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ORIGINAL ARTICLE

Sphingosine-1-phosphate restores endothelial barrier integrity in ovarian hyperstimulation syndrome

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STUDY QUESTION: Are follicular fluid (FF) sphingosine-1-phosphate (S1P) levels in patients at risk of developing ovarian hyperstimulation syndrome (OHSS) altered and in part responsible for the high vascular permeability observed in these patients.

STUDY ANSWER: FF S1P levels are lower in FF from patients at risk of OHSS and treatment with S1P may reduce vascular permeability in these patients.

WHAT IS KNOWN ALREADY: Although advances have been made in the diagnosis, and management of OHSS and in basic knowledge of its development, complete prevention has proven difficult.

STUDY DESIGN, SIZE, DURATION: A total of 40 FF aspirates were collected from patients undergoing ART. The women (aged 25–39 years old) were classified into a control group ($n = 20$) or a group at risk of OHSS ($n = 20$). The EA.hy926 endothelial cell line was used to assess the efffects of FF from patients at risk of OHSS with or without the addition of S1P. An animal model that develops OHSS in immature Sprague-Dawley rats were also used.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Migration assays, confocal microscopy analysis of actin filaments, immunoblotting and quail chorioallantoic membrane (CAM) assays of *in-vivo* angiogenesis were performed and statistical comparisons between groups were made.

MAIN RESULTS AND THE ROLE OF CHANCE: The S1P concentration was significantly lower in FF from patients at risk of OHSS ($P = 0.03$). The addition of S1P to this FF decreased cell migration ($P < 0.05$) and prevented VE-cadherin phosphorylation in endothelial cells ($P < 0.05$). S1P in the FF from patients at risk of OHSS increased the levels of VE-cadherin ($P < 0.05$), N-cadherin ($P < 0.05$) and β-catenin ($P < 0.05$), and partially reversed actin redistribution in endothelial cells. The addition of S1P in FF from patients at risk of OHSS also decreased the levels of vascular endothelial growth factor ($VEGF_{121}$; $P < 0.01$) and S1P lyase (SPL; $P < 0.05$) and increased the levels of S1PR1 ($P < 0.05$) in endothelial cells. In CAMs incubated with FF from patients at risk of OHSS with S1P, the number of vessel branch points decreased while the periendothelial cell coverage increased. Additionally, in a rat OHSS model, we demonstrated that vascular permeability and $VEGF_{121}$ and its receptor KDR expression were increased in the OHSS group compared to the control group and that S1P administration decreased these parameters.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The results of this study were generated from an *in-vitro* system. This model reflects the microvasculature *in vivo*. Even though the ideal model would be the use of human endothelial cells from the ovary, it is obviously not possible

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to carry out this kind of approach in ovaries of patients from ART. More studies will be necessary to delineate the effects of SIP in the pathogenesis of OHSS. Hence, clinical studies are needed in order to choose the most appropriate method of prevention and management.

WIDER IMPLICATIONS OF THE FINDINGS: The use of bioactive sphingolipid metabolites may contribute to finding better and safer therapeutic strategies for the treatment of OHSS and other human diseases that display aberrant vascular leakage.

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Key words: angiogenesis / ovary / OHSS / sphingolipids / vascular permeability

Introduction

Ovarian hyperstimulation syndrome (OHSS) is one the most serious iatrogenic complications of follicular growth and maturation induced by ovulation induction. It is characterized by increased vascular leakage and ovarian enlargement, which cause fast third space fluid shifts from the intravascular compartment (Golan *et al.*, 1989). OHSS affects 5–10% of patients undergoing ovulation induction treatments, and 0.5–5.0% of these patients develop a severe form (Delvigne and Rozenberg, 2002; Aboulghar and Mansour, 2003). Although advances have been made in the diagnosis and management of OHSS and basic knowledge of its development, complete prevention has proven difficult (Fiedler and Ezcurra, 2012). It is recognized that this syndrome is triggered by hCG (Gomez *et al.*, 2010) and that the predominant link between hCG and OHSS appears to be the production of angiogenic factors. In particular, members of the vascular endothelial growth factor A (VEGFA) family, angiopoietins (ANGPTs), transforming growth factors (TGFs), platelet-derived growth factor (PDGF) and sphingolipids like sphingosine-1-phosphate (S1P) are thought to be involved in the pathophysiology of OHSS (Hanahan and Folkman, 1996; Neufeld *et al.*, 1999; Carmeliet, 2000; Allende and Proia, 2002; Otrock *et al.*, 2007). Unlike VEGFA, ANGPTs, PDGF and S1P are essential for the stabilization of newly formed capillaries (Allende *et al.*, 2003; Hoch and Soriano, 2003; Murakami, 2012).

Several authors, including ourselves, have previously shown that VEGF and ANGPTs are implicated in the etiology of OHSS (Rodewald *et al.*, 2009; Scotti *et al.*, 2013, 2014, 2016). In our laboratory, in patients at risk of OHSS and in a murine model of OHSS, we have demonstrated that ANGPT1 increases pathophysiological angiogenesis by acting on endothelial cell migration and on adherens and tight junction proteins (Scotti *et al.*, 2013, 2014). We have also observed that, in ovaries from an OHSS rat model, the protein levels of PDGF ligands-B and -D decrease, whilst PDGFR-β and ANGPT2 levels do not change. (Scotti *et al.*, 2013). Nevertheless, up to now, no report has evaluated the involvement of the bioactive sphingolipid, S1P, in patients at risk of developing OHSS.

S1P is derived from sphingosine phosphorylation by sphingosine kinase (Spiegel and Milstien, 2003) and its degradation can be either mediated by S1P lyase (SPL) or by S1P phosphatases (Le Stunff *et al.*, 2002; Ogawa *et al.*, 2003). S1P is secreted by activated platelets, but also other cell types, such as erythrocytes, mononuclear cells, neutrophils and mastocytes, can also release this lipid mediator (Yatomi *et al.*, 1995; Yang *et al.*, 1999). S1P binds to specific cell surface receptors (S1PRs), which comprise a G-protein-coupled receptor family including five subcategories: S1PR1, S1PR2, S1PR3, S1PR4 and S1PR5. S1P is present in blood and plasma and is delivered by high-density lipoprotein (HDL) associated apoliprotein M to its receptors (Singleton *et al.*, 2006). S1P regulates several physiological functions in vascular and immune cells (Obinata and Hla, 2012). In endothelial cells, S1P induces cell migration (Kimura *et al.*, 2000; Liu *et al.*, 2001), cell survival (Kimura *et al.*, 2001), DNA synthesis (Kimura *et al.*, 2000) and cell barrier integrity (Garcia *et al.*, 2001). Several authors have shown that S1P possesses a strong effect on endothelial barrier protection (Dudek *et al.*, 2004; Liu *et al.*, 2009; Curry *et al.*, 2012). Dudek *et al.* (2004) and Liu *et al.* (2009) have shown that the administration of S1P in animal models with acute lung injury decreases the vascular hyperpermeability owing to the enhancement of endothelial junctional integrity (Dudek *et al.*, 2004; Liu *et al.*, 2009). Additionally, Curry *et al.* (2012) have shown that exogenous S1P stabilizes the rat endothelium via S1PR1, as it reduces acute microvascular permeability (Curry *et al.*, 2012).

In particular, S1PR1, S1PR2 and S1PR3 has been shown to be expressed in female reproductive tissues and granulosa cells (Risau, 1997; Kon *et al.*, 1999; Wang *et al.*, 2014). We and other authors have previously identified the presence of traditional (VEGF, ANGPTs, basic fibroblast growth factor (bFGF)) (Van Blerkom *et al.*, 1997; Abulafia and Sherer, 2000; Scotti *et al.*, 2013) and non-traditional angiogenic factors (S1P) in human follicular fluid (FF) (von Otte *et al.*, 2006). Regarding this, von Otte *et al.* (2006) have shown that S1P associated with HDL from human FF is a mitogenic and angiogenic factor in endothelial cells. Later, the same authors demonstrated that HDL-associated S1P promotes granulosa lutein cell migration via S1PR3 and RAC1 activation (Becker *et al.*, 2011).

It is essential for ovarian function that changes in the localization and expression of adhesion molecules are strictly controlled (Groten *et al.*, 2006; Rodewald *et al.*, 2007). This includes regulation of adherens and tight junctions, which are key components of intercellular junctions (Dejana, 2004; Schneeberger and Lynch, 2004). In this regard, S1P promotes cytoskeletal, adhesive and junctional changes, improving endothelial barrier integrity (Allende and Proia, 2002). S1P/S1PRs signaling induces the interaction of endothelial cells with pericytes via N-cadherin expression and trafficking (Paik *et al.*, 2004; McVerry and Garcia, 2005).

