

# Antiphospholipid Antibodies and Infertility: A Gene Expression Study in Decidual Stromal Cells

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**ABSTRACT:** **Background:** Antiphospholipid antibodies (aPL) have been advocated as potential mediators of unexplained female infertility, but no evidence has yet been raised to support such an association.

**Objectives:** To test the hypothesis that aPL might interfere with uterine decidualization, a gene expression study was performed on decidual stromal cells treated with different aPL preparations.

**Methods:** Decidual stromal cells were isolated from first-trimester deciduas obtained from two women undergoing elective abortion, and treated with: (i) a  $\beta$ 2GPI-dependent aPL monoclonal antibody (IS3); (ii) IS3 plus TIFI, a synthetic peptide mimicking PL-binding region of  $\beta$ 2GPI; and (iii) IgG from healthy subjects (NHS). Gene expression data were acquired using human HT-12 v3 beadchip arrays (Illumina). Differential expression analysis was performed by fitting a gene-wise linear model using the treatment group and decidual source as covariates.

**Results:** In the comparison of IS3 versus IgG NHS-treated decidual cells, gene ontology (GO) enrichment was expressed in terms relating to well-characterized aPL-mediated cellular effects: “inflammatory response,” “immune response,” “response to stress,” “oxydoreductase activity,” “metalloendopeptidase activity,” and “cytokine/chemokine activity.” As expected, almost all genes were up-regulated by IS3 treatment. The same GO categories appeared to be differentially expressed when IS3 treatment was compared to IS3 + TIFI, but with most genes being down-regulated.

**Conclusions:** Given the inflammatory response evinced on gene expression analysis of decidual stromal cells treated with a  $\beta$ 2GPI-dependent aPL monoclonal antibody, it is feasible that aPL might interfere with uterine decidualization, affecting the early stages of implantation and ultimately resulting in female infertility.

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**KEY WORDS:** antiphospholipid antibodies (aPL), infertility, gene expression, decidual stromal cells, inflammation

Infertility is the inability of a sexually active, non-contracepting couple to conceive over a 12 month period. It is a rather common condition, affecting 8–12% of couples where the woman is of childbearing age. Unfortunately, despite an appropriate diagnostic workup, approximately 20% of infertility cases remain unexplained [1]. Antiphospholipid antibodies (aPL), a family of autoantibodies reacting against phospholipid (PL)-binding proteins, have been advocated as potentially involved in unexplained female infertility. aPL, whose main antigenic target is  $\beta$ 2 glycoprotein I ( $\beta$ 2GPI), is the most common acquired risk factor for obstetric complications such as early recurrent abortions, late pregnancy loss, premature delivery due to severe pre-eclampsia, eclampsia or placental insufficiency [2]. The precise etiopathogenic mechanisms by which aPL might cause infertility have not yet been characterized. Since gametes or pre-implantation embryos never come into contact with maternal blood, some experts proposed that aPL may disrupt oocyte development after being secreted into follicular fluid. An alternative hypothesis proposed that aPL might interfere with uterine decidualization, leading to implantation impairment and ultimately causing infertility. To test such an hypothesis, we performed a gene expression study on decidual stromal cells treated with a human  $\beta$ 2GPI-dependent aPL monoclonal antibody; as a negative control, cells were treated with IgG from healthy subjects. In addition, the inhibitory effects on the aPL-mediated gene expression profile by TIFI, a synthetic peptide mimicking PL-binding region of  $\beta$ 2GPI, were investigated.

## METHODS

### TISSUE COLLECTION AND CULTURE OF DECIDUAL CELLS

First-trimester decidua were obtained from two healthy Caucasian women with clinically normal pregnancies who were scheduled for elective abortion due to social or psychological reasons. Patient 1, age 35 years, terminated the pregnancy course in the 9th gestational week; patient 2, age 37 years, underwent the procedure in the 10th gestational week. Informed consent was obtained from each patient before surgery. Samples were processed within 2 hours from collection.

