Ovarian reserve in adult patients with childhood-onset lupus: a possible deleterious effect of methotrexate?

DB de Araujo[1,2], LYS Yamakami[3], NE Aikawa[1,4], E Bonfá[1], VST Viana[1], SG Pasoto[1], RMR Pereira[1], PC Serafin[3], EF Borba[1], CA Silva[1,4]

[1]Division of Rheumatology, Faculty of Medicine, University of São Paulo, [2]Department of Rheumatology, São Paulo State Public Servants Hospital, [3]Department of Gynaecology, Faculty of Medicine, University of São Paulo, and [4]Paediatric Rheumatology Unit, Faculty of Medicine, University of São Paulo, Brazil

Objectives: To assess ovarian reserve markers and anti-corpus luteum antibodies (anti-CoL) in adult patients with childhood-onset systemic lupus erythematosus (c-SLE).

Method: Fifty-seven adult c-SLE female patients and 21 healthy controls were evaluated for anti-CoL. Ovarian reserve was assessed by: follicle stimulating hormone (FSH), luteinizing hormone (LH), oestradiol, anti-Müllerian hormone (AMH), and antral follicle count (AFC). Demographic data, menstrual abnormalities, disease activity, damage, and treatment were also analysed.

Results: The median current age was similar in adult c-SLE patients and controls (27.7 vs. 27.7 years, $p = 0.414$). The medians of AMH (1.1 vs. 1.5 ng/mL, $p = 0.037$) and AFC (6 vs. 16, $p < 0.001$) were significantly reduced in SLE patients compared to controls without significant menstrual abnormalities. Anti-CoL were solely observed in c-SLE patients (16% vs. 0%, $p = 0.103$) and were not associated with demographic data, ovarian reserve parameters, disease activity/damage, and treatment. Further evaluation of c-SLE patients treated with cyclophosphamide revealed a higher median of FSH levels compared to c-SLE patients not treated with cyclophosphamide and controls (8.8 vs. 5.7 vs. 5.6 IU/L, $p = 0.032$) and lower median AMH (0.4 vs. 1.5 vs. 1.5 ng/mL, $p = 0.004$) and AFC (4.0 vs. 6.5 vs. 16 IU/L, $p = 0.001$) levels. Nineteen patients treated exclusively with methotrexate demonstrated a negative correlation between the cumulative dose and AMH levels ($p = 0.027$, $r = -0.507$).

Conclusions: The present study demonstrated for the first time that a high cumulative methotrexate dose is a possible cause of subclinical ovarian dysfunction in adult c-SLE patients. Further studies are required to confirm this deleterious effect in other rheumatic diseases, particularly juvenile idiopathic arthritis and idiopathic inflammatory myopathy.

Female systemic lupus erythematosus (SLE) patients are living longer, including those with childhood onset (1), and transfer from paediatric to adult health care has raised concerns about their ovarian reserve and future fertility (2–6).

Ovarian reserve depends largely on the quantity and quality of primordial follicles, which are successively lost in later life. Females have about 1 to 2 million primordial follicles at birth, decreasing to 400 000 follicles at the beginning of puberty. During a menstrual cycle, hundreds of primordial follicles are recruited, but often only one follicle reaches full maturity and ovulation, and all other follicles undergo atresia (7). The assessment of ovarian reserve is performed by an indirect evaluation of tests that estimate follicle population (8).

Clovis Artur Silva, Division of Rheumatology, Faculty of Medicine, University of São Paulo, Avenida Dr Arnaldo 455, São Paulo, SP 01246-903, Brazil.
E-mail: clovis.silva@hc.fm.usp.br

Accepted 21 March 2014

Risk factors associated with the reduction of follicle population in SLE patients are hypothalamic–pituitary–gonadal (HPG) axis dysfunction (6, 8), anti-corpus luteum antibodies (anti-CoL) (9, 10), disease activity (7, 8, 11), and immunosuppressive drugs (12–16).

Reduction of ovarian reserve has been demonstrated previously in childhood-onset SLE (c-SLE) (17) and adult SLE populations (12–16, 18, 19), but most of these studies were limited to menstrual alterations and follicle stimulating hormone (FSH) levels (3, 5, 6), and a few recent reports included antral follicle count (AFC) and/or anti-Müllerian hormone (AMH) levels (5, 14–16). The contribution of the diminished follicle ovarian pool using anti-CoL (20) is, however, not available in the paediatric lupus population. In addition, there are no data regarding the impact of isolated methotrexate exposure and anti-CoL in the ovarian reserve of adult c-SLE patients.

We have therefore performed a complete assessment of ovarian reserve and anti-CoL in adult c-SLE patients and healthy controls.

