Reduced plasma fibrinolytic potential in patients with recurrent implantation failure after IVF and embryo transfer

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BACKGROUND: Recurrent implantation failure (RIF) following embryo transfer (ET) is a major continuing problem in IVF. Women with haemostatic defects may be at increased risk of miscarriage and preclinical pregnancy loss. The fibrinolytic system is considered, at present, the key to new thrombotic pathogenic mechanisms. Patients with unexplained recurrent miscarriage have an impairment of fibrinolysis, as demonstrated by prolonged clot lysis time (CLT) in association with increased plasma levels of thrombin-activatable fibrinolysis inhibitor (TAFI). In this study, we investigated fibrinolytic potential in patients with RIF.

METHODS: Three groups of patients were studied: 30 women with RIF (RIF group), 60 patients undergoing a first successful IVF–ET cycle (IVF group) and 60 healthy fertile women (FER group). Plasma CLT was measured using a global fibrinolysis assay. TAFI antigen plasma levels and polymorphisms in the TAFI gene (+505A/G and +1542C/G) were analysed using enzyme-linked immunosorbent assay and allele-specific PCR, respectively.

RESULTS: CLT was significantly longer ($P < 0.0001$ and $P < 0.0009$, respectively) and TAFI antigen levels were significantly higher (both $P < 0.0001$) in the RIF versus the IVF and FER groups. A direct relationship between CLT and TAFI antigen levels ($r = 0.40; P = 0.001$) was detected in the whole study population. There were no differences in distribution of TAFI polymorphisms between groups.

CONCLUSIONS: Patients with RIF have reduced plasma fibrinolytic potential, as shown by a prolonged CLT, and this may be explained, at least in part, by increased TAFI antigen levels.

Key words: clot lysis time / IVF / recurrent implantation failure / recurrent reproductive failure / thrombin-activatable fibrinolysis inhibitor

Introduction

Despite recent advances in reproductive medicine, human reproductive failure remains a surprisingly frequent event. Early embryonic mortality in humans is very high and it has been postulated that the largest single cause of failed pregnancy is an error of implantation (Bulletti et al., 1996; Bischof et al., 2006; Christiansen et al., 2006). The rate of spontaneous miscarriage may be as high as 60–80% if one takes into account those miscarriages occurring within the first months of conception, which usually go undetected by patients (Bulletti et al., 1996; Choudhury and Knapp, 2000).

Miscarriages affect 15% of women, primarily in the first trimester, and while most are sporadic and non-recurring, there is a subset comprising 2–5% of couples that suffers recurrent miscarriage (RM) (Clark et al., 2001). These repetitive losses suggest the presence of a specific cause, and much work has been carried out to try to identify the underlying mechanisms. However, the current medical literature suggests that causes are identified in only ~50% of patients (ACOG, 2002; Li et al., 2002). Thus, recurrent pregnancy loss is a vexing problem facing many couples and doctors.

Similarly, implantation failure following embryo transfer (ET) is a major continuing problem in IVF. Thus, it has been disappointing that only ~20% of transferred human embryos resulting from IVF implant in the uterus despite the selection of apparently normal embryos for transfer (International Committee for Monitoring Assisted Reproductive Technology (ICMART, 2009); Nyboe Andersen et al., 2009). There are some couples who failed to conceive with IVF treatment despite repeated transfers of good-quality...
embryos, and these couples are described as having recurrent implantation failure (RIF).

Remarkably, as recently stressed, it is possible that RM and RIF may, in some situations, represent different manifestations of the same pathogenic spectrum and in fact it has been documented that some abnormalities are associated with both implantation failure and miscarriage (Christiansen et al., 2006; Stern and Chamley, 2006). Thus, it is postulated that women with haemostatic defects may be at an increased risk of miscarriage and preclinical pregnancy loss, and research aiming to clarify thrombophilic causes of RM and RIF is encouraged (Christiansen et al., 2006; Stern and Chamley, 2006).

At present, the fibrinolytic system is considered the key to new thrombotic pathogenic mechanisms (Zorio et al., 2008). Routine laboratory assays of fibrinolytic factors provide static data and therefore have been of limited diagnostic use (He et al., 1999). In contrast, global coagulation assays provide a source of information that assesses changes over time in the balance of the fibrinolytic system (Curnow, 2006; Wichers et al., 2009). Clot lysis time (CLT) is a global test to evaluate fibrinolysis (Lisman et al., 2005; Wichers et al., 2009). Impaired fibrinolysis increases CLT, and elevated thrombin-activatable fibrinolysis inhibitor (TAFI) antigen levels may contribute to this in part (Wang, 1998).

