Acute immunomodulatory changes during controlled ovarian stimulation: evidence from the first trial investigating the short-term effects of estradiol on biomarkers and B cells involved in autoimmunity

Anna Ghirardello¹ · Salvatore Gizzo^{2,5} · Marco Noventa² · Michela Quaranta⁴ · Amerigo Vitagliano² · Nicoletta Gallo³ · Giorgia Pantano³ · Marianna Beggio¹ · Chiara Cosma³ · Michele Gangemi² · Mario Plebani³ · Andrea Doria¹

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Abstract

Purpose The aim of the present study was to evaluate the in vivo immunomodulatory effects of an acute short-term estradiol (E₂) increase on serum levels of B cell-activating factor (BAFF), immunoglobulins (Ig), anti-nuclear antibodies (ANA), and the peripheral B cell phenotype.

Methods We conducted, at the Infertility Center of the University of Padua, a prospective case–control study on a cohort of infertile normo-responder women (group-A, 63 patients) undergoing controlled ovarian stimulation (COS) compared with an age-matched cohort of normo-ovulatory healthy women (group-B, 39 patients). Three serial blood sample assays were conducted in both groups, at T0, hypothalamic suppression; T1, ovulation induction; and T2, βhCG test in group A, and at T0, 2nd day; T1, 14th day; and T2, 21st day of cycle in group B, and serum levels

Capsule The acute short-term estradiol (E₂) increase during controlled ovarian stimulation do not seem to significantly affect serum levels of B cell-activating factor (BAFF), immunoglobulins (Ig), anti-nuclear antibodies (ANA), and the peripheral B cell phenotype.

✉ Salvatore Gizzo
ginecologia_padova@libero.it

¹ Division of Rheumatology, Department of Medicine, University of Padua, Padua, Italy

² Department of Woman and Child Health, Gynaecologic and Obstetric Unit, University of Padua, Padua, Italy

³ Department of Laboratory Medicine, University of Padua, Padua, Italy

⁴ Department of Obstetrics and Gynaecology, University of Verona, Verona, Italy

⁵ Dipartimento di Salute della Donna e del Bambino, U.O.C. di Ginecologia e Ostetricia, Via Giustiniani 3, 35128 Padova, Italy

of E₂ and BAFF, BAFF/E₂ ratio, circulating IgM, IgG, and IgA, ANA titer, and peripheral B cell phenotype were measured. We compared group-A versus group-B in terms of absolute and E₂ normalized values of BAFF at baseline (T0) to verify for possible differences between healthy and infertile women, at T1 to verify for possible differences occurring after spontaneous ovulation versus COS, and at T2 to evaluate differences in serum BAFF levels between pregnant versus non-pregnant patients (considering only group-A) and between non-pregnant women after spontaneous versus COS cycles (group-B versus group-A). In group-A, we also evaluated IgM, IgG, IgA levels, ANA titer, and peripheral B cell phenotype at T0 versus T1 versus T2.

Results With the exception of E₂ levels at T1 (as expected), no significant differences were found between the two groups for all outcome measures. In group-A, BAFF at T0 positively correlated with IgM levels; marginal zone CD19+/CD27+/IgD+ memory B cell compartment tended to be expanded at T1 when compared with T0.

Conclusions Despite several mechanistic and clinical studies supporting a stimulatory role of E₂ on autoimmunity, the acute increase of E₂ during COS for infertility treatment does not seem to have a major impact on the immune system.

Keywords Short-term estradiol increase · Autoimmunity biomarkers · BAFF · Memory B cells · Acute immunomodulation · BLyS · Controlled ovarian stimulation · IVF

Introduction

Steroid hormones play a crucial role in the correct functioning of the reproductive system; however, they also

greatly affect many non-reproductive tissues, including the immune system [1].

17 β -Estradiol (E_2) is a naturally occurring female steroid hormone that interacts with both the innate and adaptive immune responses [2]. In experimental mouse models, it was shown that the upregulation of E_2 influences B cell development, selection, and activation, thus leading to a loss of B cell tolerance, and the promotion of B cell autoreactivity, lymphoproliferation, hypergammaglobulinemia, and autoimmunity [3]. Evidence that estrogens are implicated in the pathogenesis of B cell-mediated autoimmune diseases has been thoroughly assessed as of today [4, 5].