DOI: 10.3109/03009742.2014.908237

Method

Patients and controls

A cross-section study was performed from November 2009 to June 2012 and 113 consecutive female adult c-SLE patients (age $\leq$ 18 years) (1) were followed at the Rheumatology Division of our University Hospital. All patients fulfilled the American College of Rheumatology (ACR) SLE classification criteria (20). Exclusion criteria were: current pregnancy, HPG axis dysfunction, end-stage renal disease, contraindication or unwillingness to stop hormonal contraceptives for at least 12 consecutive months or until resumption of menses for at least three consecutive menstrual cycles, gynaecological surgery, current non-steroidal anti-inflammatory drugs, use of gonadotrophin-releasing hormone agonist (GnRH-a), and not agreeing to participate in this study. Fifty-six patients were excluded because of: current pregnancy (n = 5), lymphocytic hypophysitis (n = 1), end-stage renal disease (n = 1), contraindication or unwillingness to stop hormonal contraceptives (n = 38), previous hysterectomy (n = 3), and not agreeing to participate in this study (n = 8).

The control group included 21 healthy females. The Brazilian socio-economic classes were classified according to the the Brazilian Association of Market Research Institutes (21). The local ethics committee approved the study and informed consent was obtained from all participants (protocol 0878/09).

Study procedures

Patients' medical records were reviewed for clinical, immunological, and therapeutic findings. Blood samples were collected at the early follicular phase (between the second and fourth day of menses), except from those presenting with amenorrhoea, in which a sample was taken randomly.

Ovarian function parameters

Gynaecological evaluation. Age at menarche of patients and controls was registered based on recollection. Menstrual flow duration and cycle length were evaluated prospectively for at least 3 consecutive months. A normal cycle was defined as flow duration varying from 3 to 7 days and length from 25 to 35 days (2, 22). Menstrual disturbances were based on alterations in one or more of these parameters during evaluation. The mean cycle length and flow duration were also calculated. Amenorrhoea and sustained amenorrhoea were defined as absence of a menstrual period for at least 4 months after menarche and persisting for more than 12 months, respectively. Patients with sustained amenorrhea in whom menstruation did not resume and who had FSH levels > 40 IU/L were defined as having premature ovarian failure (POF) (2–6, 23). Secondary sexual characteristics were classified according to Tanner pubertal changes (24).

Determination of ovarian reserve. Complete ovarian function was assessed by evaluation of serum hormone levels in the early follicular phase of the menstrual cycle or randomly for those with defined amenorrhoea, blinded to the other parameters of ovarian function. FSH (reference levels: 3.5–12.5 IU/L), luteinizing hormone (LH) (reference levels: 2.4–12.6 U/L), and oestradiol (reference levels: $\leq$ 166 pg/mL) were measured by radioimmunoassay using a commercial kit (Cobas®, Roche, Mannheim, Germany). Intra- and inter-assay coefficients of variation (CVs) were recommended by the manufacturer (limited to 5.7% and 3.6%, respectively).

AMH was measured by enzyme-linked immunosorbent assay (ELISA; AMH Gen II ELISA, Beckman Coulter Inc, Brea, CA, USA) in 57 c-SLE patient and 21 controls in duplicated samples. Intra- and inter-assay CVs were limited to 5.4% and 5.6%, respectively. AMH was also evaluated by ultrasensitive AMH/Müllerian inhibiting substance enzyme-linked chemiluminescent immunoassay (US AMH/MIS AnshLabs ELISA) in duplicated samples in 53 c-SLE patient and 18 controls. Intra- and inter-assay CVs were limited to 3.1% and 2.7%, respectively. A positive correlation was observed between the AMH Gen II ELISA and US AMH/MIS AnshLabs ELISA in c-SLE patients ($r = +0.91$, $p < 0.0001$) and in healthy controls ($r = +0.94$, $p < 0.0001$). AMH values < 1.0 ng/mL were defined as reduced (25).

Detection of anti-CoL. Reactivity of serum to the 67-kDa protein from the corpus luteum was evaluated by immunoblotting in duplicated samples in patients and controls, as described previously (9). In brief, crude tissue and cell extracts obtained from bovine corpus luteum (100 μg/well) were submitted to polyacrylamide gel electrophoresis under denaturating and reducing conditions [β2-mercaptoethanol sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)]. Proteins were then electrophoretically transferred to nitrocellulose membrane. Membrane strips were further incubated with blocking buffer [5% skimmed milk in phosphate-buffered saline (PBS)] and immunoprobed by incubation with serum samples diluted 1:10. Reactivity was tagged with anti-human immunoglobulin (Ig)G alkaline phosphatase conjugate and visualized using appropriate chromogenic substrates.

