  - 32 pregnancy losses in 143 subjects.
- Intensive surveillance alone (n=147)
  - 29 losses were observed in 140 subjects.
- OR for successful pregnancy 0.91 (CI 95% 0.52-1.59) in those randomized to LMWH/LDA compared with intensive surveillance alone.
Anticoagulants for Living Fetuses (ALIFE)

- Women with a history of ≥2 unexplained miscarriages randomised to receive a) low-dose aspirin + LMWH (nadroparin), b) low-dose aspirin alone, or c) aspirin placebo.
  - aspirin started at randomization in the aspirin-only and combination-therapy groups, LMWH started at 6 weeks gestation after confirmation of viable pregnancy.
- 364 women who underwent randomization, 299 pregnancies. Trial halted early on the basis of futility.
- Neither aspirin combined with nadroparin nor aspirin alone improved the rates of live births compared with placebo.
- Live-birth rates in women who became pregnant were
  - 69.1% (relative risk, 1.03; 95% CI 0.85 to 1.25) for combination-therapy group
  - 61.6% (relative risk, 0.92; 95% CI 0.75 to 1.13) in the aspirin-only group
  - 67.0% in the placebo referent group

Heparin in pregnant women with Adverse Pregnancy outcome to improve the rate of successful Pregnancy (the HAPPY trial)

- 135 women randomized to treatment with nadroparin + surveillance, or surveillance alone
- 21% of active treatment group developed a combined end point vs 18% of controls.
- Event risk difference of 2.2 (95% CI: -11.6 to 16.0).
- Distribution of components of the composite end point (PET, eclampsia, HELLP, FGR, IUD, & abruption) was also similar.

Martinelli et al *Blood* 2012; Greer *Blood* 2012
NOH-AP & NOH-PE studies

- NOH-AP - 160 women
  - Abruption in 1st pregnancy.
    - 16.3% with thrombophilia
  - LMWH vs. no LMWH
    - LDA at discretion of the treating physician (n=48).
  - Composite outcome:
    - PE; IUGR/SGA ≤ the 5th percentile; abruption; IUFD after 20 weeks
      - **Enoxaparin 12.5%**
      - **No enoxaparin 31.3%**
- NOH-PE -224 women
  - Severe PE in 1st pregnancy.
    - 14.2% with thrombophilia
  - LMWH/LDA vs. LDA
  - **Preeclampsia**
    - **LMWH 5.8%**
    - **Control 16.7%**
  - **Severe PE:**
    - **LMWH 0.9%**
    - **Control 7.1%**
**FRUIT** - 139 women with Thrombophilia + previous delivery at <34/52 for preeclampsia/SGA

- LDA/LMWH vs LDA
- Recurrent HD at < 34 weeks lower with LMWH
  - risk difference 8.7% [CI 1.9–15.5%; P . 0.012]
- Reduced steroids but no difference to clinical outcome (De Vries et al. *J Thromb Haemost* 2012)

**TIPPS:** (Rodger et al 2014) Women with thrombophilia and previous PMPC or VTE randomised to LMWH or no treatment

- No difference between the LMWH and controls groups
Systematic review of LMWH in PMPC

LMWH and PMPC

- Rodger et al. *Blood* 2014;123:822-8
- Meta-analysis of LMWH to prevent recurrent PMPC
  - 6 studies included, question over generalisability
- Primary Outcome:
  - composite endpoint – PE, SGA(<10thC), Abruption, Pregnancy loss >20 week
- Result
  - Overall 18.7% recurrence rate with LMWH vs 42.9% controls
  - RRR 0.52 (95%CI 0.32-0.86) for composite endpoint
  - Similar effects with secondary outcomes, PE, SGA and preterm delivery
- Implications: LMWH may be of value for women with previous PMPC but corroboration required with high quality multicentre studies
LMWH and adverse pregnancy outcome: Are we missing something?

- Benefits may be limited to particular phenotypes or genotypes
  - Women with three or more recurrent miscarriages, who may represent a more homogeneous group
  - Specific thrombophilias and their interaction with disease
  - Thrombotic damage such as placental infarction
- Are there biomarkers or phenotypes to guide treatment?
- Current treatment is imprecise, generalized by disease outcome and not stratified by maternal (or paternal) characteristics or the disease mechanism.
- While awaiting randomised trials we must avoid medicine based on hope rather than evidence as we await precision medicine approaches for placental mediated pregnancy complications
Annexin A5 M2 Haplotype

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

Simon.fishel@carefertility.com
<table>
<thead>
<tr>
<th>Time</th>
<th>Session 6: Carrier screening and Personalized medicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>08:30-10:00</td>
<td>Chair: Eugene Pergament, USA</td>
</tr>
<tr>
<td>08:30-09:00</td>
<td><em>Carrier screening of recessive disease - targeted vs. expanded carrier screening</em></td>
</tr>
<tr>
<td>09:00</td>
<td>Jim Goldberg, USA</td>
</tr>
<tr>
<td>09:00-09:30</td>
<td><em>Carrier screening</em></td>
</tr>
<tr>
<td>09:30</td>
<td>Julio Martin, Spain</td>
</tr>
<tr>
<td>09:30-10:00</td>
<td>Mosaicism - a natural phenomenon of the human embryo: knowing its existence enhances IVF outcome. &quot;</td>
</tr>
<tr>
<td></td>
<td>Josh Blazek, USA</td>
</tr>
</tbody>
</table>
Reduced placental expression of Annexin A5 caused by C4/M2 carriage in males or females is responsible for a range of thrombophilia related obstetric complications.