As mentioned above, an increase in vascular permeability is the main feature observed in patients with OHSS. S1P is a bioactive lipid that maintains the integrity of the endothelial barrier, through its binding to S1PRs (McVerry and Garcia, 2004; Singleton *et al.*, 2005). We therefore postulate that S1P levels in patients at risk of developing OHSS are altered, being in part responsible for the high vascular permeability observed in these patients.

Thus, we sought to determine the levels of S1P in FF from women at risk of developing OHSS. We also analyzed the effect of the presence of S1P in FF from women at risk of OHSS, on cell migration, on expression of adherens junction protein, VEGF isoforms, and S1PR1 and SPL, on

cytoskeletal changes in endothelial cell culture, as well as on *in-vivo* angiogenesis by using chorioallantoic membranes (CAMs) of quail embryos as an experimental model. Furthermore, we evaluated the effect of S1P administration on vascular permeability and on expression of $VEGF_{121}$ and its receptor KDR in ovaries from a rat OHSS model.

Materials and Methods

Subjects and collection of FFs

A total of 40 FF aspirates were collected from patients aged 25–39 years old undergoing assisted reproductive technology at the Reproductive Medicine Center Pregna (Buenos Aires, Argentina). The patients were classified into two groups: control group ($n = 20$) and at risk of OHSS group ($n = 20$). The criteria for considering a patient at risk of developing OHSS were more than 20 oocytes retrieved and a serum E2 concentration >3000 pg/ml on the day of hCG administration (Table I) (Chen *et al.*, 1997; Aboulghar and Mansour, 2003). Patients with pelvic pathologies such as uterine fibroids, pelvic inflammatory disease or endometriosis were excluded. hCG (5000 UI/ml Pregnyl®, Organon SA) was injected when follicles reached 17 mm, and 34 h later oocyte retrieval was conducted under vaginal ultrasound guidance. During oocyte retrieval, FF was extracted from 16 to 20 mm follicles in diameter, and collected from all follicles of each patient. FF samples, which were macroscopically clear indicating lack of contamination, were considered in the study. The FF was centrifuged immediately for 10 min at 2000*g* and the supernatant was stored at −80°C until it was assayed. All the patients sign the written informed consent before recruitment. The study was approved by the ethics committee of the Institute of Biology and Experimental Medicine (IByME, Buenos Aires, Argentina).

Endothelial cells

EA.hy926 endothelial cells were donated by Dr Gareth Owen (Pontifical Catholic University of Chile, Santiago, Chile). EA.hy926 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, NY, USA/Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 mg/ml streptomycin sulfate at 37°C with 5% CO_2.

Animal model

All procedures were approved by the Committee of the Experimental Medicine and Biology Institute (IByME-CONICET), in accordance with the National Institutes of Health standards, as described in the Guide for Care and Use of Laboratory Animals. We used an animal model that develops OHSS in immature Sprague-Dawley rats (21–23 days, 60–80 g), as described by Kitajima *et al.* (2004, 2006). The control group ($n = 6$) was injected with 10UI eCG and with 10UI hCG 48 h later. The OHSS group ($n = 6$) was injected with 50UI eCG for 4 consecutive days, followed by 25UI of hCG. The OHSS + S1P group ($n = 6$) received the same doses of eCG and hCG as the OHSS group and was also treated with S1P. For the S1P administration, on the day of hCG injection, the rats were anesthetized with ketamine (70 mg/kg; Holliday-Scott S.A., Buenos Aires, Argentina) and xylazine (5 mg/kg; König Laboratories, Buenos Aires, Argentina), and the ovaries were exteriorized through an incision made in the dorsal lumbar region. The OHSS+S1P group received 5 µl S1P (1 mM) (Sigma Aldrich St Louis, MO, USA) under the bursa of both ovaries, whereas control and OHSS animals received S1P vehicle (0.8% Tween-80; 2.5% ETOH; 5% PEG). Rats were euthanized by CO_2 asphyxiation 48 h after hCG injection and the ovaries were removed, cleaned of adhering tissue in culture medium and processed for western immunoblot assays.

Measurement of S1P

The concentration of S1P in FF was determined with a human ELISA kit (MyBioSource, San Diego, CA, USA). Intra-assay and inter-assay coefficients of variation were less than 15%.

Migration assay

A wound-healing assay was performed to assess the effect of FF from patients at risk of developing OHSS on endothelial cells in the presence of S1P, as previously described by our laboratory (Scotti *et al.*, 2013). Briefly, EA.hy926 cells were placed at 3×10^5 cells per well in a 24-well plate and grown to confluence. The cell monolayers were wounded with a 1000-µl micropipette tip in one direction. The wounded cells were incubated with FF (25%) (from both control patients and patients at risk of developing OHSS) in the presence of increasing concentrations of S1P (0.05, 0.1 and 0.2 µM). Serum-free DMEM/F12 was used as a negative control. Cells were incubated for 15 h at 37°C. Cell migration was monitored at initial wounding ($t = 0$ h) and at 15 h ($t = 15$ h) under a phase-contrast microscope (Olympus CKX41, Tokyo, Japan). The result was calculated as the percentage of cell migration (cell free area at $t = 0$ h – cell free area at $t = 15$ h) with respect to the negative control. Endothelial cell migration in the negative control is arbitrarily presented as 100%. The wound area was measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Confocal microscopy analysis of actin filaments

EA.hy926 cells were grown to confluence on glass coverslips and incubated with FF (25%) from patients for 24 h and then in the presence of S1P (0.1 µM) for 30 min. Actin staining with TRITC-phalloidin (Sigma Aldrich Co. St Louis, MO, USA) was performed following manufacturer recommended procedures. In brief, the cells were fixed in 3.7% formaldehyde solution in PBS for 5 min, permeabilized with 0.1% TRITON X-100 in PBS for 5 min and stained with 50 µg/ml of TRITC-phalloidin for 40 min at room temperature. Then, the cells were mounted on glass-slides using Vectashield mounting medium (Vector Laboratories, Inc., CA, USA) and observed by a confocal laser scanning system attached to a Nikon Eclipse E800 Microscope (Nikon, Tokyo, Japan).

Table I Clinical information of control and OHSS patients.

	Control patients (n = 20)	Patients at risk of OHSS (n = 20)	P value
Estradiol (pg/ml)	1919 ± 204	3453 ± 133	<0.0001
Age (years)	35.8 ± 0.7	33.7 ± 0.9	NS
Oocytes retrieved (number)	10.0 ± 0.5	25.0 ± 1.0	<0.0001

Data are expressed as the mean ± standard error of the mean. Student's *t*-test. Statistical significance was defined as <0.05. OHSS, ovarian hyperstimulation syndrome.

Western blot

After treatment with FF with or without S1P, EA.hy926 endothelial cells were lysed in lysis buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mMN-CBZ-l-phenylalanine chloromethyl ketone, 0.025 mMN-p-tosyl-lysine chloromethyl ketone and 0.025 mM l-1-tosylamide-2-phenyl–ethylchloromethyl ketone). The cell lysates were centrifuged at 10 000g for 10 min at 4°C. Rat ovaries were resuspended in five volumes of buffer lysis and homogenized with an Ultra-Turrax (IKA Werk, Breisgau) homogenizer. Ovarian samples were then centrifuged at 4°C for 10 min at 10 000g and the resulting pellets were discarded. Protein concentration was measured by the Bradford assay. After boiling for 5 min, 40 µg of protein was applied to a 12% SDS–polyacrylamide gel, and electrophoresis was performed at 25 mA for 1.5 h. The resolved proteins were transferred for 2 h onto nitrocellulose membranes. The blot was preincubated in blocking buffer (5% nonfat milk, 0.05% Tween 20 in 20 mM TBS pH 8.0) for 1 h at room temperature and incubated overnight in blocking buffer at 4°C with appropriate primary antibodies: β-actin 1/3000 (sc-1616-R), N-cadherin 1/200 (sc-7939), vascular endothelial (VE)-cadherin 1/100 (sc-9989) and β-catenin 1/200 (sc-59737) (Santa Cruz Biotechnology, Inc., Santa Cruz, USA), phospho-VE-cadherin 1/1000 (MAB1002) (Millipore, MA, USA), VEGF 1/1000 (ab46154) (Abcam, Cambridge, USA), KDR (VEGFR-2) 1/1000 (D5B1) (Cell Signaling Technology, Inc., Danvers, MA, USA). The protein levels were analyzed by densitometry using Scion Image for Windows (Scion Corporation, Worman's Mill, CT, USA). Optical density data are expressed as arbitrary units ± SEM.