Transvaginal ultrasound. Ultrasound was performed in all sexually active SLE patients and controls by the same expert sonographer (LYSY) using a 6.5-MHz endovaginal transducer (HD3, Philips Ultrasound, Bothell, WA, USA) blinded to parameters of gonadal function at study entry. Ovaries were scanned in axial

and longitudinal planes, and at least two measurements of length (L), width (W), and thickness (T) were obtained and used to calculate the ovarian volume, using an ellipsoid calculation ($L \times W \times T \times \pi/6$), and the mean of the ovarian volumes was calculated (26). Follicles of 2 to 10 mm were considered to in the AFC (27). The number of AFCs was divided in a clinically suitable classification as: ≤ 10 but > 5 follicles (low ovarian reserve) and ≤ 5 follicles (very low ovarian reserve) (28).

Clinical, laboratory, and treatment assessments

SLE clinical manifestations were defined as: cutaneous lesions (malar or discoid rash, oral ulcers, or photosensitivity), articular involvement (non-erosive arthritis), neuropsychiatric disease (seizure or psychosis), renal involvement (proteinuria ≥ 0.5 g/24 h, presence of cellular casts, and/or persistent haematuria ≥ 10 red blood cells per high power field), serositis (pleuritis or pericarditis), and haematological abnormalities (haemolytic anaemia, leucopenia with a white blood cell count $< 4000/mm^3$, lymphopenia $< 1500/mm^3$ on two or more occasions and thrombocytopenia with platelet count $< 100\,000/mm^3$ in the absence of drugs or infection).

Disease activity was defined according to the SLE Disease Activity Index 2000 (SLEDAI-2K) (29) and cumulative damage using the SLE International Collaborating Clinics/ACR Damage Index (SLICC/ACR-DI) (30). Body mass index (BMI) was defined by weight in kilograms/height in metres^2 (kg/m^2).

Anti-double-stranded DNA (anti-dsDNA) was detected by indirect immunofluorescence using *Crithidia luciliae* as substrate. A haemagglutination assay with rabbit thymus extract was used to determine the presence of antibodies to RNP and Sm proteins. Detection of autoantibodies to saline-soluble antigens, Ro/SS-A and La/SS-B, was performed by counterimmunoelectrophoresis against dog spleen saline extract (31, 32). Non-specific reference sera were included in each assay to define autoantibody specificity. The presence of anticardiolipin antibodies (aCL) IgG and IgM was analysed by ELISA (33). Lupus anticoagulant (LAC) was detected according to the guidelines of the International Society on Thrombosis and Haemostasis (34). Anti-ribosomal P protein antibodies were detected by Western blotting using the purified ribosomal fraction isolated from rat hepatocytes as substrate (35).

Data concerning the cumulative and current dosage of prednisone, methotrexate, azathioprine, intravenous cyclophosphamide (IVCYC), cyclosporin, and mycophenolate mofetil were determined.

Statistical analysis

The frequency of diminished ovarian reserve according to the presence of anti-CoL was calculated in both groups and compared by Fisher's exact test, considering $\alpha = 0.05$. The size sample provided power of 80% to find differences of at least 27% (Graphpad StatMate 1.01). The results are presented as the mean $\pm$ standard deviation (SD) or median (range) for continuous variables and number (%) for categorical variables. Data were compared by the Mann–Whitney test in the continuous variables to evaluate differences among the SLE and control groups, and among the SLE subgroups. For categorical variables, differences were assessed by Fisher's exact test. The Kruskal–Wallis one-way analysis of variance was used to compare SLE patients with IVCYC, without IVCYC, and healthy controls, followed by a post-hoc analysis by Dunn's multiple comparison test to determine where the difference occurred between the groups. Spearman's rank correlation coefficient was used for correlations between the cumulative dose of immunosuppressive drugs and ovarian reserve parameters. The level of significance of the independent variables was set at 5% ($p < 0.05$).

Results

Patients with adult c-SLE vs. controls

Table 1 includes demographic features, menstrual cycles, ovarian reserve tests, and anti-CoL in 57 c-SLE patients and 21 healthy controls. Medians (range) of current age [27.7 (18.3–39.8) vs. 27.7 (18.1–40.0) years, $p = 0.41$] and BMI, and the frequencies of Caucasian race and socio-economic class, were similar in c-SLE patients and controls ($p > 0.05$) (Table 1). The median of disease duration in c-SLE patients was 13.3 (2.1–25.4) years.

All adult c-SLE patients and healthy controls were Tanner pattern 5 according to pubertal changes. The median of age at menarche was similar in both groups [13 (10–17) vs. 13 (11–15) years, $p = 0.21$], although the median age at menarche after disease onset was significantly higher in c-SLE compared to controls [14 (11–17) vs. 13 (11–15) years, $p = 0.03$]. No differences were detected regarding menstrual disturbances between c-SLE patients and controls (Table 1).