TAFI is a procarboxypeptidase B-like protease, which is a main inhibitor of fibrinolysis and a known contributing factor in the development of thrombotic events (Bouma and Meijers, 2003). Several polymorphisms have been identified in the TAFI gene (Franco, 2001; Henry, 2001). Among TAFI polymorphisms, +505A/G and +1542C/G (Henry, 2001) have been the most thoroughly studied, and associations have been reported between these polymorphisms and plasma concentrations of TAFI (Franco, 2001; Henry, 2001). Both TAFI levels and TAFI polymorphisms have been related to arterial and venous thrombosis. Plasma hypoﬁbrinolysis, explained by elevated plasma levels of TAFI and plasminogen activator inhibitor-1 (PAI-1), has recently been reported as a cause of venous thrombosis (Meltzer et al., 2010).

Very recently, we have reported for the first time that patients with unexplained RM have an impairment of fibrinolysis, as demonstrated by a prolonged CLT, which can at least be partly explained by higher TAFI antigen levels (Martínez-Zamora et al., 2010). Therefore, this study was undertaken to investigate CLT, TAFI antigen plasma levels and TAFI polymorphisms in patients with unexplained RIF. The CLT, a global fibrinolysis assay, is considered to be a better method for detecting the risk of venous and arterial thrombosis than traditional laboratory testing of fibrinolytic factors (Curnow et al., 2006; Meltzer et al., 2009; Wichers et al., 2009).

Three groups of patients were considered. The study group consisted of 30 women diagnosed as having RIF (RIF group) on the basis of the following criteria. All women have had ≥3 failed ET attempts (range: 3–6) replacing ≥1 high-quality (grades I and II) fresh or frozen-thawed embryos each. All patients underwent at least two fresh ET. Embryos were classified, as we previously reported (Creus et al., 2003; Puerto et al., 2003), as follows: grade 1: perfectly symmetrical with no fragmentation; grade 2: perfectly symmetrical with slight fragmentation (<20% fragmentation of the total embryonic volume); grade 3: uneven blastomeres with no fragmentation; grade 4: uneven blastomeres with gross fragmentation (>20% fragments). Embryos of grades 1 or 2 were considered high quality and they were at the 4-cell or 8-cell stage on Day 2 or 3 post-fertilization, respectively. For statistical comparison purposes and to quantify objectively the embryo quality, embryos of grades 1–4 were scored 2.5, 2, 1.5 and 1, respectively, as previously reported (Puerto et al., 2003). For the final analysis of results, the embryo score per ET was considered as the mean value of the scores given to each of the transferred embryos. All the ETs were performed by senior physicians with ultrasonographic guidance. A difficult ET was defined as previously reported (Puerto et al., 2003).

Patients in the RIF group were screened and found to be unaffected by systemic diseases, diabetes mellitus, thyroid dysfunction, polycystic ovary disease, thrombophilia (plasma levels of protein S and C, plasminogen and tissue plasminogen activator (t-PA), factor V Leiden and prothrombin G20210A mutations, acquired protein C resistance and antiphospholipid antibodies) and endometrial and cervical infection. The RIF group also showed no abnormalities in parental chromosome assessment, and uterine and endometrial morphology.

Controls included two groups of women. The IVF group (n = 60) comprised the nearest patient undergoing a first successful IVF–ET cycle before and after each ET defining a patient as an RIF (i.e. the closest IVF–ET cycles resulting in live birth and being in temporal relationship with the third failed ET for each patient in group RIF). A fertile control group (FER group) included 60 healthy women who had at least one child born at term and no history of infertility or miscarriage. The FER group was recruited from women who requested surgical sterilization at our hospital. No patient had taken medications known to affect plasma CLT for ≥6 months before the study.

To the best of our knowledge, this study is the first to investigate CLT in patients with RIF. The sample size was based on our previous study, which showed an impairment in fibrinolysis, as demonstrated by prolonged CLT, in patients with unexplained first-trimester RM compared with fertile controls (Martínez-Zamora et al., 2010). The sample size required to provide power of 80% to detect at least a similar magnitude of difference between groups using a case–control study design (1:2) was calculated to be ≥15 cases and ≥30 controls, using a two-tailed analysis with a detection limit of 5% of avoiding a type-I error in hypothesis testing. As it is the policy of our laboratory to store blood samples from IVF cycles carried out during the previous 12–14 months, at the time of designing this study, appropriate frozen blood samples were available for 30 patients fulfilling the criteria of RIF provided above. Thus, 60 subjects were included in both control groups (IVF and FER).

