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Preliminary evidence for high anti-PLAC1 antibody levels in infertile patients with repeated unexplained implantation failure

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ABSTRACT

Objective: Placenta-specific1 (PLAC1) is a trophoblast-specific gene encoding for a protein that is highly expressed in human placenta, on the surface of the syncytiotrophoblast. PLAC1 was found to elicit spontaneous antibody responses in cancer patients. We aimed to determine the levels of anti-PLAC1 antibodies in infertile women with a history of unexplained repeated implantation failure after IVF cycles as compared to fertile women.
Study design: An observational case–control clinical study.
Main outcome measure(s): Two groups of patients were analysed in two different experimental settings: 21 infertile women and 81 control patients were enrolled in the first group, 16 infertile women and 67 fertile controls in the second group.
Anti-PLAC1 antibody levels and ranking were analysed by ELISA test.
Results: In both groups of infertile patients enrolled, optical densities (OD) from ELISA test ranked significantly higher than those of controls (0.27 ± 0.2 vs. 0.13 ± 0.1 respectively; $p = 0.0009$ in the first group), (0.62 ± 0.38 vs. 0.39 ± 0.35 respectively; $p = 0.0044$ in the second experiment). In the first group about one case in four (29%) had OD levels above the 95thpercentile (0.337) for healthy controls ($p = 0.005$). In the second experiment 4 out of 16 cases (25%) had OD levels above the 95th percentile (0.878) for healthy controls ($p = 0.023$).
Conclusions: Anti-PLAC1 antibodies could represent a biomarker associated with infertility and with high probability of repeated implantation failure after ovarian stimulation and IVF-ET, greatly improving the diagnostic work up of infertile couples.

1. Introduction

Infertility affects about 15% of couples worldwide [1,2]. The overall incidence of infertility has remained stable over the past decades [2]. Treatment options and success vary with the cause of infertility [3]. Although in vitro fertilisation (IVF) is a widely spread treatment and the success rates of assisted reproductive technologies have markedly improved during the last decades, many of infertile patients experience unexplained implantation failures after IVF cycles, despite repeated transfers of morphologically normal embryos [4].

Several studies have investigated the role of embryo quality [5], of the endometrial uterine receptivity [6–8] or a genetic aetiology [9,10] as possible causes of repeated unexplained implantation failure. Since implantation is characterized by the interaction of two immunological distinct tissues, several studies have also investigated the role of autoimmune factors potentially relevant to in vitro fertilization failure such as anti-nuclear, anti-sperm, anti-ovarian, anti-endometrial and anti-phospholipid antibodies (aPLs), however the association between the recurrence of implantation failure and the above mentioned immune factors remains unproven [11].

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PLAC1 is a trophoblast-specific gene that maps to a locus on the X-chromosome, which is important to placental development [12]. PLAC1 protein is restricted primarily to the differentiated trophoblast, localizing to intracellular membranous compartment(s) in the apical region of the syncytiotrophoblast and associated with its apical, microvillous membrane surface [12]. Since PLAC1 is localized on the surface of the syncytiotrophoblast and is accessible for antibodies, it was reasoned that the development of anti-PLAC1 antibodies could possibly contribute to an impaired implantation process and consequently to infertility. Therefore the aim of this study was to compare anti-PLAC1 antibody levels in sera of women affected by infertility and repeated unexplained implantation failure after IVF cycles with those of fertile healthy controls. Moreover, we compared the frequency of high antibody levels (above the 95th percentile for healthy controls) between fertile and unfertile women.