The median of AMH was significantly decreased in 57 c-SLE vs. 21 controls according to AMH Gen II ELISA kits [1.1 (0–6.3) vs. 1.5 (0.1–5) ng/mL, $p = 0.04$]. The lower median of AMH [1.2 (0–9.0) vs. 1.3 (0–6.6) ng/mL, $p = 0.37$] using the US AMH/MIS AnshLabs ELISA kit in 53 c-SLE patients compared to 18 controls did not reach statistical significance ($p = 0.37$). The median of AFC [6 (0–27) vs. 16 (5–36), $p < 0.001$] was significantly reduced in c-SLE patients vs. controls (Table 1).

The numbers of patients with low ovarian reserve [31 (69%) vs. 4 (21%) patients with ≤ 10 follicles, $p < 0.001$] and very low ovarian reserve [9 (16%) vs. 0 (0%) patients with ≤ 5 follicles, $p < 0.001$], using the number of AFCs as a parameter, were also significantly reduced in SLE patients vs. controls. FSH, LH, and oestradiol levels were similar in adult c-SLE patients and controls ($p > 0.05$). Anti-CoL were observed solely in c-SLE patients (16% vs. 0%, $p = 0.10$) (Table 1).

Table 1. Demographic features, menstrual cycles, ovarian reserve, and anti-corpus luteum antibody (anti-CoL) in systemic lupus erythematosus (SLE) patients with onset before adulthood and healthy controls.

Variables	SLE (n = 57)	Controls (n = 21)	p
Demographic features			
Current age (years)	27.7 (18.3–39.8)	27.7 (18.1–40)	0.41
BMI (kg/m^2)	23.8 (17.2–48.8)	25.3 (19.3–35.8)	0.56
Caucasian	21 (37)	12 (57)	0.13
Socio-economic class C or D	44 (77)	15 (71)	0.77
Menstrual cycles			
Age at menarche (years)	13 (10–17)	13 (11–15)	0.21
Age at menarche after disease onset *	14 (11–17)	13 (11–15)	0.03
Time between menarche and current age (years)	14 (4.2–25.7)	16.5 (5–28)	0.53
Any menstrual disturbances	13 (37)	6 (29)	0.77
Amenorrhoea	2 (4)	0 (0)	1.0
Flow duration (days)	5 (0–15)	5 (2–10)	0.94
< 3 days	5 (9)	1 (5)	1.0
> 7 days	3 (5)	3 (14)	0.34
Cycle length (days)	30 (0–75)	30 (15–40)	0.58
< 24 days	0 (0)	1 (5)	0.27
> 35 days	3 (5)	1 (5)	1.0
POF	1 (2)	0 (0)	1.0
Ovarian reserve			
FSH (IU/L)	6.4 (0.6–80.6)	5.6 (2.2–14.4)	0.34
Elevated levels	15 (27)	2 (10)	0.13
LH (IU/L)	6.2 (0.1–47.7)	4.4 (2.1–10.3)	0.41
Elevated levels	24 (43)	5 (24)	0.19
Oestradiol (pg/mL)	45 (15–1271)	34 (24–128)	0.14
Decreased levels	4 (7)	0 (0)	0.57
AMH Gen II (ng/mL)	1.1 (0–6.3)	1.5 (0.1–5)	0.04
Decreased levels	28 (49)	5 (24)	0.07
AMH AnshLabs (ng/mL) †	1.2 (0–9.0)	1.3 (0–6.6)	0.37
Decreased levels †	24 (45)	5 (28)	0.27
Ovarian volume (mm^3) ‡	9.6 (4.3–188.9)	10.4 (4.7–34.5)	0.64
AFC §	6 (0–27)	16 (5–36)	< 0.001
AFC ≤ 10 follicles §	31 (69)	4 (21)	< 0.001
AFC ≤ 5 follicles §	18 (40)	0 (0)	< 0.001
Anti-CoL	9 (16)	0 (0)	0.10

BMI, Body mass index; POF, premature ovarian failure; FSH, follicle stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count; anti-CoL, anti-corpus luteum antibody.
* n = 14 SLE patients, † n = 53 SLE patients and 18 controls, ‡ n = 47 SLE patients and 19 controls, § n = 45 SLE patients and 19 controls. Values expressed as n (%) or median (range).

Ovarian reserve, disease activity, and immunosuppressive agents in c-SLE patients

The medians of current age [27.3 (18.3–39.8) vs. 29.4 (18.9–39.3) vs. 27.7 (18.1–40.0) years, p = 0.75] and BMI were similar in c-SLE patients treated with IVCYC, c-SLE patients not treated with IVCYC, and controls. In addition, no differences were observed in the age at menarche [13 (11–16) vs. 13 (10–17) vs. 13 (11–15) years, p = 0.23] and the time between menarche and current age [13.3 (7.3–25.7) vs. 16.8 (4.2–25.3) vs. 16.5 (5–28) years, p = 0.73] (Table 2).