In-vivo angiogenesis: quail CAM assay

Fertilized eggs of Japanese quail (*Coturnix coturnix japonica*; $n = 120$) were purchased from a local farmer and incubated *in ovo* at 37°C and 60% relative humidity for 2 days. The CAM assay was performed according to the methodology described previously in our laboratory (Scotti *et al.*, 2016). Briefly, a hole was drilled through its shell at the acute pole of the egg, and 1 ml albumen was removed with a sterile syringe. Afterwards, eggs were fenestrated with the aid of small dissecting scissors and embryos were cultured *ex ovo* in a 6-well plate for 5 days. Sterilized filter-paper disks 5 mm in diameter, which were used as a carrier for being directly loaded with FF from both control patients ($n = 20$) and patients at risk of OHSS ($n = 20$), with or without S1P (0.1 µM), were adhered on CAMs from 7-day embryos. The embryos were further incubated at 37°C for 2 days and then sacrificed by freezing. CAMs from 9-day-old embryos were fixed using a 4% paraformaldehyde/2% glutaraldehyde in PBS solution. Angiogenesis was visualized as the blood vessel branch points on a delimited surface of CAMs under a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan) at 40×.

Co-localization of Von Willebrand factor and α-actin in the CAM assay

CAMs were fixed with 4% paraformaldehyde solution in PBS for 30 min. Areas confined by filter disk were cut and placed in 12-well culture plates. CAMs were permeabilized with 1% BSA, 0.1% Triton 100X, 0.05 % Tween 20 in PBS overnight at 4°C. The CAMs were further incubated with the primary antibody rabbit polyclonal to Von Willebrand (VW, 1/200) (A0082 Dako, CA, USA) overnight at 4°C. The following day, the CAMs were incubated with the primary antibody mouse monoclonal to α-smooth muscle actin (α-SMA) (1/250) (ab18147 Abcam, Cambridge, USA) overnight at 4°C. CAMs were washed and incubated with the corresponding secondary antibodies Anti-rabbit FITC (1/1000) (81-6111 Zymed Laboratories Inc., CA, USA) and anti-mouse Dylight®549 (1/1000) (DI-2549, Vector Laboratories, CA, USA) for 2 h at room temperature. The CAMs were washed, placed on slides and mounted with glycerol:PBS (1:1) and observed under a Nikon Eclipse E800 confocal microscope.

Evaluation of vascular leakage

Ovarian permeability was measured by extravasation of sodium fluorescein (NaFl), a low molecular mass molecule (376 Da). A previously described protocol has been adapted (Phares *et al.*, 2006; Seelbach *et al.*, 2010). Rats were injected i.p. with 0.1 ml of 10% NaFl (Biopack, Argentina) in saline solution. After 45 min, animals were anesthetized and then transcardially perfused with 40 ml of saline solution. Ovaries were removed, embedded completely in OCT compound Cryoplast® (Biopack, Argentina) and then cut at 20 µm in a cryostat. Images showing the vascular cross-sections with fluorescent extravasation were obtained using a Nikon Eclipse E800 confocal microscope.

Data analysis

All the experiments were performed in triplicate and repeated three times. Data are expressed as the mean ± SEM of three independent experiments using 20 control patients and 20 patients at risk of OHSS, or six rats per group. The normally distributed data were subjected to one-way ANOVA followed by Tukey's or Dunnett's test for statistical comparison of the groups. Student's *t*-test was used to compare single means. Statistical significance was defined as $P < 0.05$. For statistical analysis of data, we used the statistical program Prism v5.0.

Results

S1P concentration in FF from patients at risk of OHSS and control patients

The concentration of S1P in FF was measured using an ELISA. S1P concentration was significantly lower in FF from patients at risk of OHSS than in FF from control patients ($P = 0.03$) (Table II).

Effect of FF from patients at risk of OHSS, in the presence of S1P, on endothelial cell migration

Endothelial cell migration in the presence of FF from patients at risk of OHSS was higher than that in the presence of FF from control patients ($P < 0.05$). The addition of S1P (0.05 or 0.1 µM) to the FF from patients at risk of OHSS decreased endothelial cell migration compared to FF from patients at risk of OHSS without treatment ($P < 0.05$) (Fig. 1).

Table II S1P concentration in FF from control and OHSS patients.

	FF control (n = 20)	FF OHSS (n = 20)	P value
S1P (µg/L)	23.15 ± 0.58	18.80 ± 0.55	0.03

Sphingosine-1-phosphate (S1P) was measured by ELISA. Data are expressed as the mean ± standard error of the mean. Student's *t*-test. Statistical significance was defined as <0.05. FF, follicular fluid.

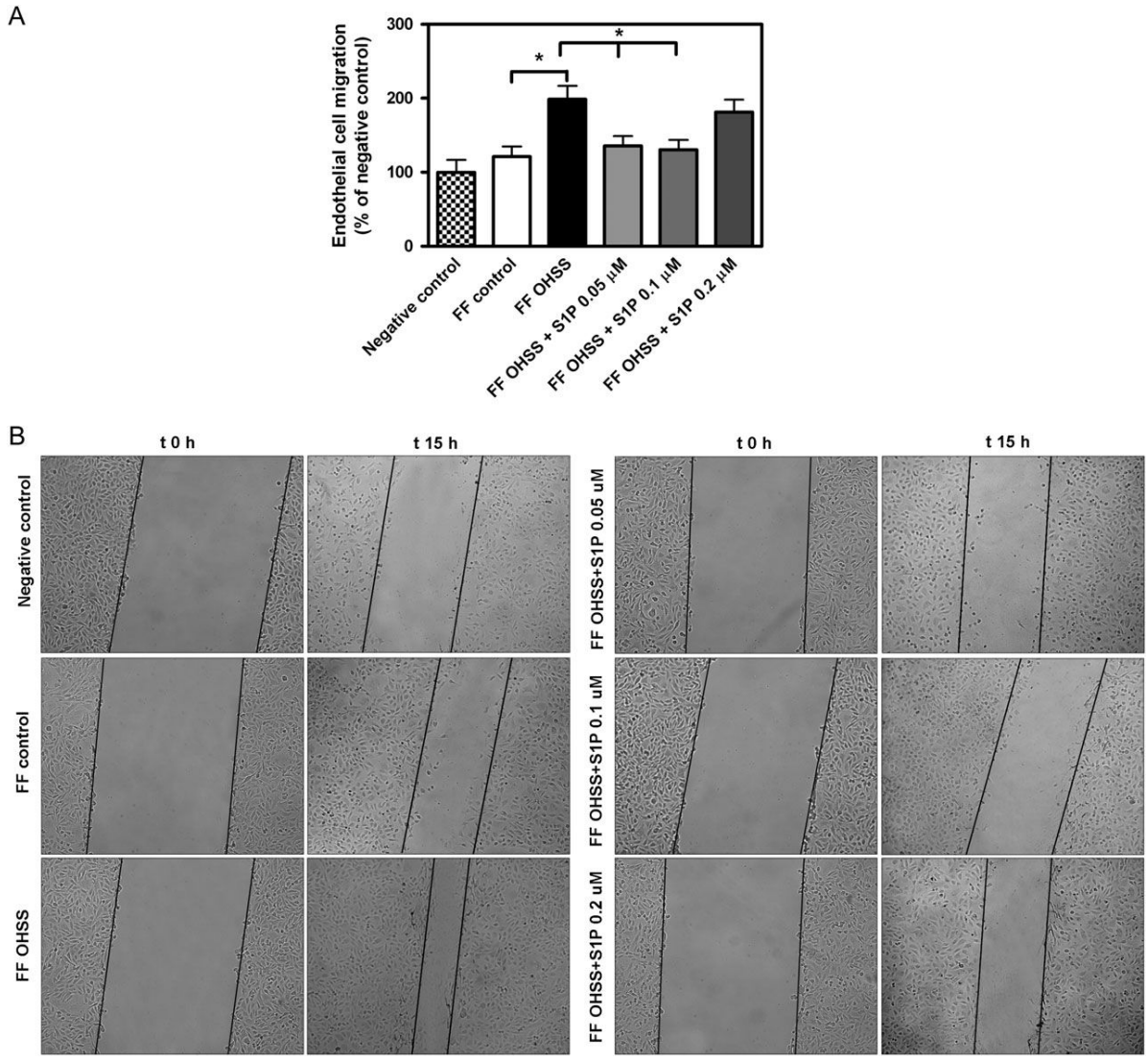

Figure 1 Effect of sphingosine-1-phosphate (S1P) on endothelial cell migration in the presence of follicular fluid (FF) from patients at risk of ovarian hyperstimulation syndrome (OHSS). (**A**) Quantification of the wound-healing assay. The columns show the percentage of endothelial cell migration respect to the negative control. Endothelial cell migration in the negative control is arbitrarily presented as 100%. Data are expressed as means ± SEM (Control: $n = 20$, OHSS: $n = 20$; *$P < 0.05$). Values represent the mean of three independent experiments. (**B**) Representative images of the induction of endothelial cell migration in a wound-healing assay by FF preincubated for 1 h at 37°C with or without S1P. Images were taken immediately after scratching the cultures (t0) and 15 h later (t15). Original magnification ×400.