The gender bias of autoimmune diseases occurring in humans has been universally accepted, being as they target chiefly women, predominantly in childbearing age [6]. Despite that the mechanisms by which estrogens may induce autoimmunity in humans are poorly defined, certain evidence supports a role of the physiologic shift from Th1 to Th2 immune response (typically occurring during pregnancy) in triggering B cell-mediated humoral autoimmunity [7–10].

B cell-activating factor (BAFF), alternatively called B lymphocyte stimulator (BLyS), is a member of the tumor necrosis factor (TNF) ligand superfamily. BAFF is primarily a myeloid-derived cytokine, either soluble or cell surface expressed, which physiologically promotes immature and mature B cell survival in the periphery [11, 12]. It is involved in autoantibody production as well as in T cell co-stimulation. The majority of the biological effects of BAFF can be attributed to its soluble form [13]. BAFF is upregulated mainly in diseases characterized by lymphoid neogenesis. In fact, increased levels of BAFF were documented in patients with B cell malignancies, chronic viral infections, and allergic diseases. Increasing evidence suggests that BAFF is essentially implicated in the pathogenesis of B cell-mediated autoimmune diseases [14]. Indeed, increased serum levels of BAFF were reported in both non-organ-specific autoimmune diseases (such as systemic lupus erythematosus and Sjögrens's syndrome) and organ-specific conditions [15, 16].

Intriguingly, by measuring BAFF in serum derived from patients affected by various systemic autoimmune diseases, we observed higher levels in the female as opposed to the male patients, while this sex-related dimorphism was not observed in healthy subjects. These findings support the notion that both E_2 and BAFF are implicated in B cell-mediated autoimmunity.

To our knowledge, no existing studies have reported data on the *in vivo* short-term immunomodulatory effects of E_2 on BAFF levels and other immunological related changes. The aim of the study was to evaluate if the short-term increase in E_2 levels (and subsequent establishment of pregnancy) may modulate serum levels of BAFF, immunoglobulins (Ig), anti-nuclear antibodies (ANA), and peripheral B cell phenotype in women without any prior history of clinical or biochemical features of autoimmune disease.

To create a human *in vivo* model, we assigned women affected with infertility and undergoing assisted reproduction treatment by controlled ovarian stimulation (COS) to the experimental cohort and normo-ovulatory healthy women monitored for the period of a single menstrual cycle to the control arm.

The final goal of our study was to evaluate whether significant differences exist between BAFF serum levels of healthy normo-ovulatory women as opposed to infertile women undergoing *in vitro* fertilization evaluated by repeat measurements in the following moments: baseline (second day of menstrual cycle), ovulation (spontaneous versus induced), and late luteal phase. We further discriminated women belonging to the infertile cohort in those who achieved pregnancy as opposed to those who did not.

Patients and methods

We conducted a prospective case control study on infertile women scheduled for fresh non-donor *in vitro* fertilization treatment at the Assisted Reproduction Unit of Gynecology and Obstetrics Clinic, Department of Women's and Children's Health, University of Padua, between January 2011 and March 2014. The study was performed in conjunction with the Rheumatology Unit and the Department of Laboratory Medicine, University of Padua, Italy, for clinical and biochemical evaluations, respectively.

The study was approved by the Local Ethics Committee (Comitato Etico per la Sperimentazione dell'Azienda Ospedaliera di Padova, Prot. Nr. 2313P). All women included in this study were properly counseled regarding the aim and were included only upon obtaining written informed consent for participation in the study and use of relevant data in accordance with Italian privacy laws.

All eligible patients were affected with unexplained infertility and defined normo-responders according to biochemical and sonographical features collected during ovarian reserve testing [17]; age ranged between 18 and 43 years (group-A: treatment group).