The median of the last IVCYC dose of 21 SLE patients was 45 months (0–119.9) and only two patients used IVCYC before menarche. Regarding ovarian reserve, the median of FSH levels was significantly higher in c-SLE patients treated with IVCYC compared to c-SLE patients not treated with IVCYC and controls [8.8 (0.6–80.6) vs. 5.7 (2.3–15.8) vs. 5.6 (2.2–14.4) IU/L, p = 0.03]. The median of oestradiol levels was significantly reduced [35 (15–1271) vs. 47.5 (19–127) vs. 34 (24–128) pg/mL, p = 0.03] between c-SLE patients with IVCYC and controls when compared to c-SLE patients without IVCYC. The median of AMH (Gen II ELISA) [0.4 (0–3.5) vs. 1.425 (0–6.3) vs. 1.5 (0.1–5) ng/mL, p = 0.004] and AMH (US AMH/MIS AnshLabs ELISA) [0.45 (0–5.2) vs. 1.8 (0–8.7) vs. 1.35 (0–6.6) ng/mL, p = 0.017] levels were significantly lower in c-SLE patients treated with IVCYC compared to those not treated with IVCYC and healthy controls. The median of AFC was significantly lower in c-SLE patients with and without IVCYC compared to healthy controls [4 (0–27) vs. 6.5 (1–23) vs. 16 (5–36), p = 0.001] (Table 2).

With regard to other immunosuppressive agents, 19 patients were treated with methotrexate and had never used IVCYC. A negative correlation was observed in this group between cumulative methotrexate dose and AMH levels [Gen II ELISA, p = 0.03, r = –0.507 (Figure 1); US AMH/MIS AnshLabs ELISA, p = 0.03,

Table 2. Demographic features, menstrual cycles, and ovarian reserve in systemic lupus erythematosus (SLE) patients with onset before adulthood according to intravenous cyclophosphamide (IVCYC) use.

Variables	SLE with IVCYC (n = 21)	SLE without IVCYC (n = 36)	Controls (n = 21)	p *
Demographic features				
Current age (years)	27.3 (18.3–39.8)	29.4 (18.9–39.3)	27.7 (18.1–40.0)	0.75
BMI (kg/m^2)	23.9 (19.4–48.8)	23.9 (17.2–37.9)	25.3 (19.3–35.8)	0.73
Menstrual cycles				
Age at menarche (years)	13 (11–16)	13 (10–17)	13 (11–15)	0.23
Time between menarche and current age (years)	13.3 (7.3–25.7)	16.8 (4.2–25.3)	16.5 (5–28)	0.73
Ovarian reserve				
FSH (IU/L)	8.8 (0.6–80.6)	5.7 (2.3–15.8)	5.6 (2.2–14.4)	0.03
LH (IU/L)	7.6 (0.1–47.7)	5.9 (2.8–14.5)	4.4 (2.1–10.3)	0.13
Oestradiol (pg/mL)	35 (15–1271)	47.5 (19–127)	34 (24–128)	0.03
AMH Gen II (ng/mL)	0.4 (0–3.5)	1.42 (0–6.3)	1.5 (0.1–5)	0.004
AMH AnshLabs (ng/mL) †	0.45 (0–5.2)	1.8 (0–8.7)	1.35 (0–6.6)	0.017
Ovarian volume (mm^3) ‡	9.0 (4.3–188.9)	9.7 (5.2–42.6)	10.4 (4.7–34.5)	0.67
AFC §	4 (0–27)	6.5 (1–23)	16 (5–36)	0.001

BMI, Body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count.
* p calculated by the Kruskal–Wallis one-way analysis of variance.
† n = 53 SLE patients and 18 controls, ‡ n = 16 SLE patients with IVCYC, 31 without IVCYC, and 19 controls, § n = 17 SLE patients with IVCYC, 28 SLE without IVCYC, and 19 controls.
Values expressed as median (range).

r = –0.504], with no difference in AFC (p = 0.10). This negative correlation between cumulative methotrexate dose and AMH levels by Gen II ELISA (p = 0.012, r = –0.504) and US AMH/MIS AnshLabs ELISA (p = 0.044, r = –0.479) remained even with the exclusion of the outlier patient treated with 15 g of methotrexate. No correlation was observed between methotrexate and AFC (p = 0.11). In addition, no correlations were observed between cumulative doses of prednisone, IVCYC, mycophenolate mofetil, and azathioprine and AMH (using both assays) and AFC levels (p > 0.05).

The median of AMH (Gen II ELISA) was similar in patients who received mycophenolate mofetil compared those who did not receive this medication [0.6 (0–3.4) vs. 1.3 (0–6.3) ng/mL, p = 0.14], as well as in SLE patients treated with azathioprine [0.8 (0–6.3) vs. 1.7 (0–3.4) ng/mL, p = 0.64] and methotrexate [1.4 (0–5.9) vs. 0.65 (0–6.3) ng/mL, p = 0.53]. Similar results were observed with the other AMH kit (US AMH/MIS AnshLabs ELISA) (p > 0.05).