2. Materials and methods

2.1. Patients

Serum samples were drawn from a total of 37 infertile patients with a history of repeated unexplained implantation failure after ovarian stimulation and IVF-ET (infertile group), and a total of 148 fertile control women (control group). ELISA, tested serum samples in two different experimental settings, in order to evaluate anti-PLAC1 antibody levels and ranking. In the first experiment sera of 21 infertile women (Group 1) and 81 control patients, enrolled from January to June 2010, were analysed (Creative Bio Labs, Shirley, New York, USA). In the second experiment, sera of 16 infertile women (Group 2) and 67 fertile controls, enrolled from September to November 2011, were analysed in a separate independent laboratory (PRIMM labs, Milan, Italy) in order to confirm our preliminary results. Inclusion criteria were: age <42 years, at least 6 good quality embryos transferred in 3 or more previous IVF/ICSI cycles without signs of implantation, normal response with at least 6 oocytes retrieved with standard induction protocol, normal uterine cavity as shown by sonohysterography or hysteroscopy, normal parental peripheral karyotype. Exclusion criteria for both groups of patients were as follows: FSH day 3° >10 mUI/mL, BMI > 30 mUI/mL, history of clinical repeated pregnancy loss, less than 3 previous IVF/ICSI cycles without signs of implantation, severe male factor infertility (total progressive motile count < than 1,000,000 sperms/ejaculate), testicular or frozen sperms, previous surgery for myoma and/or endometriosis, clinical or ultrasound diagnosis of endometriosis, corticosteroids treatments or other medical treatments known to interfere with immune system, known clinical autoimmune disease, antiphospholipid syndrome, thrombophilic condition requiring anticoagulant therapy, presence of anti-sperm, antibodies, unwilling to give informed consent.

Written informed consent was obtained from all patients enrolled in the study, which was approved by the local ethical committee. All infertile women underwent the following examinations recommended for the basic infertility evaluation [13]: semen analysis of the partner, testing for detection of ovulation (mid luteal progesterone, LH kit), assessment of ovarian reserve, transvaginal ultrasound, and hysterosalpingography. Unexplained infertility was diagnosed by exclusion, after all of the standard investigations revealed no abnormality [13,14]. Repeated unexplained implantation failure was defined by at least three IVF failures despite good hormonal reserve, after fresh embryo transfers with at least two embryos of good quality. In the first experimental setting, among controls, serum samples of 72 patients were drawn within 72 h after delivery, 9 serum control samples were obtained from healthy fertile patients referred to our centre for a gynaecological examination. In order to avoid any bias due to a possible interference of the placenta on circulating antibodies, in the second experimental setting all the control sera were obtained from healthy women referring to our department for a gynaecological examination.

2.2. Cloning of human PLAC1 gene

The region between nucleotides 67 and 636 of the coding sequence of the gene (HUGO Gene Nomenclature Committee ID: HGNC: 9044) corresponding to amino-acid 23 to 212 of PLAC1 protein, was cloned, from previously frozen human placenta tissues, into the vector p2N in which 10 His TAG is present at the N-terminus (Primm, Milan, Italy). The protein was then purified through Ni+ Sepharose High Performance column as follows: after packing the column and equilibrating it with buffer, the clarified lysate was applied to the column at 1 mL/min and then eluted. Every fraction was assayed by SDS-PAGE.

The cloned region of the gene encodes for the entire protein that has a molecular weight of about 21 kDa. The DNA fragment was obtained by PCR amplification using human cDNA deriving from placenta and bone marrow samples. The oligos used in the PCR reaction were named FORlic and REVlic and their sequence is:

5′-caccaccacggcgtcCAAAGTCCAATGACTGTGCTGTGC-3′ and 5′-cgagcgaaggcgtcagattaTCACATGGACCCAATCATATCATCTGTGTG-3′ respectively. Cloning was then performed into vector p2N using the LIC technique (Ligation Independent Cloning) [15,16].

2.3. ELISA kits construction

We employed PLAC1 expression in BL21 (DE3) host strain. Protein expression was induced overnight at 37 °C. The target protein was expressed into inclusion bodies and purified by standard method. Finally PLAC1 protein was used for ELISA kits construction.

In particular the antigen was diluted to a final concentration of 16 μg/mL in order to coat the wells of a PVC microtiter plate (100 μL per well).

We used blocking buffer (150 μL 10%FBS-PBS) and incubated sera for 2 h at 37 °C. The washing solution was PBST (Phosphate Buffered Saline with Tween20).

The secondary antibody was HRP-conjugated anti-human IgG (Abcam, Cambridge, UK). It was detected by direct horse-radish peroxidase. The final colour was yellow after adding the stop solution. After the colour reaction, the optical density (OD) was recorded at either 450 nm (in the first group) using Multiskan MK3 (Thermo Fisher Scientific, MA, USA) or 492 nm (in the second group) with microtiter plates reader 3912 (Beckton Dikinson; CA, USA). Sera (both form patients and controls) were diluted 1:100 in the first experiment and 1:20 in the second experiment. In order to check for reproducibility we tested two different batches of ELISA plates produced four weeks apart. We picked out 11 samples, which contain 9 tested negative sera, and 2 tested positive sera, then we performed kit reproducibility tests. The coincidence rate of reproducibility was 100%.