Blood collection

In our IVF programme, women have blood samples routinely drawn during the early follicular phase of their cycle within 3 months of the IVF attempt. For the specific purpose of this study, the time point of blood sampling was the same in fertile controls. Thus, in all subjects included in the current investigation, blood samples were obtained on menstrual cycle days 2–4, between 0800 and 1000 h after overnight fasting from food, liquids and cigarettes (only water was allowed). Venous blood samples

Materials and Methods

Study populations and design

This study was a retrospective analytic investigation of frozen blood samples prospectively collected from 150 women at the Hospital Clinic of Barcelona. A total of 90 infertile patients undergoing IVF treatment at the Assisted Reproduction Unit of our hospital and 60 healthy women fulfilling inclusion criteria reported below were included. All the women involved gave informed consent to participate in the present study, which was approved by the Ethics Committee of our hospital.
for coagulation and fibrinolysis studies were collected in tubes containing 3.8% trisodium citrate (1/9 volume/volume; Becton Dickinson, Rutherford, NJ, USA), and platelet-free plasma was immediately obtained by double centrifugation, first at 2000g for 10 min at 22°C, and then at 5000g for 10 min at 4°C. Plasma was aliquoted, snap-frozen in a mixture of dry ice/ethanol (1/2 volume/volume) and stored. For genotype studies, samples were drawn in trisodium EDTA tubes (Becton Dickinson) and 100 μl of whole blood was immediately transferred into tubes containing lysis buffer [5 M guanidine thiocyanate, 1.3% (weight/volume) Triton X-100 and 50 mM Tris–HCl, pH 6.4] and frozen at −80°C.

Fibrinolysis parameters

TAFI antigen levels were determined by an enzyme-linked immunosorbent assay that is known to detect all the isoforms of TAFI (Gils, 2003) (Asserachrom TAFI, Diagnostica Stago, Asnieres, France).

The CLT, which is the lysis of a thrombin-induced fibrin clot by exogenous t-PA, was studied by monitoring changes in turbidity during clot formation and subsequent lysis. Plasma CLT was measured as previously described, with modifications (Lisman, 2002). Briefly, 75 μl of a mixture containing thrombin (0.2 U/ml), 40 ng/ml of t-PA (Acytilyse, Boehringer Ingelheim, Germany), 12.5 mM CaCl2 and HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 3 mM CaCl2, 0.1% bovine serum albumin, pH 7.4) was added to 75 μl of citrated platelet poor plasma. After thorough mixing, turbidity at 405 nm was measured in time (minutes) at 37°C in a Multiskan Ascent (Thermolab Systems). Changes in optical density at 405 nm were monitored every 3 min. CLT was defined as the time from the midpoint of the clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbidity to clear transition, which represents clot lysis. Samples were tested in duplicate.

Genetic analyses

For detection of TAFI polymorphisms, genomic DNA was extracted from 100 μl of whole blood by a silica gel column method (QIAamp DNA Blood Mini Kit, Qiagen GmbH, Hilden, Germany).

TAFI +505A/G and +1542C/G polymorphisms were determined by allele-specific PCR as previously reported (Henry, 2001) with minor modifications. For the +1542C/G polymorphism, the following primers were used: forward primer: 5′-CCA GCA AGA CCA AAT CA-3′; reverse primer: 5′-ATT ACC GTG GAG CAA AC-3′; C allele (reverse) primer: 5′-AGT CAA ACA AGG TGC AAA CT-3′; G allele (reverse) primer: 5′-AGT CAA ACA AGG TGC AAA CT-3′. PCR was carried out in 50 μl samples, with 40 cycles at 95°C for 60 s, 55°C for 60 s and 72°C for 60 s. PCR products were separated by electrophoresis in a 2.5% agarose gel and visualized under UV light after staining with ethidium bromide. The expected size of the products was a common 408-bp band and a 238-bp band specific for the C or the G allele.