No differences were observed in ovarian reserve parameters with regard to disease activity (Table 3).

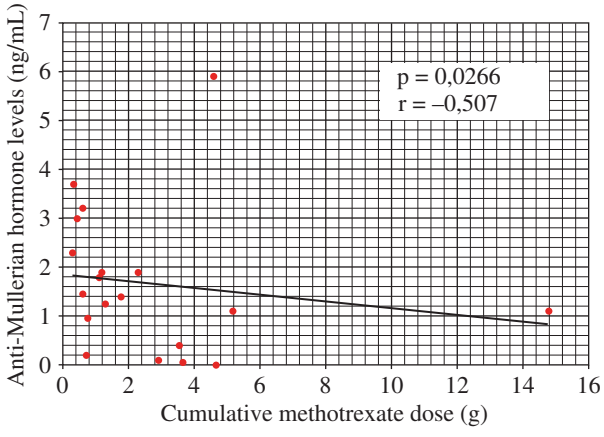

Figure 1. Correlation between anti-Müllerian hormone (AMH) levels and cumulative methotrexate dose.

Ovarian reserve and anti-CoL in adult c-SLE patients

The median of current age, age at disease onset, and disease duration were similar in patients with and without anti-CoL (Table 4). Menstrual cycle parameters were similar in both groups (p > 0.05). The assessment of ovarian reserve according to FSH, LH, and oestradiol levels did not show any difference between c-SLE patients with and without anti-CoL (p > 0.05, Table 4). The lower median AMH (Gen II ELISA, p = 0.39; US AMH/MIS AnshLabs ELISA, p = 0.33) and AFC (p = 0.08) levels did not reach statistical significance (Table 4). Clinical and immunological features, as well as the median of SLEDAI-2K [2.0 (0–8) vs. 0 (0–13), p = 0.08] and SLICC-ACR/DI [0.7 (0–2) vs. 0.9 (0–4), p = 0.69], were similar in both groups. The frequencies of treatments were similar in both groups (p > 0.05) (Table 5).

Discussion

To our knowledge, this is the first study to identify that adult c-SLE patients may have a subclinical impaired ovarian reserve related to a high dose of methotrexate, and to confirm the deleterious effect of IVCYC use.

Table 3. Disease activity in systemic lupus erythematosus (SLE) patients with onset before adulthood according to ovarian reserve parameters.

Ovarian reserve parameters	SLEDAI-2K $\geq$ 6 (n = 15)	SLEDAI-2K < 6 (n = 42)	p
FSH (IU/L)	6.9 (1–81)	5.85 (2–67)	0.45
Elevated levels	5 (33)	10 (24)	0.51
AMH Gen II (ng/mL)	1.25 (0–5.7)	1.02 (0–6.3)	0.89
Decreased levels	7 (47)	21 (50)	1.0
AMH AnshLabs (ng/mL) *	0.67 (0–8.7)	1.2 (0–5.2)	0.42
Decreased levels *	7 (47)	17 (41)	0.76
AFC †	6.5 (1–13)	5 (0–27)	0.66
AFC $\leq$ 10 follicles †	6 (40)	25 (59)	0.23
AFC $\leq$ 5 follicles †	3 (20	15 (36)	0.34

SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; FSH, follicle stimulating hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count.
* n = 53 SLE patients, † n = 45 SLE patients.
Values expressed in n (%) or median (range).

Table 4. Demographic features, menstrual cycles, and ovarian reserve in systemic lupus erythematosus (SLE) patients with onset before adulthood according to the presence of anti-corpus luteum antibody (anti-CoL).