We excluded patients with the following: history of personal or familial autoimmune and/or other immunological disorders, diagnosis or suspicion of endometriosis based on clinical symptoms associated with ultrasound features and an increasing Ca125 serum value [18–20], abnormalities in karyotype, mutations of the cystic fibrosis gene, acquired or inherited thrombophilia, previous chemotherapy and/or radiotherapy for neoplasia, and cancellation of COS prior to oocyte retrieval due to poor ovarian response. In order to avoid bias, we also excluded women who, in the 6 months prior to COS, underwent any treatment which may potentially affect the immune system such as inositol, statins, and human papilloma-virus vaccine [21–23]. We also excluded patients who received low-dose aspirin during COS [24]. Initial evaluation

of all study patients included a detailed family and personal history specifically aimed to exclude any suspicion of autoimmune disorder. Moreover, we performed a general serum screening for markers of the most common autoimmune disorders. In detail, thyroid autoantibody screening (anti-tireoglobulin antibodies, anti-tiroperoxidase antibodies, anti-TSH receptor antibodies) [25], anti-nuclear antibodies (ANA), anti-cardiolipin antibodies, β 2-glycoprotein antibodies, lupus anti-coagulant (LAC), anti-neutrophil cytoplasmic antibodies (ANCA), extractable nuclear antigens (ENA), anti-DNA antibodies, and rheumatoid factor (RF). In addition to the women assigned to group-A, we recruited a cohort of normo-ovulatory age-matched healthy women (group-B: control group) who spontaneously adhered to the aim of the study. Their serum samples were used for time-matched comparison with sera of group-A women.

Clinical intervention

All patients recruited in group-A underwent pre-treatment basal ovarian reserve testing by biochemical assays of follicular-stimulating hormone (FSH) and anti-mullerian hormone (AMH) levels in association with sonographic antral follicular count (AFC). COS cycles were performed by a long protocol using gonadotropin-releasing hormone agonist (Decapeptyl, Ipsen, Paris, France), 0.1 mg daily initiated in the mild-luteal phase of the previous cycle, and recombinant follicle-stimulating hormone (rFSH; Gonal-F, Merck-Serono, Geneva, Switzerland) at a starting dose of 200 IU daily administered on confirmation of correct hypothalamic inhibition. The subsequent gonadotropin dose adjustments were performed by clinicians according to the biochemical and ultrasound features of ovarian response, starting from day 5 of stimulation. When at least three follicles with mean diameter larger than 16 mm (or at least one follicle bigger than 18 mm) were observed at transvaginal sonography, we administrated rhCG 250 μ g (Ovitrelle, Merck-Serono, Geneva, Switzerland) for ovulation induction. Oocyte retrieval took place 35 h after hCG administration. All oocytes were fertilized by ICSI technique. When obtained, one or two embryos were transferred 3 days after pickup. All patients received high-dose progesterone supplementation (600 mg vaginally and 100 mg intramuscular per day) for luteal phase support until β -hCG assay was performed 14 days after embryo transfer (ET) [26].

Pregnancy was confirmed by a positive serum β -hCG test (>5 IU/ml) performed 2 weeks after embryo transfer.

Timing

We considered T0 as baseline: For group-A patients, it coincided with the initiation day of COS and for group-B women the second day of a spontaneous menstrual cycle. T1 was the periovulatory period, which for group-A patients was

represented by the day of oocyte retrieval and for group-B women the 14th day of the menstrual cycle. T2 was the luteal period: for group-A patients, the day of β hCG assay and for group-B the 21st day of the menstrual cycle.

Laboratoristic intervention

We collected blood samples at T0, T1, and T2 for all patients in both groups. In detail, we quantified serum 17 β estradiol levels (E_2 , nmol/l) at T0 and T1 by electrochemiluminescent immunoassay (ECLIA, Roche Diagnostics, Rotkreuz, Switzerland) using a Cobas E601 analyzer (Roche Diagnostics); beta human chorionic gonadotropin (β hCG) levels (IU/l) at T2 were quantified by automatized chemiluminescent immunoassay (Architect System, Abbott Ireland Diagnostics Division, Sligo, Ireland).