For the +505A/G polymorphism, the following primers were used: a allele primer: 5′-GTT TCT GGA AAA GAA CAA A-3′; G allele primer: 5′-GTT TCT GGA AAA GAA CAA G-3′; common reverse primer: 5′-ATG GCC TAT GGA CCA AAT C-3′. PCR was carried out in 50-μl samples, with 40 cycles at 95°C for 60 s, 58°C for 60 s and 72°C for 60 s. PCR products were analysed by electrophoresis in a 4% agarose gel. The expected size of the products was 105 bp.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences software, Release 15.0 for Windows (SPSS, Chicago, IL, USA). Comparison of quantitative variables was performed using analysis of variance with Bonferroni’s post hoc analysis. Comparison of qualitative variables was carried out using the χ² test. Correlation between quantitative variables was assessed by the Pearson’s test. Statistical significance was defined as $P < 0.05$. Results are presented as mean ± SD or median and range.

Results

Clinical characteristics of subjects

Clinical characteristics of the three groups studied are presented in Table I. There were no differences among groups for age, BMI and smoking habit. Also, causes and duration of infertility, the day of ET, the number and quality of embryos replaced, and the number of difficult ETs were similar in the RIF and IVF groups.

CLT, TAFI antigen levels and TAFI polymorphisms

CLT was significantly longer in the RIF group compared with the IVF and FER groups and, similarly, TAFI antigen levels were significantly higher in the study group when compared with both control groups (Fig. 1). A significant direct relationship was found between CLT values and TAFI antigen levels ($r = 0.40; P = 0.001$) in the whole study population (Fig. 2).

Allele distribution of TAFI polymorphisms did not differ among the groups (Table II). Plasma TAFI antigen levels were significantly higher in patients with RIF when compared with IVF and FER groups,

| Table I Clinical characteristics of the three groups of patients analysed. |
|-----------------|----------------|----------------|----------------|
| Parameter       | RIF group (n = 30) | IVF group (n = 60) | FER group (n = 60) | P  |
| Age (years)     | 34.1 ± 2.3        | 33.8 ± 3.1        | 32.1 ± 3.9        | NS |
| BMI (kg/m²)     | 23.9 ± 4.2        | 24.3 ± 2.7        | 24.1 ± 2.4        | NS |
| Smoker          | 12 (40)           | 28 (47)           | 20 (33)           | NS |
| Duration of infertility (years) | 4.9 ± 1.2 | 4.2 ± 1.9 | NS |
| Infertility factor |               |               |               |    |
| Male factor     | 6 (20)            | 12 (19)          | NS              |    |
| Tubal factor    | 13 (43)           | 28 (47)          | NS              |    |
| Unexplained     | 5 (17)            | 10 (17)          | NS              |    |
| Endometriosis   | 6 (20)            | 10 (17)          | NS              |    |
| Day of ET       |                  |                  |                 |    |
| +2              | 23 (76.6)         | 48 (80)          | NS              |    |
| +3              | 7 (23.4)          | 12 (20)          | NS              |    |
| No. of embryos per replacement | 2.5 ± 0.5 | 2.3 ± 0.4 | NS |
| High-quality embryos replaced | 1.6 ± 0.2 | 1.5 ± 0.2 | NS |
| Embryo score/ replacement | 2.1 ± 0.2 | 2.2 ± 0.3 | NS |
| Difficult ET    | 2 (6.7)           | 2 (5)            | NS              |    |

RIF group: patients with recurrent implantation failure; IVF group: infertile patients achieving a live birth on the first IVF/ET attempt; FER group: fertile controls. Values are mean ± SD or n (%).
irrespective of polymorphisms investigated (Fig. 3). As expected, TAFI antigen levels were significantly higher in women with +1542 G/G and +505 A/A versus other genotypes (Franco, 2001; Henry, 2001) (Fig. 3).

**Table II** Allele distribution of TAFI polymorphisms in the three groups of patients studied.

<table>
<thead>
<tr>
<th>Group</th>
<th>TAFI +505A/G</th>
<th>TAFI +1542C/G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>GA</td>
</tr>
<tr>
<td>RIF (n=30)</td>
<td>3 (10)</td>
<td>15 (50)</td>
</tr>
<tr>
<td>IVF (n=60)</td>
<td>6 (10)</td>
<td>25 (42)</td>
</tr>
<tr>
<td>FER (n=60)</td>
<td>7 (12)</td>
<td>26 (43)</td>
</tr>
</tbody>
</table>

Values are n (%).