Effect of FF from patients at risk of OHSS, in the presence of S1P, on the expression of endothelial adherens junction proteins

We evaluated the expression of the adherens junction proteins VE-cadherin, N-cadherin and β-catenin in endothelial cells incubated with FF from patients at risk of OHSS in the presence of S1P (Fig. 2). The levels of phospho-VE-cadherin in endothelial cells incubated with FF from patients at risk of OHSS were higher than those in endothelial cells incubated with FF from control patients ($P < 0.05$). The addition of S1P to the FF from patients at risk of OHSS prevented VE-cadherin phosphorylation (Fig. 2A). VE-cadherin levels in endothelial cells incubated with FF from patients at risk of OHSS were decreased as compared to those in the control group ($P < 0.05$) but were restored to control levels in the presence of 0.2 µM S1P (Fig. 2B). N-cadherin levels in endothelial cells incubated with FF from patients at risk of OHSS were also lower than those of the control group ($P < 0.05$). The addition of S1P to the FF from patients at risk of OHSS restored N-cadherin levels to control values (Fig. 2C). Similarly β-catenin levels in endothelial cells incubated with FF from patients at risk of OHSS were lower than in control patients ($P < 0.05$) but similar to control values in the presence of S1P (Fig. 2D).

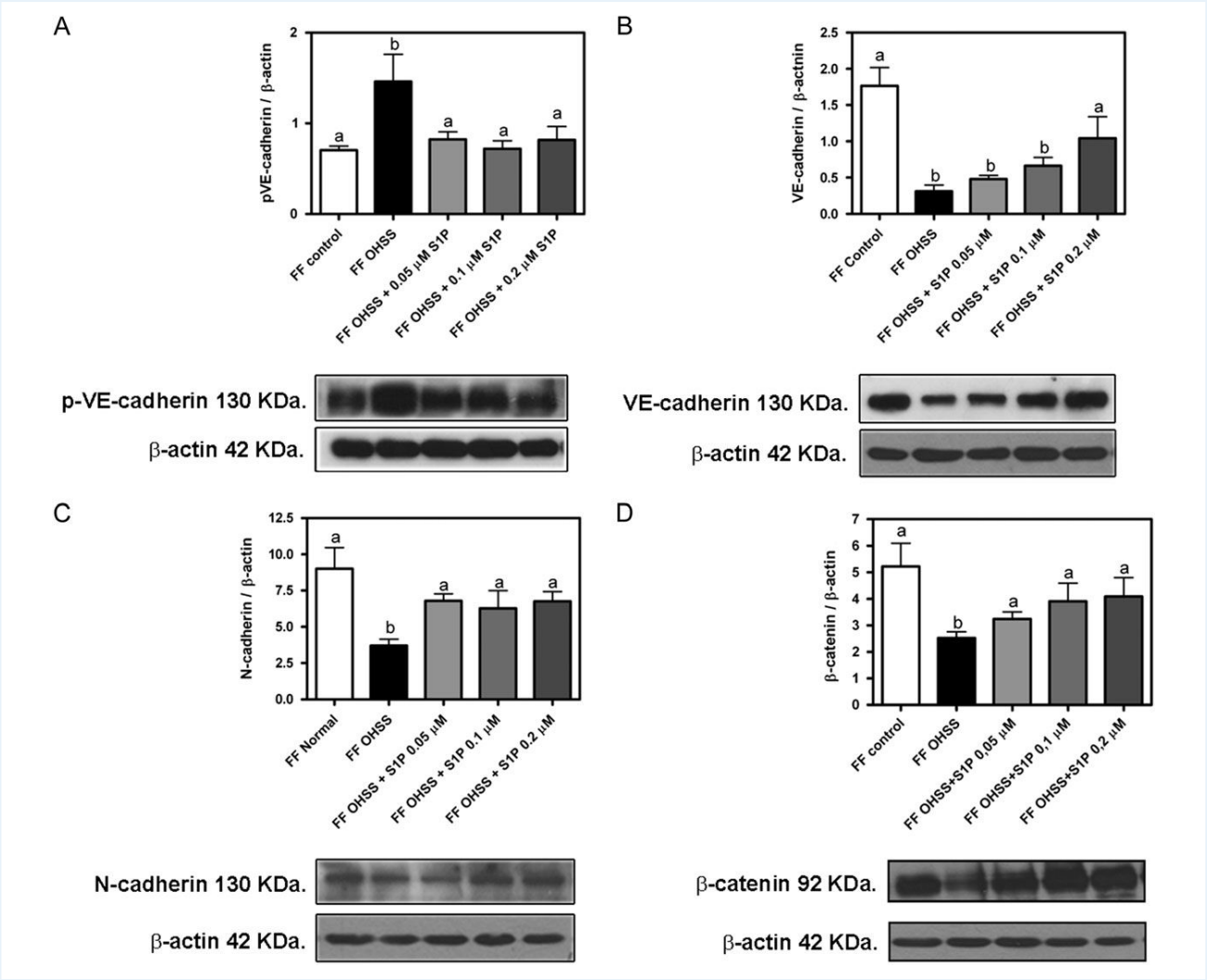

Figure 2 Effect of S1P on the expression of endothelial adherens junction proteins in endothelial cells in the presence of FF from controls and patients at risk of OHSS. Densitometric quantification of (**A**) phospho-vascular endothelial (p-VE)-cadherin, (**B**) VE-cadherin, (**C**) N-cadherin and (**D**) β-catenin. Representative immunoblots are shown in the lower panel. Data are expressed as means ± SEM normalized to β-actin of three independent experiments using 20 control patients and 20 OHSS patients at risk of OHSS (*$P < 0.05$).

Effect of FF from patients at risk of OHSS, in the presence of S1P, on actin polymerization of endothelial cells

The actin filament polymerization of endothelial cells cultured in different conditions was evaluated using confocal microscopy (Fig. 3). The incubation of endothelial cells with FF from patients at risk of OHSS caused a dramatic rearrangement of actin filaments (characterized by the formation of long stress fibers which traversed the cells) compared to the FF from control patients (Fig. 3A and B). In addition, paracellular gap formation was induced in endothelial cells incubated with FF from patients at risk of OHSS compared to FF from control patients. In the presence of S1P, FF from patients at risk of OHSS induced the formation of a cortical band near the plasma membrane, essential to the maintenance of endothelial barrier function, and decreased the paracellular gap formation compared to the OHSS group without treatment (Fig. 3C).

Effect of FF from patients at risk of OHSS, in the presence of S1P, on the expression of VEGF isoforms

We next analyzed the expression of $VEGF_{121}$ and $VEGF_{165}$ isoforms in endothelial cells incubated with FF from patients at risk of OHSS either alone or preincubated with S1P. $VEGF_{121}$ levels in endothelial cells incubated with FF from patients at risk of OHSS were higher than those of the control group ($P < 0.01$). The addition of S1P in the FF from patients at risk of OHSS decreased $VEGF_{121}$ levels compared to the OHSS group without treatment (Fig. 4A). The treatment of endothelial cells with FF from patients at risk of OHSS had no significant effect on the basal expression of $VEGF_{165}$ (Fig. 4B).

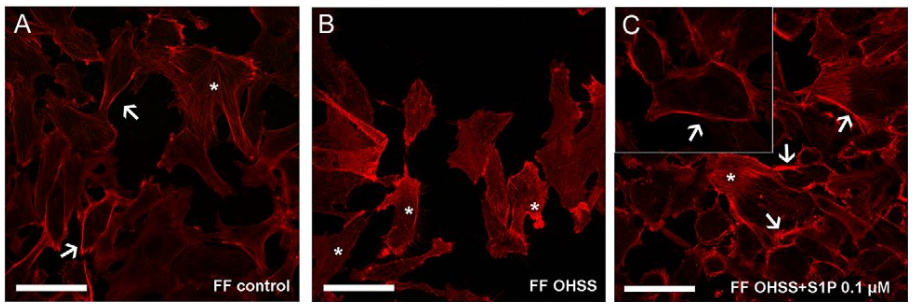

Figure 3 Effect of S1P on actin polymerization of endothelial cells in the presence of FF from controls and patients at risk of OHSS. The panel shows representative images of actin filaments stained with TRITC-phalloidin in endothelial cells incubated with (**A**) FF from control patients; (**B**) FF from patients at risk of OHSS; and (**C**) FF from patients at risk of OHSS with S1P. Asterisks indicate stress fibers and arrows indicate cortical bands. Original magnification ×60. The insert shows a cortical band at high magnification.