Serum BAFF (ng/ml) was measured in 20-fold diluted serum samples by sandwich ELISA (Human BAFF Construction Kit, Antigenix America Inc., NY, USA). The assay was optimized for the recognition of both glycosylated and non-glycosylated soluble BAFF isoforms by using polyclonal goat anti-human BAFF as “capture” antibody (1 μ g/ml) and biotin-labeled polyclonal goat anti-human BAFF as “detection” antibody (0.25 μ g/ml), according to recommendations by Le Pottier et al. [27]. A standard curve was constructed using serial dilutions of recombinant human BAFF (Antigenix); the lowest limit of detection was 0.04 ng/ml, and range of measurability was 0.049–50 ng/ml.

Briefly, 96-well microplates (Nunc Maxisorp, Roskilde, Denmark) were coated overnight with goat affinity purified polyclonal anti-human BAFF (Antigenix America Inc., NY, USA) diluted in 0.05 M phosphate-buffered saline (PBS), pH 9.0, to concentration of 1.0 μ g/ml and then blocked with 1 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS). One hundred microliters of serially diluted standard recombinant human BAFF (Antigenix), starting from 50 ng/ml to zero in diluent (0.1 % BSA/0.05 % Tween-20 in PBS), was added to each ELISA well in duplicate for 2 h at room temperature (RT). After washing with PBS-0.05 % Tween-20 (PBS-T), 100 μ l/well biotin-labeled goat polyclonal anti-human BAFF (Antigenix) diluted to concentration of 0.25 μ g/ml in PBS-T was incubated at RT for 1–2 h. After extensive washing, streptavidin horseradish peroxidase conjugate (Antigenix) approximately 1:2000 in PBS-T was added and incubated for 30 min at RT. After extensive washing, TMB substrate solution was added and the reaction was terminated by the addition of 100 μ l 2 N sulfuric acid and measured at a wavelength of 450 nm in an ELISA microplate reader (Multiskan EX, Labsystems Italia, Milano, Italy).

Serum immunoglobulin (IgG, IgA, IgM) levels (g/l) were quantitated by automatized immunonephelometry (Dimension Vista System, Siemens Healthcare Diagnostics, Milano, Italy).

Serum anti-nuclear antibodies (ANA) were semiquantitatively detected by indirect immunofluorescence on HEp-2 cells (NOVA Lite Hep-2 ANA Kits, Inova Diagnostics, USA), and ANA titer was determined considering titer 80 as the cutoff value (work serum dilution 1:80).

Flow cytometry analysis of B cell surface markers was performed according to standard protocols. Fifty microliters of EDTA-anti-coagulated blood was stained for 15 min at RT with a combination of optimal aliquots of the following fluorochrome-conjugated monoclonal antibodies: anti-human CD45-V450 (Becton Dickinson GmbH, Milano, Italy), anti-human CD19-phycoerythrin Texas Red-X (ECD) (Beckman Coulter, Milano, Italy), anti-human CD268 (BAFF receptor, also known as BR3)-fluorescein isothiocyanate (FITC) (Bender Medsystems, eBioscience, Germany), anti-human IgD-phycoerythrin (PE) (Dako, Milano, Italy), anti-human CD27-allophycocyanin (APC) (Beckton Dickinson), and anti-human CD21-phycoerythrin-cyanin 7 (PE-Cy7) (Becton Dickinson). After incubation and lysis of erythrocytes, immunophenotyping was performed by flow cytometer Navios (Beckman Coulter) and data analyzed with Kaluza software (Beckman Coulter).

Endpoints

Primary endpoint was to compare group-A versus group-B in terms of absolute and normalized for E_2 values of BAFF at baseline (T0) in order to evaluate whether differences may exist between healthy and infertile women.

Second endpoint was to compare group-A versus group-B in terms of absolute and normalized for E_2 values of BAFF at T1, in order to evaluate if differences may exist between spontaneous ovulation versus COS.