The specimens were minced and incubated with type IV collagenase (53,000 U/100 ml, Sigma-Aldrich, St. Louis, MO, USA) for 90 minutes at 37°C. After centrifugation at 15,000 rpm for 15 minutes at 4°C, the pellet was resuspended in complete culture medium (DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, Gibco-Life Technologies, Gronigen, Netherlands). Cells were seeded in a culture dish and incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator. To further stimulate the decidualization of endometrial stromal cells, cells near confluence were treated for 7 days with culture medium to which estradiol was added (E2, 10<sup>-8</sup>M, Sigma-Aldrich) and medroxyprogesterone acetate (MPA, 10<sup>-7</sup>M, Sigma-Aldrich) [3]. To rule out potential contamination, an aliquot of cell suspension was stained for 30 minutes at room temperature with the following human monoclonal antibodies: CD45/CD14 (markers of leukocytes), CD146 (marker of endothelial cells) and cytokeratin (marker of epithelial cells). Data were acquired using a four-color cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). Results were expressed as a percentage of gated events, using CELLQuest software (BD Biosciences). Cell cultures with a positivity rate for the above cited markers > 2% were not used in the experiments.

#### CELL TREATMENTS

Decidual stromal cells were stimulated for 24 hours with different treatments: a) human monoclonal anti- $\beta$ 2GPI antibody IS3 (40  $\mu$ g/ml), b) anti- $\beta$ 2GPI antibody IS3 (40  $\mu$ g/ml) + TIFI (20  $\mu$ g/ml), c) TIFI (20 mg/ml), and d) IgG from normal healthy sera (NHS). IS3 is an IgG anti-human  $\beta$ 2GPI-dependent aPL monoclonal antibody purified from supernatants of B cell clones from an APS patient [4]. Whole IgG fractions from NHS were purified on protein G-Sepharose (Mab Trap-GII, Pharmacia-Biotech, Uppsala, Sweden) as previously described [5]. The sterile-filtered IgG fractions were determined to be free of endotoxin contamination by the limulus amoebocyte lysate gel-clot test (Pyrosate kit, Associates of Cape Cod Incorporated, East Falmouth, MA, USA; sensitivity 0.25 U/ml). The antibody concentration was evaluated by spectrophotometry, and the specific reactivity with CL- and  $\beta$ 2GPI-coated plates was confirmed as previously described [6]. TIFI, a 20 amino acid synthetic peptide derived from cytomegalovirus, was demonstrated to share similarities with the PL-binding region of  $\beta$ 2GPI: by competing with  $\beta$ 2GPI, it inhibited the interaction of anti- $\beta$ 2GPI antibodies with cell membrane [7,8].

#### GENE EXPRESSION ANALYSIS

Total RNA from decidual stromal cells cultured in different experimental conditions was extracted using Trizol Reagent (Life Technologies), following the manufacturer's instructions, and then purified by RNeasy Mini Kit (Qiagen, Hilden, Germany). Amplification Grade DNase I (Life Technologies) was used to eliminate residual genomic DNA from RNA