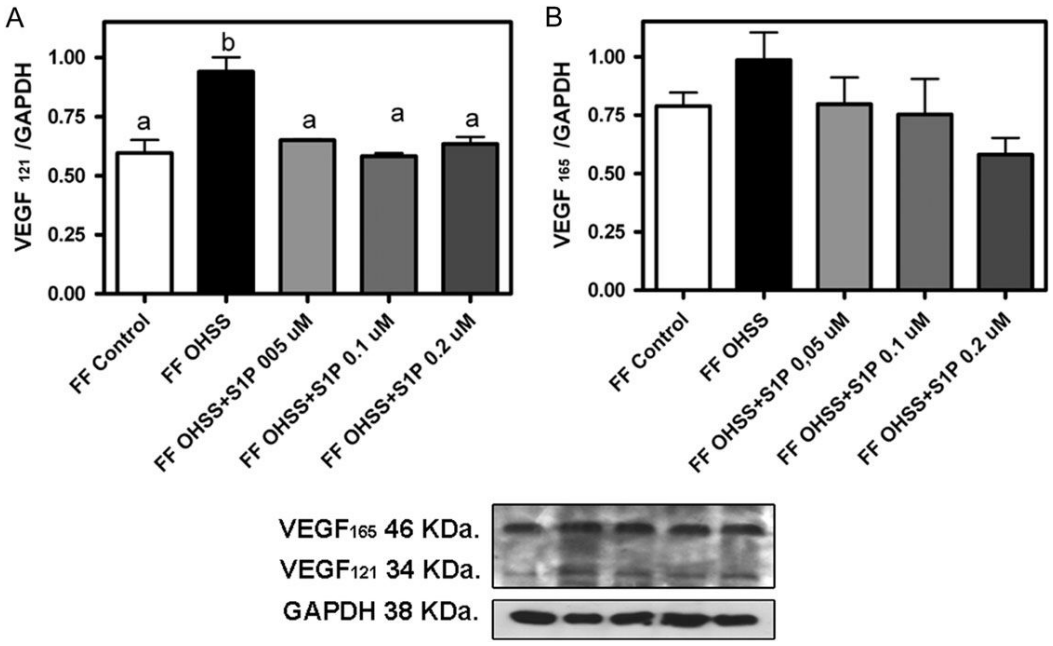

Figure 4 Effect of S1P on the expression of vascular endothelial growth factor (VEGF) isoforms in the presence of FF from controls and patients at risk of OHSS. Densitometric quantification of (**A**) $VEGF_{121}$ and (**B**) $VEGF_{165}$. Representative immunoblots are shown in the lower panel. Data are expressed as means ± SEM normalized to β-actin of three independent experiments using 20 control patients and 20 OHSS patients at risk of OHSS (*$P < 0.05$).

Effect of FF from patients at risk of OHSS, in the presence of S1P, on S1PR1 and SPL expression

The levels of S1PR1 in endothelial cells incubated in the presence of FF from patients at risk of OHSS were lower than in the presence of FF from control patients ($P < 0.05$). S1P (0.1 µM) increased S1PR1 levels similar to the control group (Fig. 5A). We next analyzed the expression of SPL, the enzyme that degrades S1P. The levels of SPL in the presence of FF from patients at risk of OHSS were increased compared to FF from control patients ($P < 0.05$) but were restored to control levels in the presence of S1P (0.1 and 0.02 µM) (Fig. 5B).

Effect of FF from patients at risk of OHSS, in the presence of S1P, on ovarian angiogenesis

We evaluated the effect of FF from patients at risk of OHSS in the presence of S1P using the CAM assay, an *in-vivo* model of angiogenesis. The CAMs incubated with FF from patients at risk of OHSS revealed an increase in the number of vascular branch points compared to that

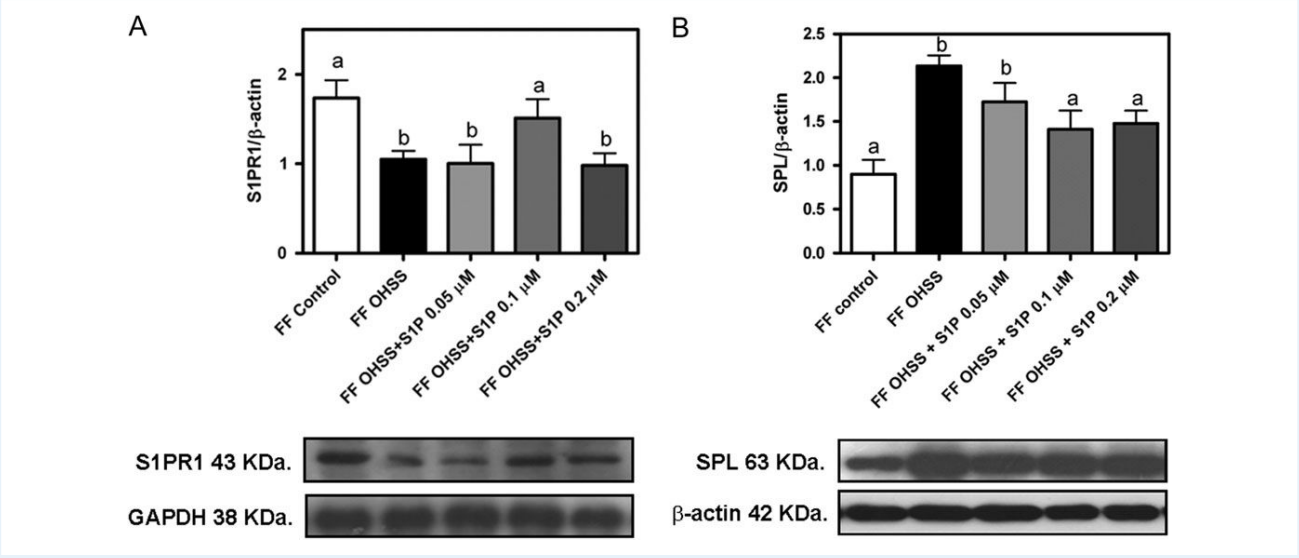

Figure 5 Effect of S1P on S1PR1 and SPL expression in endothelial cells in the presence of FF from control and patients at risk of OHSS. Densitometric quantification of (**A**) S1PR1 and (**B**) SPL. Representative immunoblots are shown in the lower panel. Data are expressed as means ± SEM normalized to GAPDH or β-actin of three independent experiments using 20 control patients and 20 OHSS patients at risk of OHSS (*$P < 0.05$).

from control patients (Fig. 6A and B). The presence of S1P (0.1 μM) in the FF from patients at risk of OHSS decreased the vascular branch points compared to the OHSS group without treatment (Fig. 6C).

We also analyzed the effect of S1P (0.1 μM) on the maturation and stabilization of newly formed blood vessels in the presence of FF from patients at risk of OHSS. To this end, we performed immunofluorescence and evaluated the co-localization of endothelial cells (VW) and periendothelial cells (α-SMA) in the capillary plexus of the CAMs. In the presence of FF from patients at risk of OHSS, we observed few α-SMA-positive vessels compared to the same CAMs incubated in the presence of FF from control patients (Fig. 6D–I). The addition of S1P (0.1 μM) in the FF from patients at risk of OHSS increased the α-SMA-positive periendothelial cell coverage in the CAMs compared to the FF from patients at risk of OHSS without treatment (Fig. 6J–L).

Effect of S1P administration on ovarian capillary permeability and expression of VEGF and KDR proteins in the rat OHSS model

The images detecting vascular permeability corresponding to slices from control group (Fig. 7A) showed a diffuse pattern commonly associated without NaFl background without a clear vessel morphology identity. However, the images from OHSS group (Fig. 7B and insets C and D) exhibited the typical vascular pattern associated with increased permeability, where transversal and longitudinal vessels were distinguished. Notably, after the S1P treatment, the appearance of the blood vessels did not differ from the control group, showing appropriate vascular integrity (Fig. 7E). Additionally, the ovarian levels of $VEGF_{121}$ and its receptor KDR were higher in the OHSS group than in the control group ($P < 0.05$), while local administration of S1P decreased the levels of these proteins in the OHSS group ($P < 0.05$) (Fig. 7F and G).

Discussion

OHSS characteristics include marked ovarian enlargement owing to ovarian stimulation and overproduction of ovarian hormones and angiogenic factors, which cause an increase in vascular permeability, leading to ascites in the patients. Previously, we have shown that the inhibition of VEGF (TRAP: soluble form of VEGF receptor-1) and ANGPT1 (neutralizing antibody) both prevents the early development of OHSS and decreases its degree of severity (Scotti *et al.*, 2013, 2014, 2016).