Third endpoint was to evaluate whether differences exist in BAFF levels between pregnant versus non-pregnant patients in group-A and between non-pregnant women conceiving after spontaneous versus COS cycles (group-B versus group-A) at T2.

Finally, in the group-A women, we evaluated for variations in immunoglobulin serum levels (considering IgG, IgA, IgM) and ANA titer, at T0 versus T1 versus T2, and for peripheral blood B cell subpopulation status (considering the proportion of transitional, mature naïve, and memory $CD19^+BR3^+$ B cells) at T0 versus T1.

Statistical analysis

Parametric and non-parametric statistics were applied, when appropriate. Since BAFF and ANA titer did not follow a normal distribution, non-parametric statistical analysis was used using Mann–Whitney *U* test, Spearman's rank test, and Wilcoxon's test for paired data. Normally distributed parameters were analyzed using Fisher's exact test and paired or

unpaired Student's *t* tests. All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) for Windows. A *p* value <0.05 was considered statistically significant.

Results

Sixty-three patients eligible for inclusion in study group-A and 39 healthy women for study group-B were recruited. Mean age ($\pm$ standard deviation, SD) of group-A versus group-B was 35 ± 4.3 versus 33 ± 8.5 years (p =not significant (n.s.)).

T0, baseline

At T0, the comparison between women in group-A versus group-B showed no significant differences in terms of absolute value of E_2 (mean $\pm$ SD, 0.06 ± 0.04 vs 0.08 ± 0.06 nmol/l; p =n.s.) as well as absolute value of BAFF (median, 95° percentile, 0.85, 5.85 vs 0.53, 4.34 ng/ml; p =n.s.) (Fig. 1)

Considering the BAFF/ E_2 ratio, no significant difference was observed between group-A and group-B: (median, 95° percentile, 19.0, 104.33 vs 16.3, 29.60; p =n.s.).

T1 evaluation

At T1, the comparison between group-A versus group-B women showed a significant difference in terms of absolute value of E_2 (mean $\pm$ SD 4.63 ± 3.19 vs 0.94 ± 0.11 nmol/l; $p < 0.0001$), but no difference in terms of absolute value of BAFF (median, 95° percentile, 0.74, 10.60 vs 0.44, 4.15 ng/ml; p =n.s.) (Fig. 1).

Considering the BAFF/ E_2 ratio no difference was observed between group-A and group-B: (median, 95° percentile, 0.17, 1.84 vs 0.48, 4.15; p =n.s.).

T2 evaluation

Of the 63 women comprising group-A, 16 (25.4 %) had hematic β hCG levels ≥ 100 (mIU/ml), and 15/16 (93 %) were confirmed pregnant.

The comparison between the non-pregnant women of group-A versus those of group-B showed no differences in terms of absolute values of BAFF levels (median, 95° percentile, 0.68, 4.52 vs 0.81, 4.34 ng/ml; p =n.s.). The comparison between the non-pregnant versus the pregnant women of group-A showed no differences in terms of absolute values of BAFF levels (median, 95° percentile, 0.68, 4.52 vs 1.24, 3.20 ng/ml; p =n.s.)

Considering only group-A patients, IgG, IgA, and IgM immunoglobulin levels and ANA titers at T0 versus T1 versus T2 are summarized in Table 1. Notably, a correlation was

Fig. 1 Comparison of soluble E₂ and BAFF levels in group-A patients versus group-B controls at **a** baseline and **b** T1 evaluations

observed between BAFF levels at T0 and IgM at T0 ($\rho=0.401$; $p=0.009$), T1 ($\rho=0.496$; $p=0.002$) and at T2 ($\rho=0.406$; $p=0.061$) (Fig. 2)

Additionally, in Table 2, we reported peripheral B cell subpopulation status in this cohort of patients. The frequency of CD19⁺ and CD19⁺/BR3⁺ B cells was similar to those observed in healthy adult controls (data not shown) and did not significantly vary over the observation period ($p=n.s.$). Of note, a tendency was observed toward an expansion of the mature marginal zone CD19⁺CD27⁺IgD⁺ of memory B cells at T1 in comparison with T0 ($p=0.083$), whereas the

proportion of immature “transitional” type 2 B cells CD19⁺CD27[−]IgD^{hi}CD21^{hi} was lower at T1 than at T0 ($p=0.074$). No significant difference was observed in the proportion of transitional type 1 B cells CD19⁺CD27[−]CD21^{lo}IgD^{lo} at T1 versus T0, although a tendency toward an expansion at T1 was observed (data are summarized in Table 2). Notably, the amount of transitional type 1 B cells at T0 resembled that reported in healthy peripheral blood (approximately 2 %) [28].