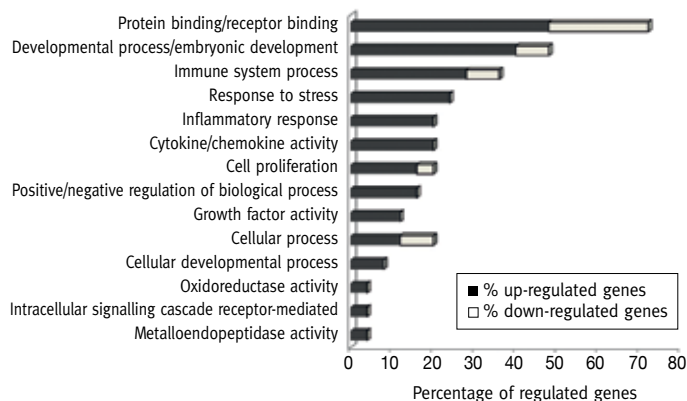
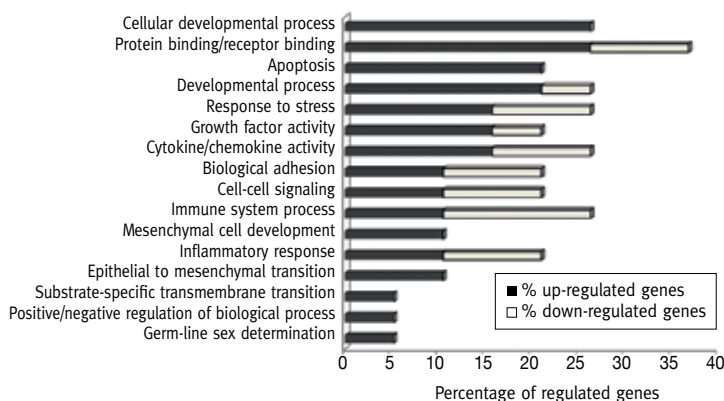
samples. RNA concentration was evaluated by the nanophotometer P-300 (Implen, Westlake Village, CA, USA). Samples with an A260/A280 ratio between 1.8 and 2.1 were considered for experiments. The Illumina TotalPrep RNA Amplification kit (Ambion, Foster City, CA, USA) was employed using 200 ng of total RNA as starting material. Labelled cRNA (750 ng) was hybridized to human HT-12 v3 beadchip arrays (Illumina, San Diego, CA, USA) for 16 hours at 58°C. Following hybridization, beadchip arrays were washed and stained with streptavidin-Cy3 (GE Healthcare, Little Chalfont, UK). Fluorescent images were obtained with a Beadarray reader and processed with the BeadScan software (Illumina).

#### STATISTICAL ANALYSIS

Background correction, quantile normalization and log<sub>2</sub> transformation were applied to standardized signal among samples. Intensity data were filtered on probe annotation, removing probes whose sequence had undesirable properties. Differential expression analysis was performed by fitting a gene-wise linear model using the treatment group and decidual source as covariates and exploring relevant contrasts, evaluated using an empirical Bayes moderated *t*-test. Multiple tests were corrected with a 0.05 false discovery rate (FDR) procedure. Gene expression analysis was performed using R software (Limma package). Gene ontology (GO) enrichment was performed on differentially expressed genes and on genes with an absolute fold change  $\geq 1.5$ , if FDR > 0.05, to investigate the over-represented GO terms with the platform GOSTAT, available at <http://gostat.wehi.edu.au> [9]. We considered biological processes and molecular functions as GO pathways; minimal length of considered GO pathways was 3 and maximal *P* value was 0.1. FDR was used for multiple testing corrections.

#### RESULTS

Probes with a "bad" or "no match" quality score were filtered out, resulting in 34,362 out of 48,803 genes. According to the probe-wise linear models, the comparison between IS3- and IgG NHS-treated samples showed 28 differentially expressed genes, while 22 genes emerged when comparing IS3 versus IS3 + TIFI group. To gain insight into the potential biological significance of these genes, GO enrichment was performed to identify statistically over-represented GO terms. Interestingly, significant differences in the comparison of IS3 versus IgG NHS-treated decidual cells concerned GO categories related to inflammation. In particular, enrichment was found in terms belonging to well-characterized aPL-mediated cellular effects: "inflammatory response," "immune response," "response to stress," "oxydoreductase activity," "metalloendopeptidase activity," "cytokine/chemokine activity" and "developmental process." As expected, almost all these genes were up-regulated by IS3 treatment [Figure 1, Panel A]. The same GO categories

**Figure 1.** Selected gene ontology groups in IS3 versus IgG NHS contrast [Panel A] and IS3 versus IS3 + TIFI [Panel B]**A** IS3 versus IgG NHS contrast**B** IS3 versus IS3 versus IS3 + TIFI contrast

appeared to be differentially expressed when IS3 treatment was compared to IS3 + TIFI. However, most genes belonging to IS3 treatment-related terms were down-regulated by IS3 plus TIFI [Figure 1, Panel B].