2.4. Statistical analysis

Data were described as number and percentage or mean and standard deviation (SD), where appropriate. Wilcoxon analysis or chi square test with Fisher correction, as appropriate, were performed to compare clinical features of both groups enrolled. The Mann–Whitney test was used to compare ODs between study groups. Fisher's exact test was used to compare frequencies above and below the value of the 95th percentile for the fertile (control) group. Tests were two-sided and a $p < 0.05$ was considered significant. All analysis was performed with Stata11 (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP.)

3. Results

Clinical features of women of both groups enrolled are reported in Table 1. There was no significant difference in terms of age, number of first (I) trimester voluntary interruption of pregnancy (VIP) between patients and controls. Controls showed a significantly higher number of pregnancies and of live births compared to infertile patients, which showed a significantly higher number of I trimester spontaneous abortion (SA). Unexplained infertility was assessed in 10 (27%) out of 37 patients. Tubal factor was assessed in 4 (10.8%) out of 37 patients and a mild moderate male factor was diagnosed in 23 (62.1%) out of 37 patients enrolled. The analysis of ODs from ELISA test showed in both groups a statistically significant difference between patients and controls. More in details in the first group of patients enrolled infertile women's mean (and SD) ODs from ELISA test ranked significantly higher than controls (0.27 ± 0.2 vs. 0.13 ± 0.1 respectively; $p = 0.0009$) (Fig. 1, I group). Similarly, in the second experiment the mean (and SD) ODs were significantly higher in infertile patients enrolled than controls

Table 1
Clinical features of infertile patients and controls enrolled. Data are expressed as means ± SD; p value referred to Wilcoxon analysis, or chi square test with fisher correction, as appropriate.

	Global population	Controls	Cases	p value
	185	148	37	
Age	41 ± 13	42 ± 14	37 ± 3	0.461
No of pregnancies	1.82 ± 1.72	2.16 ± 1.74	0.51 ± 0.73	<0.001*
No of live births	1.39 ± 1.22	1.74 ± 1.13	0.05 ± 0.23	<0.001*
No I trimester SA	0.19 ± 0.47	0.15 ± 0.43	0.35 ± 0.59	0.012*
No of I trimester VIP	0.28 ± 0.91	0.32 ± 1.00	0.11 ± 0.31	0.353
OD	0.28 ± 0.30	0.25 ± 0.28	0.42 ± 0.34	0.001*
OD positive	16 (8.65%)	6 (4.05%)	10 (27.03%)	<0.001*

A $p < 0.05$ was considered significant (*).
VIP: Voluntary interruption of pregnancy.
SA: Spontaneous abortion.

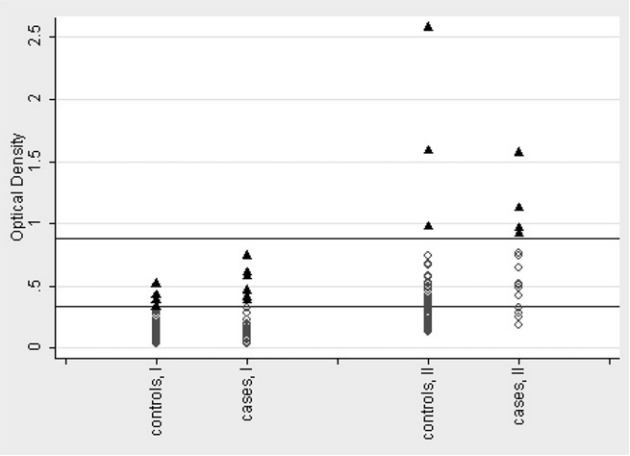

Fig. 1. Anti-PLAC1 antibody levels infertile women (cases) and controls of group I and II, triangle indicate subjects with ODs above the 95th percentile for relative fertile controls.