Discussion

Various epidemiological and immunological studies suggest that reproductive and sex hormones play a fundamental role in the etiology and progression of various autoimmune diseases [29–31].

Despite strongly held opinions, there is presently a lack of scientific evidence able to support most empirical statements regarding autoimmunity, autoimmune diseases, and assisted reproductive technologies (ART), at least in the human model. It is not likely that autoimmunity causes infertility, nor is it implicit that patients with autoimmune diseases must suffer from infertility [32]. In patients suffering from autoimmune disorders with coexistent infertility issues, ART represents a valid option for women attempting to achieve pregnancy.

Table 1 Circulating IgG, IgA, and IgM levels and ANA positivity and titer in group-A patients at T0, T1, and T2 evaluations

	Observation periods			p
	T0	T1	T2	
IgG (g/l) mean±SD	11.9±2.56	11.4±2.15	11.9±2.74	n.s.
IgA (g/l) mean±SD	2.0±0.90	1.9±0.82	1.9±0.74	n.s.
IgM (g/l) mean±SD	1.5±0.64	1.4±0.53	1.6±0.77	n.s.
ANA positivity number (%)	42 (67 %)	38 (61 %)	41 (66 %)	n.s.
ANA titer median (range)	80 (0–320)	80 (0–320)	80 (0–320)	n.s.

ANA anti-nuclear antibodies, SD standard deviation, T0 baseline, T1 pre-ovulatory period, T2 luteal period

Fig. 2 Correlations between soluble BAFF and IgM levels in group-A patients at **a** baseline, **b** T1, and **c** T2 evaluations

Unresolved issues pertaining to the application of ART in patients affected with autoimmune disorders have arisen specifically in regards to the safety profile of rapidly increasing levels of E2 observed in a COS cycle [33].

The observed increase in autoimmune activity following ART treatment has been associated with different potential mechanisms chiefly correlated with an increase in circulating estrogen levels and their effects on the immune system [34].

It is widely known that estrogens are immune system modulators, which influence cytokine production and the induction of autoimmune disease processes, primarily systemic lupus erythematosus or lupus-like diseases [3, 35, 36]. The plasma concentration of BAFF is upregulated in many autoimmune diseases and is essentially implicated in B cell abnormalities typically associated with autoimmune clinical manifestations [4]. In autoimmune experimental animal models, it

Table 2 Circulating B cell subpopulations in group-A patients at basal (T0) and periovulatory (T1) evaluations

	Observation periods		
	T0	T1	<i>p</i>
Lymphocytes (106/ml, mean±SEM)	2.15±0.1	2.12±0.1	n.s.
CD19+ B cells (% , mean±SEM)	10.36±0.8	10.31±0.8	n.s.
B lymphocytes (106/ml, mean±SEM)	0.23±0.3	0.22±0.3	n.s.
CD19+BR3+ B cells (% , mean±SEM)	97.08±0.5	98.07±0.4	n.s.
MFI BR3+ B cells (mean±SEM)	3.76±0.1	3.73±0.1	n.s.
Marginal zone memory B cells	14.35±3.0	26.39±8.1	0.083
CD19+CD27+IgD+ (% , mean±SEM)			
Switched memory B cells	15.15±1.3	13.96±1.3	n.s.
CD19+CD27+IgD- (% , mean±SEM)			
Naïve B cells	65.79±3.9	65.53±5.7	n.s.
CD19+CD27-IgD+ (% , mean±SEM)			
Transitional B cells (% , mean±SEM)			
–Type 1 (CD19+CD27-IgDloCD21lo)	2.16±1.0	3.04±1.0	n.s.
–Type 2 (CD19+CD27-IgDhiCD21hi)	91.04±2.2	77.70±7.9	0.074

SEM standard error of the mean, MFI mean fluorescence intensity

has been well established that both E_2 and BAFF potently exacerbate B cell autoimmunity by promoting the expansion and activation of autoreactive marginal zone immunocompetent B cells which are consequently induced to secrete antibodies and undergo class-switch [37].