In the present study, we demonstrated for the first time that the levels of sphingolipid S1P in FF from women at risk of developing OHSS are lower in comparison with FF from women who are not at such risk. We also show evidence that the addition of S1P to the FF alters endothelial cell migration, expression of $VEGF_{121}$ isoform, S1PR1, SPL and endothelial cell–cell junction protein, and distribution of actin filaments. By means of an *in-vivo* assay, we found that FF from patients at risk of OHSS in the presence of S1P decreased the angiogenic activity and restored the vascular integrity in CAMs. Additionally, we demonstrated that intrabursal *in-vivo* administration of S1P decreased ovarian capillary permeability and the expression of $VEGF_{121}$ isoform and its receptor KDR in the ovaries from an OHSS rat model.

As several authors have shown, increased levels of VEGFA constitute one of the pivotal etiologic factors leading to OHSS-associated vascular dysfunction (McClure *et al.*, 1994; Artini *et al.*, 1998; Molskness *et al.*, 2004). In addition, we have previously demonstrated that an increase in ANGPT1 levels in patients' FF, as measured on the day of oocyte retrieval, could prove an important nonsteroidal marker

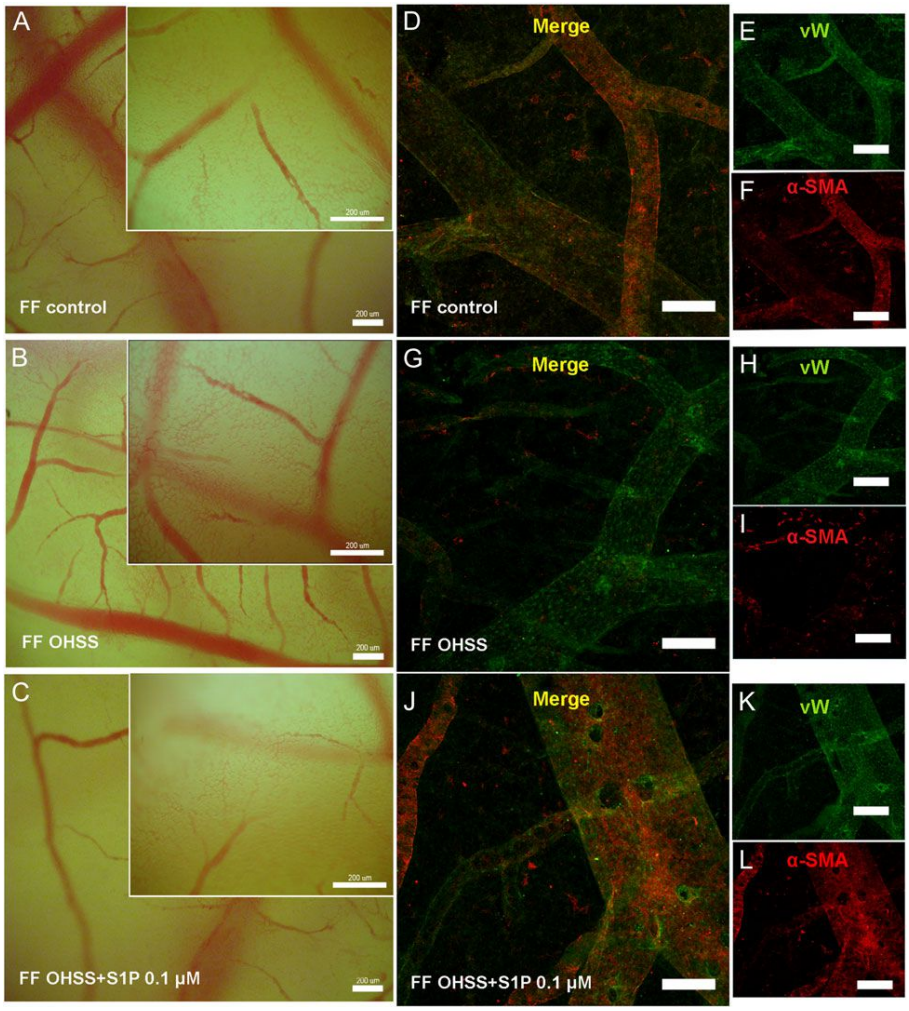

Figure 6 Effect of S1P on angiogenesis of chorioallantoic membranes (CAMs, **A–C**) Representative images of blood vessel of CAMs incubated with (A) FF from control patients, (B) FF from patients at risk of OHSS and (C) FF from patients at risk of OHSS with S1P (0.1 μM). Original magnification ×40, insert ×100. Scale bars represent 200 μm. (**D–L**) Pictures show immunostaining for Von Willebrand (VW, green) and α-smooth muscle actin (α-SMA, red) on CAMs incubated with (D, E and F) FF from control patients; (G, H and I) FF from patients at risk of OHSS, and (J, K and L) FF from patients at risk of OHSS with S1P (0.1 μM). Original magnification ×100. Scale bars represent 200 μm.

of OHSS (Scotti *et al.*, 2013). In this study, we observed a decreased in S1P concentration in FF from patients at risk of OHSS. These results suggest that there is a smaller availability of S1P in FF from patients with symptoms of OHSS, which would cause a loss in endothelial barrier protection and, in turn, an increase in the vascular permeability observed in these patients.

Since the angiogenic process requires the proliferation and migration of vascular endothelial cells, we studied the specific effect of S1P on ovarian angiogenesis in patients at risk of OHSS. To achieve this, we assessed the effect of FF in the presence of the S1P on endothelial cell migration. Although FF from patients at risk of OHSS induced cell migration to a larger extent than that from control patients, incubating FF from patients at risk of OHSS with S1P produced a decrease in cell migration when compared to that without the sphingolipid. We also showed that S1P was able to restore the levels of $VEGF_{121}$ in endothelial cells incubated with FF from patients at risk of OHSS to those of control patients. These results suggest that S1P decreases, at least in part, endothelial cell migration through the reduction of $VEGF_{121}$ levels in OHSS. It is important to mention that several authors, including ourselves, have previously demonstrated that VEGFA is strongly implicated in the increase in endothelial migration and vascular permeability that characterizes various female angiogenesis-related reproductive disorders, like OHSS (Villasante *et al.*, 2007; Scotti *et al.*, 2014). Although in many biological systems S1P promotes cell migration (Lee *et al.*, 1999b; Kimura *et al.*, 2003), there is evidence that S1P also triggers the reorganization of the cytoskeleton, leading to inhibition of cell migration (Okamoto *et al.*, 2000; Olivera *et al.*, 2003). Hence, we can hypothesize based on these findings that the reduced levels of S1P in