Figure 1 Box plot showing CLT and TAFI antigen levels in RIF patients (RIF group, n = 30), infertile patients achieving a live birth with their first IVF/ET attempt (IVF group, n = 60) and fertile controls (FER group, n = 60). Each box represents the middle 50% of the data (25–75% range). The central horizontal line represents the median. Vertical lines represent the 10–90% range of data, as indicated by the small horizontal lines. Statistical comparisons between groups are indicated (NS, not significant).

Figure 2 Relationship between CLT values and TAFI antigen levels ($r = 0.40; P = 0.001$) in the whole study population (n = 150).

Figure 3 TAFI antigen levels (mean ± SD) according to the genotypes of two TAFI polymorphisms (+1542C/G and +505A/G) in the three groups of patients. Results with common superscripts were significantly different ($^{a-h, p, y, z} P < 0.0001; P = 0.001$) (TAFI antigen levels were significantly higher in genotypes +1542 G/G and +505 A/A compared with the respective patient group with the other genotypes, $P < 0.001$).

irrespective of polymorphisms investigated (Fig. 3). As expected, TAFI antigen levels were significantly higher in women with +1542 G/G and +505 A/A versus other genotypes (Franco, 2001; Henry, 2001) (Fig. 3).
Discussion

The haemostatic pathways are intimately involved in ovulation, implantation and placentation and thus, the hypothesis has been developed that many cases of recurrent reproductive failure (i.e. RM and RIF) are caused by a defective maternal haemostatic response leading to thrombosis of the uteroplacental vasculature (Rai, 2003; Christiansen et al., 2006; Stern and Chamley, 2006). The precise mechanism by which thrombophilia may affect RIF, however, is as yet undetermined. Women with a history of RM are in a prothrombic state even outside pregnancy and coagulation changes precede pregnancy loss in pregnant women with a history of RM (Regan and Rai, 2002; Rai, 2003; Sebire et al., 2003). While the mechanisms by which thrombophilic factors impact on the frequency of RM are thought to be mainly related to clotting in placental vessels, the methods involved in RIF appear to involve the effects of hypofibrinolysis on trophoblast migration (Coulam et al., 2006; Coulam and Jeyendran, 2009).

The potential implication of fibrinolytic defects in recurrent reproductive failure was first suggested in the early 1990s, but research in this field has only recently undergone a systematic review (Sotiriadis et al., 2007). The fibrinolytic system includes a broad spectrum of proteolytic enzymes with physiological and pathophysiological functions in several processes such as haemostatic balance, tissue remodelling, tumour invasion, angiogenesis and reproduction (Zorio et al., 2008). As early as implantation, the fibrinolytic system participates in the regulation of early human trophoblastic migration and invasion (Coulam et al., 2006; Sotiriadis et al., 2007). Trophoblastic invasion during implantation involves extracellular matrix (ECM) degradation, which is facilitated by matrix metalloproteinases. Expression of metalloproteinases at the implantation site is stimulated by the serine protease plasmin (Coulam et al., 2006; Sotiriadis et al., 2007). Plasmin promotes trophoblastic invasion also by directly degrading certain components of the ECM of decidua (Sotiriadis et al., 2007). Therefore, fibrinolytic abnormalities are likely to result in decreased trophoblast invasion and implantation failure.

Plasmin is the main enzyme of the plasminogen activator system and it is responsible for the degradation of fibrin into soluble degradation products. The activation of plasminogen into plasmin is mediated by two types of activators, urokinase-type plasminogen activator and tissue-type plasminogen activator, and the activity of both types is regulated by specific PAIs. Plasmin can be inhibited by specific plasma inhibitors (mainly α2-antiplasmin and also α2-macroglobulin). Fibrinolysis is also reduced by TAFI, which acts as an inhibitor of tissue-type plasminogen activator (Zorio et al., 2008).