Our aim was to evaluate the short-term effects of an E_2 increase on BAFF levels and the immunological effects linked to its activities.

Our data suggested that COS in infertile women in the absence of immunological disorders does not exert significant immunomodulatory short-term effects on circulating BAFF and B cell biomarkers and phenotype in comparison with healthy normo-ovulatory women. In accordance with what was observed in estradiol-treated mice and in autoimmune experimental models [37], our findings suggest that a short-term in vivo E_2 increase might lead to a physiological expansion of marginal zone mature B cells, as observed in group-A patients at T1, which however does not influence biomarkers of B cell dysfunction, including BAFF, immunoglobulins, or ANA titer. Conversely, our results confirmed that BAFF is constitutively involved in the activation of antigen-stimulated B cells, as demonstrated by the correlation between BAFF levels and IgM. Indeed, IgM is the first antibody produced by B cells after antigen stimulation [38]. Yet, such BAFF-induced immunomodulation seems to be independent from the short-term surge in E_2 levels.

To our knowledge, our study was the first performed with the aim of clarifying the immediate effect of ART on one of the most important pathways involved in the development of immunological disorders. We are conscious that our data will require further confirmation in a large population, but we believe that it represents a useful starting point from which to develop further studies involving women affected with immunological disorders and infertility in order to evaluate the effects of ART on the immunological parameters in this cohort of patients. Certainly, it will be interesting to evaluate in the near future, in a cohort of women with proved autoimmunity, possible modifications involving the same pathways comparing infertile patients undergoing COS to fertile ones. One of the goals will be to comprehend whether the immunological profile of these patients may explain the different outcomes in terms of both spontaneous pregnancy and in vitro fertilization success rates.

We stress the fact that all infertile patients undergoing COS were defined as normo-responder according to Bologna criteria, and therefore certainly comparable to fertile women in terms of ovarian reserve and likely in terms of COS response. Certainly, the discrimination between type of infertility (such as male factors, anovulation, tubal factor, and endometriosis) and different ovarian reserve status (estimated high, normo, and poor response) would likely represent relevant data that certainly warrants in-depth analysis by future well-defined prospective studies. Since our data showed that, in

patients without immunological disorders, E_2 has no short-term effects on BAFF, the analysis and comparison of data in patients affected by immunological disorders undergoing ART may contribute to better comprehend the mechanisms involved in the short-term effects of E_2 on BAFF and may better clarify whether or not ART may represent a safe option in this subgroup of patients.

Considering that women affected by autoimmune diseases and candidate to receive potentially gonadotoxic treatments are at increased risk for primary ovarian insufficiency [39], it is mandatory that the scientific community finally prove the safety profile of ART treatments for fertility preservation in women with immune system disorders in order to adequately counsel the patient about her potential reproductive outcome and implement the most appropriate strategy for fertility preservation.

Our data strongly supports the call for further studies on this topic since additional clarity and definitive evidences are urgently required.

We report the following as strengths of our study: originality in aim, study design, precise inclusion criteria, exclusion of autoimmune disease in both cohorts, the administration of the same treatment protocol for COS, and the same luteal phase support after embryo transfer for all group-A women.

Unfortunately, some limitations affect our study such as small sample size, the fact that all infertile patients were affected by unexplained infertility, the relatively low pregnancy rate in 35-year-old women (probably related to the small sample size), the absence of evidences regarding the effects of different COS protocols, and the lack of data regarding pregnancy after spontaneous cycle. All these above stated limitations suggest caution in the interpretation of data.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

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