(0.62 ± 0.38 vs. 0.39 ± 0.35 respectively; $p = 0.0044$) (Fig. 1, II group). In the first experiment about one case in four (29%) had OD levels above the 95th percentile (0.337) for healthy controls ($p = 0.005$). Notably, also in the second experiment 4 out of 16 cases (25%) had OD levels above the 95th percentile (0.878) for healthy controls ($p = 0.023$) (see Supplementary table).

Interestingly, in the first group of infertile patients, after one year of follow up, among the 5 infertile patients positive to anti-PLAC1 antibodies, 4 did not achieve a pregnancy after subsequent IVF attempts. On the contrary among the 7 infertile patients with anti-PLAC1 antibody level below the threshold value, 4 patients achieved a pregnancy in the following IVF attempts. Finally we analysed differences of clinical features among positive (OD value above the 95th percentile for fertile controls) and negative subjects (OD value below the 95thpercentile for fertile controls) enrolled and we found a significantly higher number of I trimester SA among women with OD levels above the threshold value (Table 2). On the other hand women with OD levels within the threshold value showed a significantly higher number of live births, although the number of total pregnancies did not differ between OD positive and negative subjects.

4. Discussion

A large number of women affected by infertility and undergoing IVF, still experience unexplained implantation failures, despite repeated transfers of morphologically normal embryos. This can have devastating psychological consequences on infertile couples and it remains a relevant social and medical problem [4,17,18]. Our results show that anti-PLAC1 antibody levels are significantly higher in a subset of infertile patients with repeated unexplained implantation failure when compared to control fertile women (Fig. 1), confirming data recently reported by other authors [19]. The process of implantation involves the interaction of the human blastocyst and the uterine epithelium. Several autoimmune factors have been implicated to have an influence on implantation failure. Studies in literature, showed a higher prevalence of several antibodies, in women with repeated implantation failure [20–28].

Although the published series support the conclusion that some autoantibodies are present more often in infertile patients, none of these antibodies were found to be associated with an altered prognosis for infertile women undergoing IVF [27,29,30]. Moreover most of the studies published so far either involved a small number of patients, were poorly conducted or without control groups, such that meaningful conclusions cannot be drawn [11]. A meta-analysis of seven eligible studies on aPLs and IVF outcome showed that there was no significant association between aPLs and either clinical pregnancy or live-birth in women undergoing IVF cycles [31,32] and they are more significantly associated with late pregnancy losses than early losses [27].

When we consider anti-trophoblast antibodies, the specificity in ELISA has yet to be determined, since trophoblast samples contain many allotypic proteins and maternal immunity to these proteins cannot be excluded. Moreover, most importantly, there is little consensus regarding the subpopulation of trophoblast to be used as antigens [33].

In the present study we identified a novel specific anti-trophoblast antibody, anti-PLAC1, in infertile women with repeated unexplained implantation failure. Compared to the other anti-trophoblast antibodies reported in literature, PLAC1 shows placenta-specific expression and is localized primarily in the syncytiotrophoblast. The gene encoding PLAC1 is X-linked, maps 65 kb telomeric to *HPRT* at Xq26 and has been completely sequenced at the cDNA and genomic levels [34,35]. In situ hybridization studies with the antisense mRNA during mouse embryogenesis detect PLAC1 expression from 7.5 to 14.5 days postcoitum (dpc), making PLAC1 a marker for placental development, with a possible role in the establishment of the mother–fetus interface [34]. Since PLAC1 is localized, in the apical, microvillous membrane surface [12], the development of anti-PLAC1 antibody response could possibly lead to trophoblast damage at the mother–fetus interface and in turn to impairment of the implantation process. This hypothesis seems to be supported by the analysis of clinical features of women enrolled in our study, that showed a significant higher number of I trimester SA not only in infertile patients compared to controls (Table 1), but also in the total population with OD levels above the threshold value compared to OD negative subjects (Table 2). It is not surprising that PLAC1 is immunogenic since other authors have recently shown that PLAC1, is expressed in a range of human tumours, and can elicit a spontaneous antibody response [36,37]. Moreover, recent studies reported that PLAC1 mRNA is distributed differently in pregnant women with preeclampsia compared to controls [38], suggesting a possible association of increased levels of PLAC1 mRNA and clinical conditions associated to trophoblast damage [39].