It is a biomarker for placenta mediated pregnancy complications (PMPC).
Slide 34 is of note as it is the yardstick table showing where untreated M2 patients lose their pregnancies and how the pattern differs from control patients. i.e they implant better, have a better clinical pregnancy rate but then lose them.

- I have removed SGA and left in PGD.
- Note we have **also updated slide 19 showing where clinical pregnancies are lost** – mainly between 5 to 12 weeks Range 5 -23 (few after 15 weeks)
- General properties

Member of the annexin protein family

Ca\(^{2+}\)-dependent binding to anionic phospholipids

Highest levels in:

Extracellular presence, anticoagulant properties
Immunostaining for annexin A5 protein in placental tissue sections

Chorionic villi were stained with a mouse anti-human annexin A5 monoclonal antibody.

A. Non-M2-carrying placentas from a non-M2-carrying mother.
B. Non-M2-carrying placentas from an M2-carrying mother.
C. M2-carrying placentas from a non-M2-carrying mother.
D. M2-carrying placentas from an M2-carrying mother.

Scale bars, 100 μm.
Fig. 5

Blood M2(-)
Placenta M2(+)  Blood M2(+)
Placenta M2(-)
Fig. 6

### Maternal blood

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N/N</th>
<th>N/M2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/N</td>
<td>3.4 (26)</td>
<td>4.0 (2)</td>
<td>3.7 (28)</td>
</tr>
<tr>
<td>N/M2</td>
<td>12.0 (3)</td>
<td>8.0 (3)</td>
<td>10.0 (6)</td>
</tr>
<tr>
<td>Total</td>
<td>7.7 (29)</td>
<td>6.0 (5)</td>
<td></td>
</tr>
</tbody>
</table>

**A**

**B**

- $P=0.004$
- $P=0.162$
- Placental expression of ANXA5

Schematic representation of the chorionic villi. FC fetal circulation, CT cytotrophoblast, ST syncytiotrophoblast, M mesenchymal cells, ECT extravillous cytotrophoblast, DC decidual cells, UA Uterine artery, IVS intervillous space.

Summary of Case and Control Genotyping Conducted to date:

Total cases 4,592
Total controls 2,577

1) Muenster
Affected patients 2,750 (includes German, French, Bulgarian, Dutch, Italian, Spanish and Mahgreb - African ethnicities)
Controls: 1,100 German
200 Bulgarian
360 Malay

2) Italian Case control Studies
(Chinni et al 09, Tiscia et al 09, Grandone et al 2010, Tiscia et al 2012)
Total cases 502
Total controls 195

3) UK (Imperial College)
Cases RPL (male and female early and late loss) 996
Controls male and female placentas (trios) 482

4) Japan - Miyamura et al 2011 Ota et al 2013
RPL cases 243 M2 Placentas and blood samples 101
Controls 119 Controls 121
Spiral artery remodelling: Remodeling of the uterine arteries is a key event in early pregnancy that begins after implantation.

Beneath the syncytiotrophoblasts are the cytotrophoblasts. These cells are the stem cells for syncytiotrophoblasts.

Cytotrophoblasts continually differentiate into syncytiotrophoblasts during villous formation and development.

Cytotrophoblast invasion into the uterine spiral arteries is accompanied by loss of the endothelial lining and musculoelastic tissue in these vessels.

This process of invasion is necessary for placental vascular remodelling in the early stages of the implantation process.

Trophoblastic invasion of uterine spiral arteries normally starts at around 8–10 weeks and completes by 20-22 weeks of gestation in normal pregnancy.
- The annexin A5 gene variants
- initially found through systematic sequence analysis of the ANXA5 gene in 70 patients of Northern German origin with RPL (defined as more than two fetal losses), non-carriers of PTm and/or FVL mutations. In the meantime, since the original publication, more than 4500 patients of different ethnicities and various obstetric complications have been genotyped, where the associated risk in C4/M2 carriers is between 2.4 and 4 for the various thrombophilia related phenotypes.

**ANXA5**

- **N** (-19G, 1A, 27T, 76G)
- **M1** (-19G, 1C, 27C, 76G)
- **M2** (-19A, 1C, 27C, 76A)