Variables	With anti-CoL (n = 9)	Without anti-CoL (n = 48)	p
Demographic features			
Current age (years)	27.3 (18.9–36.9)	27.6 (18.3–39.8)	0.86
Age at disease onset (years)	16.6 (11–18)	16.1 (6–18)	0.66
Disease duration (years)	13.4 (5.6–20.3)	13.2 (2.1–25.4)	0.74
BMI (kg/m^2)	22.5 (19.7–34.1)	24 (17.2–48.8)	0.58
Caucasian	3 (33)	18 (38)	1.0
Socio-economic class C or D	8 (89)	36 (75)	0.67
Menstrual cycles			
Any menstrual disturbances	2 (22)	11 (23)	1.0
Amenorrhoea	0 (0)	2 (4)	1.0
Flow duration (days)	5 (1–7)	5 (0–15)	0.80
< 3 days	2 (22)	3 (6)	0.17
> 7 days	0 (0)	3 (6)	1.0
Cycle length (days)	30 (28–30)	30 (0–75)	0.78
> 35 days	0 (0)	3 (6)	1.0
POF	0 (0)	1 (2)	1.0
Ovarian reserve	1 (11)	0 (0)	0.16
FSH (IU/L)	7 (4.3–18.6)	6.9 (0.6–80.6)	0.38
Elevated levels	4 (44)	11 (23)	0.22
LH (IU/L)	7.6 (2.8–14.2)	5.9 (0.1–47.7)	0.41
Elevated levels	5 (56)	19 (40)	0.47
Oestradiol (pg/mL)	48.0 (26–133)	43.5 (15–1271)	0.22
Decreased levels	0 (0)	4 (8)	1.0
AMH Gen II (ng/mL)	0.5 (0–2.9)	1.15 (0–6.3)	0.39
Decreased levels	5 (56)	23 (47)	0.73
AMH AnshLabs (ng/mL) *	0.8 (0–2.6)	1.3 (0–9.0)	0.33
Decreased levels *	4 (44)	20 (42)	1.0
Ovarian volume (mm^3) †	7.5 (4.7–14.3)	9.6 (4.3–188.9)	0.09
AFC ‡	2 (1–11)	6 (0–27)	0.08
AFC $\leq$ 10 follicles ‡	6 (86)	25 (66)	0.41
AFC $\leq$ 5 follicles ‡	4 (57)	15 (40)	0.43

BMI, Body mass index; POF, premature ovarian failure; FSH, follicle stimulating hormone; LH, luteinizing hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count.
* n = 53 SLE patients, † n = 7 positive and 40 negative anti-CoL SLE patients, ‡ n = 7 positive and 38 negative anti-CoL SLE patients.
Values expressed as n (%) or median (range).

The main strength of the present study was a complete ovarian reserve assessment in post-pubertal lupus patients and controls at the early follicular phase of the menstrual cycle, which provided a more accurate estimation of follicle population (7, 8). The rigorous selection criteria of our patients and controls without recent

Table 5. Clinical and immunological features, disease activity and damage, and treatment in systemic lupus erythematosus (SLE) patients with onset before adulthood according to the presence of anti-corpus luteum antibody (anti-CoL).

Variables	With anti-CoL (n = 9)	Without anti-CoL (n = 48)	p
Clinical features			
Cutaneous	8 (88)	42 (88)	1.0
Articular	8 (88)	43 (90)	1.0
Serositis	1 (11)	17 (35)	0.25
Renal	4 (44)	26 (54)	0.72
Neuropsychiatric	2 (22)	13 (27)	1.0
Haematological	7 (78)	30 (63)	0.47
APS	1 (11)	8 (17)	1.0
Immunological features			
Anti-Sm	4 (44)	15 (31)	0.46
Anti-Ro	4 (44)	22 (45)	1.0
Anti-La	0 (0)	4 (8)	1.0
Anti-P	3 (33)	19 (40)	1.0
Anti-RNP	2 (22)	14 (29)	1.0
Anti-dsDNA	5 (55)	35 (73)	0.43
Lupus anticoagulant	2 (22)	7 (15)	0.62
IgG anticardiolipin	1 (11)	8 (17)	1.0
IgM anticardiolipin	0 (0)	3 (6)	1.0
SLE activity and damage			
SLEDAI-2K	2 (0–8)	0 (0–13)	0.08
SLICC-ACR/DI	0.7 (0–2)	0.9 (0–4)	0.69
Treatment			
Prednisone	9 (100)	48 (100)	1.0
Current dose (mg/day)	12.5 (5–40)	15 (2.5–40)	0.47
Cumulative dose (g)	37.6 (4–161)	47.3 (9.5–199.5)	0.37
Intravenous cyclophosphamide	2 (22)	19 (40)	0.46
Current dose (g/day)	–	1	–
Cumulative dose (g)	6.4 (5.8–7)	13.4 (1–26.4)	0.07
Time since last dose (months)	22.8 (20.9–24.7)	47.9 (0–119.9)	0.23
Azathioprine	6 (67)	37 (77)	0.67
Current dose (mg/day)	150 (100–200)	150 (100–200)	0.93
Cumulative dose (g)	67.4 (4.8–676.4)	157 (2.7–863)	0.53
Mycophenolate mofetil	3 (33)	18 (38)	1.0
Current dose (g/day)	2.5 (2–3)	2 (1–3.5)	0.56
Cumulative dose (g)	1936 (899–3992)	1962 (29.5–13 414)	1.0
Methotrexate	5 (56)	20 (42)	0.49
Current dose (mg/week)	17.5 (15–50)	16.3 (15–20)	0.69
Cumulative dose (g)	1.3 (0.3–4.7)	1.2 (0.3–14.8)	0.87

APS, Antiphospholipid syndrome; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; SLICC/ACR-DI, Systemic Lupus International Collaborating Clinics/ACR Damage Index.
Values expressed as n (%) or median (range).

gynaecological surgeries, hypothalamic–pituitary–ovary axis dysfunction, and end-stage renal disease are important because these abnormalities may influence ovarian reserve tests (8, 23).