## DISCUSSION

To our knowledge, this is the first study to perform a gene transcriptional profile on decidual stromal cells treated with different aPL preparations. According to our gene expression analysis, treatment with a monoclonal  $\beta$ 2GPI-dependent aPL antibody resulted in the up-regulation of several genes involved in inflammation and immune responses. Interestingly, treatment with IS3 in combination with TIFI, an inhibitor of aPL binding to  $\beta$ 2GPI, partially reversed the up-regulation of pro-inflammatory genes. The finding that  $\beta$ 2GPI-dependent aPL monoclonal preparations can modulate the gene expression profile in decidual stromal cells implies that aPL might impair female fertility through interference with the process of endometrial decidualization, which provides a necessary step for embryo implantation. Decidualization consists in the acquisition, under the influence of female hormones in the mid-secretory phase of the menstrual cycle, of a secretory endometrial profile. Once implantation occurs, the endometrial lining evolves to a newly formed tissue, the decidua, which is the maternal side of human placenta.

There is solid evidence that aPL exert a pathogenic role in mediating obstetric manifestations of antiphospholipid syndrome (APS). Based on the earliest histopathologic report of APS placentas, aPL were first thought to interfere with pregnancy physiology by inducing spiral artery thrombosis, leading to placental infarction [10]. In recent years, it has been increasingly acknowledged that non-thrombotic etiologies might also

be implicated in aPL-mediated pregnancy complications. In particular, aPL have been shown to exert a direct effect on trophoblasts, with a down-regulation of chorionic gonadotropin and an abnormal expression of integrins and cadherins, resulting in a reduced trophoblast proliferation, syncytialization, and invasion [11]. Most importantly, polyclonal aPL have been shown to react in vitro with human decidual stromal cells, inducing a pro-inflammatory phenotype with reduced release of eicosanoids and insulin-like growth factor binding protein-1 (IGF BP-1), critical players in cell growth and differentiation [12,13]. In addition, aPL were observed to bind endometrial endothelial cells, impairing the angiogenesis both in vivo and in vitro [14]. Consistent evidence of the interaction of aPL with decidual tissues has been acquired in in vivo models: at histologic examination, mice treated with aPL IgG displayed decidual necrosis, with prominent intravascular decidual IgG and fibrin deposition [15]. Furthermore, the decidua was reported to provide the ground for complement deposition upon aPL IgG treatment: immunohistochemical analyses of deciduas obtained from 8 day pregnant mice showed extensive deposits of C3 and C5 [16-18]. In a previous gene expression study performed on endometrial tissues obtained from women with recurrent pregnancy losses, mRNA expression profiles differed depending on the presence of circulating aPL, with APS women expressing lower levels of decidual markers such as prolactin and tissue factor, and DAF/CD55, a complement regulatory protein which confers cellular protection against complement-mediated lysis [19]. As a whole, all these lines of evidence appointed decidual tissues as a preferential target of the detrimental effects of aPL. Therefore, the pro-inflammatory modulation of mRNA profile induced by aPL treatment in decidual stromal cells evinced at our gene expression study is consistent with previous data. In addition, the specificity of our

findings is further confirmed by the observation that the pro-inflammatory effects of IS3 were partially reversed by TIFI, a synthetic peptide that competes with  $\beta$ 2GPI for aPL binding.

This study was biased by several limitations, in particular the limited number of decidual samples. Nevertheless, we believe that it might impact current knowledge about the relationship between aPL and female infertility. From a clinical point of view, the association of aPL with infertility is still much debated. Even though a higher aPL positivity rate is described among infertile women, aPL profile was reported not to affect the outcome in cases of assisted reproduction [20,21]. Unfortunately, the literature in the field is biased by several limitations, related mainly to patients' inclusion criteria and aPL testing, which prevent the drawing of meaningful conclusions. However, given the inflammatory response observed at gene expression analysis on decidual stromal cells treated with a  $\beta$ 2GPI-dependent aPL monoclonal antibody, we propose that aPL might interfere with uterine decidualization, thus affecting the early stages of implantation and ultimately resulting in female infertility. The potential link between aPL and infertility warrants further confirmation in future larger studies.