According to the above evidence, there is a biological basis for a causative role of fibrinolytic disorders in recurrent reproductive failure. The current investigation is the first report showing increased TAFI antigen levels in patients with RIF, and this was associated with an impairment of fibrinolysis, as demonstrated by an increased CLT. Therefore, our results are in agreement with previous studies suggesting that hypofibrinolysis may be a parameter involved in early reproductive failure, either in the form of RM or RIF (Gris et al., 1997; Coulam et al., 2006; Sotiriadis et al., 2007; Martinez-Zamora et al., 2010). This notwithstanding, it should be stressed that different determinants of CLT exist. Thus, a recent study (Meltzer et al., 2010) showed that PAI-1 levels were the main determinants of CLT, followed by TAFI and other fibrinolytic factors: a clear increase in CLT with increasing plasma levels of PAI-1, TAFI, α2-antiplasmin and decreasing levels of plasminogen was found. In simple regression analyses, all fibrinolytic factors except plasminogen were associated with CLT, the strongest association being found with PAI-1, which, as an independent factor, explained 40% of the variation in CLT followed by TAFI levels, while including all fibrinolytic factors in a multiple regression model increased the explained variance to 53% (Meltzer et al., 2010). In addition, as stressed by the authors themselves (Meltzer et al., 2010), while the role of PAI-1 in venous thrombosis is controversial, TAFI (which defines the molecular connection between the coagulation and fibrinolytic cascades) is clearly an independent risk factor for venous thrombosis. Finally, studies using a clot lysis assay and a model of thrombus lysis (Mutch et al., 2007) have shown a substantial and complementary, but approximately equal, role for PAI-1 and TAFI in the regulation of thrombus lysis. Therefore, although PAI-1 levels were not measured in the current investigation, the above evidence and the direct relationship found between CLT values and TAFI antigen levels in our study allow us to conclude that the impairment in fibrinolysis observed in RIF patients may be explained, at least in part, by increased TAFI antigen levels.

A feature of the present investigation is that we included only patients who had at least three failed ETs in which reasonably high-quality embryos were transferred. Including patients after only two failed attempts may represent a bias (El-Toukhy and Tararissi, 2006), while an increasing number of authors consider that inability to produce good-quality embryos is itself an important predisposing factor for RIF (Ola and Li, 2006). Also, the case–control study design used (where each case was sampled with two controls at the date when the corresponding case was entered into the IVF programme) helps us to preclude any bias owing to possible changes in IVF laboratory techniques. In addition, women were only recruited for this study if the screening investigations reported above excluded a possible contributing factor for their recurrent reproductive failure (Quenby et al., 2009). This notwithstanding, it could be argued that embryonic aneuploidy, even in couples with normal karyotype, may be a cause of RIF (Margalloith et al., 2006) and preimplantation genetic screening (PGS) was not performed in our study. However, at present, it is widely accepted that there is no evidence that routine PGS is beneficial for patients with RIF (Practice Committee of Society for Assisted Reproductive Technology and The Practice Committee of American Society for Reproductive Medicine, 2008; ACOG, 2009; Harper et al., 2010). Finally, if fibrinolysis was critical to implantation it could be postulated that correction of the supposed defect should reduce miscarriage rates. Recent RCTs showed no benefit of aspirin or heparin in the maintenance of pregnancy in women with idiopathic reproductive failure (Laskin et al., 2009; Clark et al., 2010; Kaandorp et al., 2010). However, it has been previously reported that heparin is unable to stimulate fibrinolysis through a TAFI-dependent mechanism (Colucci et al., 2002) while chronic high-dosing (650 mg every 12 h) with aspirin is necessary to increase susceptibility of fibrin clots to lysis in human subjects (Bjornsson et al., 1989). In contrast, in the RCTs the patients received low-dose (75–80 mg daily) aspirin treatment. In addition, while we have previously reported that aspirin treatment is an independent and significant prognostic factor associated with favourable outcome in patients with fetal losses related to thrombophilia only when used before conception (≥1 month before attempting conception).
(Carmona et al., 2001), in the RCTs (Laskin et al., 2009; Clark et al., 2010; Kaandorp et al., 2010) patients started treatment usually once pregnancy was confirmed by ultrasonography at 6–7 weeks’ gestation.

In conclusion, patients with RIF have a reduced plasma fibrinolytic potential, as shown by a prolonged CLT. Analysis of CLT is considered as a better approach to detecting the risk of thrombosis than traditional laboratory testing of fibrinolytic factors. The prolonged CLT may be explained, at least in part, by increased TAFI antigen levels.

**Authors’ roles**

M.A.M.-Z. participated in study design and execution, interpretation of data, manuscript drafting and critical discussion. M.C. has contributed to study execution, analysis and interpretation of data and critical revision. D.T. and J.C.R. have contributed to study design and execution, and analysis and interpretation of data. S.C. participated in study execution, analysis of data and critical revision. F.C. has contributed to study design, interpretation of data, critical revision and provided statistical analysis. J.B. developed the idea for the paper, formulated the study design, participated in the analysis and interpretation of data and wrote the manuscript. All authors have approved the final version of the manuscript. The authors declare no financial or commercial interests involved in this study.

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