Notably, there are several reasons why we may have underestimated the fraction of patients bearing elevated levels of anti-PLAC1 antibodies. In fact, although the patients group was selected according to strict criteria, it cannot be excluded that some of the women failed at least 3 IVF cycles because of the limits of the technique, the estimated live-birth rates per cycle varying between 13% and 28% [4]. Moreover the antibodies may recognize conformational epitopes in vivo but not in the recombinant protein

Table 2
Differences among clinical features of women with OD values above (positive) and below (negative) the threshold value. Data are expressed as means ± SD; *p* value referred to Wilcoxon analysis.

	Global population	Positive	Negative	*p* value
	185	16	169	
Age	41 ± 13	40 ± 7	41 ± 13	0.590
No of pregnancies	1.82 ± 1.72	1.31 ± 1.14	1.87 ± 1.76	0.258
No of live births	1.39 ± 1.22	0.63 ± 0.96	1.46 ± 1.22	0.007*
No of I trimester SA	0.19 ± 0.47	0.56 ± 0.73	0.15 ± 0.43	0.001*
No of I trimester VIP	0.28 ± 0.91	0.19 ± 0.40	0.28 ± 0.94	0.760
OD	0.28 ± 0.30	0.89 ± 0.59	0.22 ± 0.16	<0.001

A $p < 0.05$ was considered significant (*).
VIP: Voluntary interruption of pregnancy.
SA: Spontaneous abortion.

(in vitro). It is not known, at this stage, which specific dilution would prove to be of clinical relevance and, since we found that in the majority of the "positive" cases the OD values were moderately increased while the control background values were definitively low, we decided to reduce the dilution factor in the second group in the attempt not to miss any further positive case. It remains to be established whether the few putative fertile women with high levels of antibodies can become pregnant since they may have developed the immune response at the end of their last pregnancy.

Furthermore, it remains to be elucidated whether the increased levels of anti-PLAC1 antibodies detected in the present study in infertile patients, could be directly associated to implantation failure and the pathogenetic mechanisms involved, which immunoglobulin isotypes are elicited, the kinetics of antibodies appearance, the role, if any, of cellular immunity in this setting and whether immune responsiveness is associated with a particular genetic background (HLA haplotype).

5. Conclusions

In conclusion the present study demonstrated the presence of a new anti-trophoblast antibody, against the placenta-specific antigen PLAC1, in infertile women with repeated unexplained implantation failure. In this study anti-PLAC1 levels in infertile patients were compared with a large group of control women and resulted significantly higher in two different experimental settings and laboratories. Should this preliminary data be confirmed, anti-PLAC1 antibodies could represent a biomarker associated with infertility and with high probability of repeated implantation failure after ovarian stimulation and IVF-ET greatly improving the diagnostic work up of infertile couples prior to IVF cycles.

Authors' contributions

Maria Matteo: I declare that I was responsible for the study concept and I participated in designing the study, interpreting data, in writing and reviewing the manuscript and that I have seen and approved the final version.

Arcangelo Liso: I declare that I was responsible for the study concept and I participated in designing the study, interpreting data and in writing and reviewing the manuscript and that I have seen and approved the final version.

Pantaleo Greco: I declare that I participated in interpreting data; in writing and reviewing the manuscript and that I have seen and approved the final version.

Paolo Emanuele Levi Setti: I declare that I participated in interpreting data, in writing and reviewing the manuscript and that I have seen and approved the final version.

Emanuela Morenghi: I declare that I participated in interpreting data, in performing statistical analysis, in writing the statistical analysis section, and that I have seen and approved the final version.

Francesca Massenzio: I declare that I participated in designing and performing PCR analysis and that I have seen and approved the final version.

Filomena De Rosario: I declare that I participated in interviewing and selecting patients, in samples collecting and in reviewing the manuscript. I declare that I have seen and approved the final version.

Elena Albani: I declare that I participated in samples collecting and in reviewing the manuscript. I declare that I have seen and approved the final version.

Pasquale Totaro: I declare that I participated in interviewing and selecting patients, in samples collecting. I declare that I have seen and approved the final version.

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Arcangelo Liso and Maria Matteo applied for a patent covering the clinical use of anti-PLAC1 antibodies. All others have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2013.01.006.

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