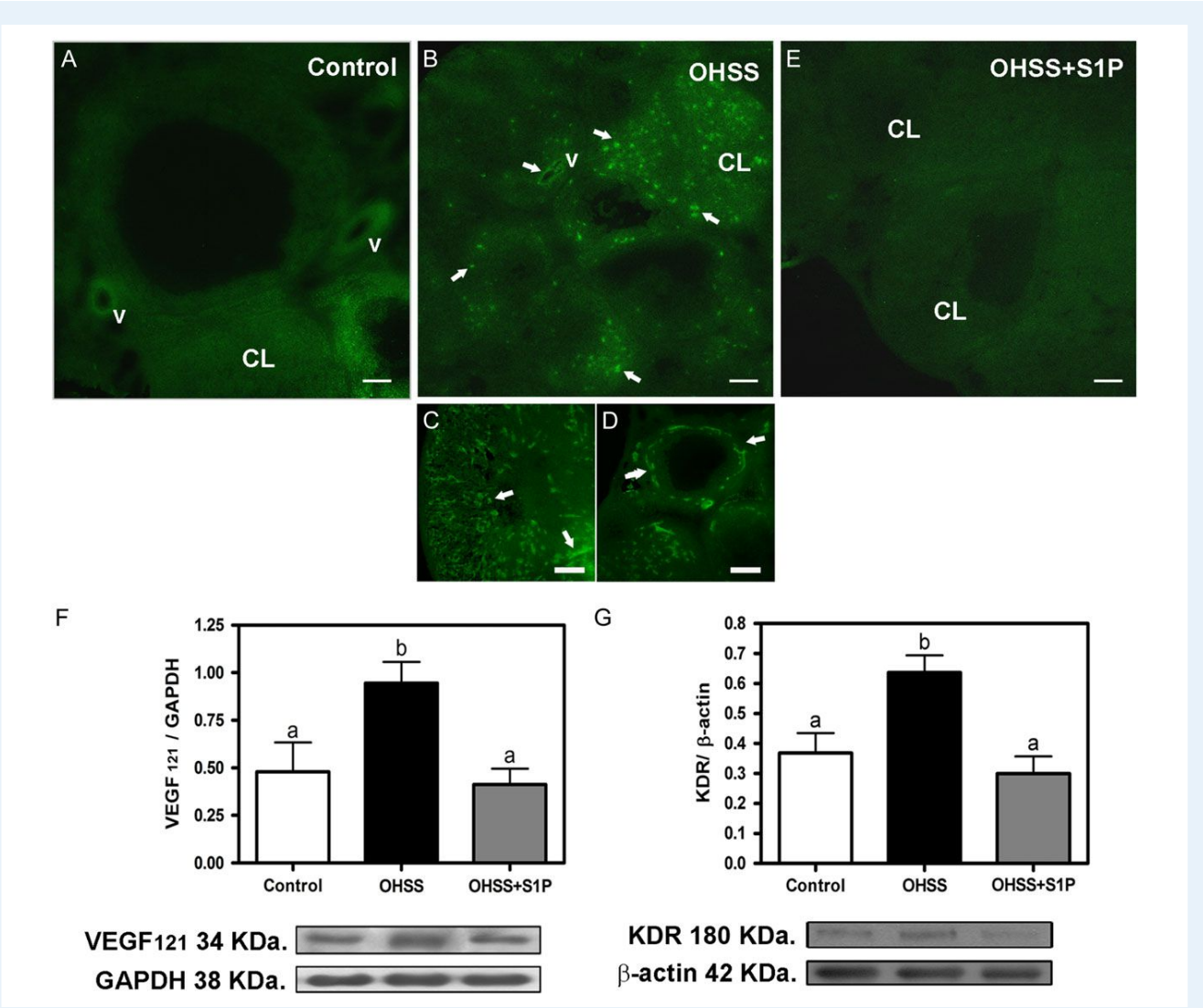

Figure 7 Effect of S1P administration on capillary permeability and the expression of $VEGF_{121}$ and KDR proteins in the rat OHSS model. (**A–E**) Representative images of ovarian permeability measured by the extravasation of sodium fluorescein (NaFlu) assay. (A) Control group, (B–D) OHSS group and (E) OHSS+S1P group. Arrows indicate the extravasation areas; CL: corpus luteum; V: blood vessel. Original magnification ×100, inserts ×200. Scale bars represent 100 μm. (**F–G**) Densitometric quantification of (F) $VEGF_{121}$ (Control vs. OHSS $P < 0.05$; OHSS vs. OHSS+S1P $P < 0.05$) and (G) KDR (Control vs. OHSS $P < 0.05$; OHSS vs. OHSS+S1P $P < 0.01$). Representative immunoblots are shown in the lower panel. Data are expressed as means ± SEM normalized to GAPDH or β-actin of three independent experiments using six rats per group.

OHSS generate a deficient regulation of the angiogenic system balance and, subsequently, alters angiogenesis in this syndrome.

Vascular maturation, which is defined as the investment of mural cells to the endothelial cell layer of neovessels, is essential for the maintenance of the established vasculature (Jain, 2003). As endothelial hyperpermeability generally comes along with junction protein reorganization, this favors a transient opening of the endothelial junctions, together with a subsequent increase in vascular permeability. Accordingly, we propose based on this notion that adherens junction proteins are downstream targets of S1P in endothelial cells in OHSS. As we have showed in this study, N-cadherin levels were significantly decreased in endothelial cells incubated with FF from patients at risk of OHSS compared with control patients. The addition of S1P increased N-cadherin levels compared with FF from patients at risk of OHSS without the sphingolipid. It is worth noting that a study by Paik *et al.* showed that S1P induces N-cadherin trafficking to the plasma membrane domains in endothelial cells, promoting the interaction with mural cells (Paik *et al.*, 2004). Therefore, we propose that the decrease in S1P levels in patients at risk of OHSS could be partly responsible for the decreased expression of N-cadherin expression in endothelial cells.

Besides N-cadherin, another adherens junction protein involved in the regulation of endothelial permeability is VE-cadherin. VE-cadherin has a key role in maintaining vascular integrity and permeability

(Dejana et al., 2009). In our study, we evaluated the effect of S1P on VE-cadherin expression and its phosphorylation in endothelial cells with FF from patients at risk of OHSS. We showed that FF from patients at risk of OHSS decreased the expression of VE-cadherin in endothelial cells compared to the FF from controls. The addition of S1P increased the expression of this junction protein. Our results also demonstrated that endothelial cells incubated in the presence of FF from women at risk of OHSS decreased the expression of β-catenin compared to endothelial cells incubated with the FF from controls. This effect was reversed by the addition of S1P to FF from patients at risk for OHSS. These results agree with the observation by Lee et al. (1999a,1999b), who demonstrated that administration of S1P to human umbilical vein endothelial cells (HUVEC) increased the localization of VE-cadherin and β-catenin in junctions (Lee et al., 1999a). It is known that changes in cytoskeletal dynamics and phosphorylation of VE-cadherin mediate adhesion between endothelial cells. Phosphorylation of VE-cadherin at tyrosine 731 leads to separation of β-catenin in its cytoplasmic tail and subsequent internalization (Potter et al., 2005). In this study, we observed enhanced phosphorylation of VE-cadherin in endothelial cells incubated with FF from patients at risk for OHSS compared to control patients. Interestingly, the addition of S1P to FF from patients at risk for OHSS decreased phosphorylation of VE-cadherin to values similar to those of controls. It is established that the integrity of adherens junctions is regulated by phosphorylation of VE-cadherin and its internalization in response to stimulation by VEGF (Gavard and Gutkind, 2006; Wallez et al., 2007). Binding of VEGF to its KDR receptor initiates a cascade of events that lead to phosphorylation of VE-cadherin and the disassembly of adherens junctions (Mukherjee et al., 2006). Gaengel et al. (2012) evaluated the communication between the VEGF and S1P systems and showed that when co-stimulating HUVEC with VEGF and S1P, VE-cadherin remained stable in endothelial junctions and was insensitive to the internalization induced by VEGF (Gaengel et al., 2012). Furthermore, *in-vivo* administration of an agonist of S1PR1 blocks VEGF-induced vascular permeability, suggesting that S1RP1 can regulate vascular permeability (Sanchez et al., 2003). Taking all these findings into consideration, we show evidence that S1P could restore the levels of N-cadherin and β-catenin and prevent VE-cadherin phosphorylation, contributing to a reduction in ovarian vascular permeability.

The cytoskeleton of endothelial cells is a critical determinant of vascular integrity and regulation of the endothelial barrier (Stevens et al., 2000; Garcia et al., 2001). Distribution of actin to a peripheral pattern is a main regulatory mechanism to maintain cell junction integrity and it leads to stress fiber formation. Here we showed that FF from patients at risk of OHSS promoted strong actin filament remodeling, forming stress fibers. This result is consistent with previously obtained data in our laboratory (Scotti et al., 2016). In this study, S1P was able to increase the polymerization of actin filaments, forming a ring of cortical actin in endothelial cells incubated with FF from patients at risk of OHSS. Furthermore, S1P prevented formation of stress fibers transversely located along the cytoplasm of the cells observed in the OHSS group. It is known that Rac1 activation by S1P is critical to the formation of the cortical ring (Wang and Dudek, 2009). This result is consistent with the increase in N-cadherin expression in endothelial cells observed in the presence of S1P, since polymerization of actin filaments induced by this sphingolipid causes an increase in the levels of this cadherin in the plasma membrane. This event, essential for a proper interaction between endothelial cells and mural cells, is necessary for vascular stabilization (Paik et al., 2004). The effects of S1P in endothelial cells, such as modulation of chemotaxis and vascular integrity, depend on cytoskeletal reorganization. Therefore, our results would indicate that S1P is able to lead to the formation of the cortical ring in endothelial cells, being the phenotype for vascular stabilization and, in turn, contributing to a decrease in ovarian vascular permeability.