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### References

- de Jesus GR, Agmon-Levin N, Andrade CA, et al. 14th International Congress on Antiphospholipid Antibodies Task Force report on obstetric antiphospholipid syndrome. *Autoimmun Rev* 2014; 13: 795-813.
- Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295-306.
- Reis FM, Viganò P, Arnaboldi E, Spritzer PM, Petraglia F, Di Blasio AM. Expression of prolactin-releasing peptide and its receptor in the human decidua. *Mol Hum Reprod* 2002; 8: 356-62.
- Lin WS, Chen PC, Yang CD, et al. Some antiphospholipid antibodies recognize conformational epitopes shared by beta2-glycoprotein I and the homologous catalytic domains of several serine proteases. *Arthritis Rheum* 2007; 56: 1638e47.
- Zhu M, Olee T, Le DT, et al. Characterization of IgG monoclonal anti-cardiolipin/anti-beta2GPI antibodies from two patients with antiphospholipid syndrome reveals three species of antibodies. *Br J Haematol* 1999; 105: 102-9.
- Meroni PL, Peyvandi F, Foco L, et al. Anti-beta 2 glycoprotein I antibodies and the risk of myocardial infarction in young premenopausal women. *J Thromb Haemost* 2007; 5: 2421-8.
- Ostertag MV, Liu X, Henderson V, Pierangeli SS. A peptide that mimics the Vth region of beta-2-glycoprotein I reverses antiphospholipid-mediated thrombosis in mice. *Lupus* 2006; 15: 358-65.
- De la Torre YM, Pregnolato F, D'Amelio F, et al. Anti-phospholipid induced murine fetal loss: novel protective effect of a peptide targeting the  $\beta$ 2 glycoprotein I phospholipid-binding site. Implications for human fetal loss. *J Autoimmun* 2012; 38: 209-15.
- Beissbarth T, Speed TP. GStat: find statistically overrepresented gene ontologies within a group of genes. *Bioinformatics* 2004; 20: 1464e5.
- Viall CA, Chamley LW. Histopathology in the placenta of women with antiphospholipid antibodies: a systematic review of the literature. *Autoimmun Rev* 2015; 14: 446-71.
- Chighizola CB, Gerosa M, Trespidi L, et al. Update on the current recommendations and outcomes in pregnant women with antiphospholipid syndrome. *Expert Rev Clin Immunol* 2014; 10: 1505-17.
- Pierro E, Cirino G, Bucci MR, et al. Antiphospholipid antibodies inhibit prostaglandin release by decidual cells of early pregnancy: possible involvement of extracellular secretory phospholipase A2. *Fertil Steril* 1999; 71: 342-6.
- Pierro E, Andreani CL, Lazzarin N, et al. Effect of anticardiolipin antibodies on prolactin and insulin-like growth factor binding protein-1 production by human decidual cells. *Am J Reprod Immunol* 1999; 41: 209-16.
- Di Simone N, Di Nicuolo F, D'Ippolito S, et al. Antiphospholipid antibodies affect human endometrial angiogenesis. *Biol Reprod* 2010; 83: 212-19.
- Branch DW, Dudley DJ, Mitchell MD, et al. Immunoglobulin G fractions from patients with antiphospholipid antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss. *Am J Obstet Gynecol* 1990; 163: 210-16.
- Holers VM, Girardi G, Mo L, et al. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med* 2002; 195: 211-20.
- Girardi G, Berman J, Redecha P, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003; 112: 1644-54.
- Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nature Med* 2004; 10: 1222-6.
- Francis J, Rai R, Sebire NJ, et al. Impaired expression of endometrial differentiation markers and complement regulatory proteins in patients with recurrent pregnancy loss associated with antiphospholipid syndrome. *Mol Hum Reprod* 2006; 12: 435-42.
- Chighizola CB, de Jesus GR. Antiphospholipid antibodies and infertility. *Lupus* 2014; 23: 232-8.
- Ostensen M, Andreoli L, Brucato A, et al. State of the art: reproduction and pregnancy in rheumatic diseases. *Autoimmun Rev* 2015; 14: 376-86.