We had also excluded subjects using non-hormonal anti-inflammatory drugs in the past 3 months because these drugs could be related to luteinized unruptured follicle syndrome (8, 36). Additionally, we evaluated hormonal parameters without the effect of any exogenous hormone to avoid possible bias, as recent studies indicate that AMH levels can be decreased in women using contraceptives (37, 38). These populations required hormonal contraceptive washout for at least 12 months or resumption of up to three consecutive menstrual cycles, and for those patients we provided contraceptive barrier methods. However, the restricted exclusion criteria resulted in a limited number of c-SLE patients and controls, hampering the statistical analysis, which is the main limitation of this study.

The reduced follicle quantity and/or quality may be explained by autoimmune oophoritis (7). Indeed, ovary specific autoantibody production was previously reported by our group in adult SLE patients, and confirmed by FSH levels (9). By contrast, anti-CoL was not identified as a marker of gonadal dysfunction and menstrual disturbances in another study of adult SLE when evaluated by this same hormone (10). Of note, anti-CoL was detected here solely in adult c-SLE patients and, as in a previous study (9), the only patient in our series with premature ovarian failure was negative for anti-CoL, probably because of lower expression of the target antigen in atrophic ovaries. However, the small representation of

this group of patients precludes a definitive conclusion about their role in ovarian dysfunction.

In male patients, anti-sperm antibodies (against parts of the spermatozoa such as the head, mid-piece, and/or tail) were not associated with sperm abnormalities in our cohort of adult SLE and c-SLE patients (39–43).

The novel findings regarding the cumulative dose of methotrexate and reduction in AMH levels identified in the current study suggest that a high dose of this immunosuppressive drug may be associated with follicular atresia. In the past decade, a high dose of methotrexate was still recommended for refractory paediatric SLE (44) and may account for this finding. In fact, a single injection of high-dose methotrexate (5.0 g/m^2) could induce destruction of primordial follicles in mice ovaries (45), whereas a low dose has no effect on reproductive fitness (8). Juvenile idiopathic arthritis treated with a high dose of methotrexate should therefore be investigated for this possible complication. Nevertheless, azathioprine and mycophenolate mofetil do not seem to impair fertility in females (5, 7, 8).

Although basal serum FSH is widely used as an ovarian reserve marker, it has demonstrated to have low accuracy in the early diagnosis of diminished ovarian reserve (7). AMH is produced by granulosa cells of early stage follicles and has the advantage of being relatively stable during the whole menstrual cycle, and hence is considered as an early and sensitive marker of ovarian reserve (8). The use of two different AMH kits provided a more accurate assessment of this hormone level, minimizing the chances of instability recently reported for the AMH Gen II ELISA kit (46). Nowadays, AMH and AFC are the best non-dynamic tests to predict ovarian performance in human reproductive treatment (5, 8). However, the clinical value of AFC is more restricted because of its low sensitivity, despite its high specificity (47).

This study also confirmed previous data that the ovary is highly susceptible to the toxic effects of IVCYC. This therapy indeed induces a persistent or long-lasting damage to the follicle population leading to significant alterations of fertility parameters as detected in our adult c-SLE patients. The median cumulative IVCYC dose was very high in our adult c-SLE patients, contrasting with the current recommendations of low doses of this gonadotoxic agent in lupus treatment. This alkylating agent affects reproductive function (48) because of ovarian primordial follicle damage, impairment of follicle maturation, follicle depletion, and eventual exhaustion (8). Our findings are also in accordance with previous studies in adult SLE and c-SLE using FSH, LH, oestradiol (2, 3, 5, 6), AMH (14–16, 18), and/or AFC (5).

In conclusion, the present study demonstrated for the first time that a high cumulative methotrexate dose is a possible additional relevant cause of subclinical ovarian dysfunction in adult c-SLE patients. Further studies are required to confirm this deleterious effect in other rheumatic diseases, particularly juvenile idiopathic arthritis and idiopathic inflammatory myopathy.

Acknowledgements

We thank Elaine P. Leon for technical support and Ulysses Doria Filho for statistical analysis. This study was supported by grants from the São Paulo Research Foundation (FAPESP 11/12471-2 to CAS), the National Council for Scientific and Technological Development (CNPQ 301411/2009-3 to EB, 303165/2008-1 to EFB, and 302724/2011-7 to CAS), and the Federico Foundation (to EB, EFB, and CAS), and by Centre Research Support 'Health of Children and Adolescents' USP (NAP-CriAd) to CAS.

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