The S1P/S1PR1 signaling in endothelial cells can be considered a mechanism of vascular stabilization, since it prevents aberrant angiogenic responses (Gaengel et al., 2012). Deletion of S1PR1 in mice causes embryonic death due to severe bleeding due to a defect in vascular stabilization (Liu et al., 2000). In particular, in the human ovary, S1PR1 is slightly expressed in the stroma and endothelial cells surrounding the vessels (Bradaric et al., 2011). Our results showed that S1PR1 expression was significantly lower in endothelial cells incubated with FF from patients at risk of OHSS compared to FF from control patients. These results indicate that unidentified factors in FF from OHSS patients may be responsible for the indirect/direct down-regulation of S1PR1 expression in endothelial cells. Several authors and our group have previously shown altered levels of angiogenic factors (VEGF, ANGPT1 and PDGF) in OHSS (Artini et al., 1998; Molskness et al., 2004; Scotti et al., 2011, 2013). Based in these observations, VEGF or ANGPT1 binding to the receptor tyrosine kinase would initiate transduction cascade, decrease the expression of S1PR1 and lead to loss of endothelial barrier maintenance. Further experiments are needed to elucidate the involvement of these angiogenic factors in regulating S1PR1 receptor expression in endothelial cells. Indeed, the addition of S1P (0.1 μM) restored S1PR1 expression to normal values. As reported by several authors, S1P presents biphasic effects in the regulation of cellular processes such as differentiation and proliferation in many cell types (Wang et al., 2005; Meyer zu and Jakobs, 2007). These effects can respond to differences amongst specific cell types, regarding distribution and expression levels of S1P receptors, as well as concentration and period of S1P stimulation. In particular, in the ovary, Hernandez-Coronado et al. (2016) have observed that S1P has a biphasic effect on granulosa cell number in culture (Hernandez-Coronado et al., 2016). Based on the information above and our results, these differences may be due to the biphasic effect of the S1P depending on the time of stimulation and cellular function that is regulating. The mechanism responsible for the increased expression of S1PR1 following S1P treatment is currently unknown. Thus, S1PR1 up-regulation caused by S1P may represent a significant positive feedback mechanism for S1P action in vascular barrier integrity improvement in OHSS.

SPL is an enzyme induced by intracellular stress, responsible for the irreversible degradation of S1P to phosphoethanolamine and hexadecanal. Therefore, this enzyme is considered to be a crucial checkpoint to regulate S1P cellular concentrations (Aguilar and Saba, 2012). Indeed, SPL-deficient transgenic mice exhibit a marked increase in S1P levels in tissues and serum (Bandhuvula et al., 2011; Zhao et al., 2011). In this study, we showed that SPL expression increased in endothelial cells incubated with FF from women at risk of OHSS compared to FF from controls. However, the addition of S1P was able to decrease the expression of SPL. Recently, several studies in animal models have showed that one of the possible causes of the decrease in S1P in various pathologies such as cardiac ischemia (Bandhuvula et al., 2011),

injury acute lung (Zhao *et al.*, 2011), human breast cancer (Ling *et al.*, 2011) and Alzheimer's disease (Ceccom *et al.*, 2014) is the increase in SPL expression. Bandhuvula *et al.* (2011) have shown that cardiac ischemia induces activation of SPL, and that inhibition of this enzyme reduces its activity, increasing levels of S1P and reducing infarct size (Bandhuvula *et al.*, 2011). In another report, in an animal model of acute lung injury, SPL levels were increased and S1P levels decreased (Zhao *et al.*, 2011). Based on these observations, our results suggest that the increased expression of SPL in endothelial cells incubated with FF from patients at risk of OHSS leads to decreased levels of S1P, and could contribute in part to the high vascular permeability characteristic of this syndrome. Furthermore, the addition of S1P to FF from patients at risk of OHSS decreases endothelial expression of its own enzyme that degrades S1P irreversibly. Oskouian *et al* (2005) have shown that the human SPL gene is regulated by GATA transcription factors (Oskouian *et al.*, 2005). Later, Ito *et al* (2011) showed in human lung cancer cell lines that the GATA-4/Sp1 complex binds to SPL promoter and, in turn, regulates the transcription of SPL (Ito *et al.*, 2011). On the other hand, it is well known that S1P produces receptor-mediated activation of RhoA, which in turn induces local activation of the actin cytoskeleton and focal adhesions, leading to endothelial barrier enhancement (Zhang *et al.*, 2016). Additionally, RhoA has been implicated in transcriptional modulation of GATA, AP-1 and NFkB (Charron *et al.*, 2001; Marinissen *et al.*, 2001; Anwar *et al.*, 2004). It is important to mention that there is evidence that GATA transcription factors activate, repress and/or spatially restrict the expression of several genes (Koh *et al.*, 2002; Fukushige *et al.*, 2003). Based on the information above and our results, S1P could activate RhoA (Rho family GTPases), affect the expression of GATA family proteins and modulate the expression of the SPL and thereby restore the vascular homeostasis in OHSS. To elucidate the roles of the GATA proteins on the regulation of SPL expression by S1P in endothelial cells, further experiments are required. It is worth noting that several reports have demonstrated the pathophysiological importance of SPL in human diseases (Oskouian *et al.*, 2005; Ceccom *et al.*, 2014) and its modulation could be a possible strategy in treating cancer and other disorders characterized by altered angiogenesis such as OHSS.

The CAM assay can be considered an intermediate stage between *in-vitro* culture and *in-vivo* animal experiments (Kurz *et al.*, 2008; Isachenko *et al.*, 2012), which has proven useful to evaluate angiogenic processes in tumors (Berube *et al.*, 2005) and endometrial tissues (Nap *et al.*, 2004). Here we used the Japanese quail CAM to study angiogenesis in OHSS and we found that FF from patients at risk of OHSS increased the angiogenic activity in CAMs in comparison with FF from control patients. This result corroborate observations previously made by our laboratory using the CAM system (Scotti *et al.*, 2016). Our results also showed that the presence of S1P in FF from patients at risk of OHSS decreased the number of vascular branches and the width of the vessels of the capillary plexus. On the other hand, by confocal microscopy, we detected less presence of α-SMA-positive cells in the CAM microvasculature incubated with FF from patients at risk of OHSS compared to control patients. However, the addition of S1P to this FF strongly increased the presence of α-SMA-positive cells in the blood vessels. All these findings suggest that the limited availability of S1P in the OHSS ovary could be associated with exacerbated vascular permeability and that this bioactive lipid is able to restore the vascular barrier integrity through the recruitment of mural cells (smooth muscle cells and pericytes) to the endothelium, in turn, promoting the cross-talk between both cell types and enhancing vessel maturation. More studies are required to elucidate whether the effect of S1P on mural cells is direct or indirect. In this regard, it has been proposed that PDGFRβ couples to S1PR1, generating a platform for integrative signaling by both receptors (Alderton *et al.*, 2001). Tanimoto *et al.* (2004) have shown that S1P transactivates PDGFRβ and EGF receptors in vascular smooth muscle cells, causing its proliferation and expansion, and improving vascular barrier integrity (Tanimoto *et al.*, 2004).

In our study, we have used an animal model that develops OHSS in immature rats to corroborate our observations in the *in-vitro* assays. This OHSS experimental model is useful owing to the similarity between the rat and human VEGF systems (Phillips *et al.*, 1990; Levin *et al.*, 1998), which is one of the reasons why it has been employed by several authors and our group (Gomez *et al.*, 2002; Kitajima *et al.*, 2004, 2006; Ozcakir *et al.*, 2005; Scotti *et al.*, 2011, 2014). The present results showed that vascular permeability and the expression of both $VEGF_{121}$ isoform and its receptor KDR were increased in the OHSS group compared to the control group, while S1P administration decreased all of these parameters. These results suggest that S1P was able to enhance vascular barrier function by modulation of the VEGF/VEGFR-2 system and, consequently, reduce the vascular permeability in OHSS.

Our results from *in-vivo* and *in-vitro* angiogenesis assays allow an improved understanding of some of the biochemical and molecular mechanisms by which the S1P/S1PR1 signaling pathway promotes affects vascular permeability in OHSS. Our findings suggest that the limited availability of S1P in FF from patients at risk of developing OHSS would be partly responsible for the loss not only of the integrity of the connection between endothelial cells, but also of the interaction of these cells with pericytes, thus causing an increase in vascular permeability. It is worth mentioning that several clinical studies have demonstrated the efficiency of the use of the S1P agonist FTY720 for the treatment of patients with multiple sclerosis and immunosuppressed organ transplant patients (Budde *et al.*, 2006; Mehling *et al.*, 2011).

In conclusion, the results described for the first time in the present study indicate that the decrease in S1P levels may contribute to the pathogenesis of OHSS, leading to an increase in vascular permeability. The treatment with S1P could both prevent the early onset of OHSS and attenuate the severity of this syndrome. Thus, the use of bioactive sphingolipid metabolites may contribute to better and safer therapeutic strategies for the treatment of OHSS and other human diseases that display aberrant vascular leakage.

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Authors' roles

L.S. and M.D.P. performed the experiments and contributed to data analysis and interpretation. N.P. performed the experiments and helped in drafting of the manuscript. G.I. and M.T. contributed to data interpretation and discussed the results. I.Z. and M.G.P. collected

follicular fluids aspirates from patients and discussed the results. C.P. and F.S. performed the experiments on vascular permeability and helped draft the manuscript. D.A. analyzed and discussed the results and helped draft the manuscript. F.P. conceived the concept, designed the experiments, supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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