# Subclinical impairment of ovarian reserve in juvenile systemic lupus erythematosus after cyclophosphamide therapy

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## Summary

### Objective

*To perform systematic assessment of ovarian reserve markers using a combination of tests in juvenile systemic lupus erythematosus (JSLE) patients without amenorrhoea.*

### Methods

*Twenty-seven consecutive JSLE female patients and 13 healthy controls without amenorrhoea were evaluated for 6 months. Ovarian reserve was assessed during early follicular phase by serum levels of follicle stimulating hormone (FSH), luteinising hormone (LH), estradiol, inhibin A, inhibin B and anti-Mullerian hormone (AMH). Ovarian size was measured by abdominal ultrasonography. Demographic data, disease activity, damage and treatment were also analysed.*

### Results

*The median of current age was similar in JSLE patients and controls (16.5 vs. 15years, p=0.31) with a significantly higher age at menarche (13 vs. 12years, p=0.03). A trend of lower median total antral follicle count was observed in JSLE compared to controls (9 vs. 14.5, p=0.062) with similar median of other ovarian reserve parameters (p>0.05). Further evaluation of patients treated with cyclophosphamide and those without this treatment revealed a higher median FSH levels (6.4 vs. 4.6 IU/L, p=0.023). Inhibin B, AMH levels and ovarian volume were also lower but did not reach statistical significance (10.8 vs. 27.6 pg/mL, p=0.175; 0.6 vs. 1.5 ng/mL, p=0.276; 3.4 vs. 5 $cm^3$, p=0.133; respectively). LH (2.7 vs. 2.9 IU/L, p=0.43), estradiol (50 vs. 38 pg/mL, p=0.337) and inhibin A (1.1 vs. 0 pg/mL, p=0.489) levels were comparable in both groups.*

### Conclusions

*Our study suggests that ovarian reserve after cyclophosphamide treatment may be hampered in spite of the presence of menstrual cycles emphasising the relevance of gonadal protection during the use of this alkylating agent.*

**Key words**

juvenile systemic lupus erythematosus, ovarian reserve, inhibin A, inhibin B, anti-Müllerian hormone

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## Introduction

Immunosuppressive agents, especially intravenous cyclophosphamide (IV-CYC), could induce premature ovarian failure and transient amenorrhoea in juvenile systemic lupus erythematous (JSLE) patients (1-4). Complete assessment of ovarian function including new markers of ovarian reserve, particularly inhibin A, inhibin B, anti-Mullerian hormone (AMH) levels and ovarian ultrasound, is therefore of great interest in these patients and may allow a more accurate prediction of immunosuppressor-related risk to future fertility, as previously reported in Hodgkin´s lymphoma (5). However, a systematic evaluation of ovarian reserve in JSLE patients, especially without amenorrhoea, is lacking in the literature.

We have therefore performed a complete assessment of ovarian function, including complete hormonal profile and ovarian ultrasound, in JSLE patients and healthy controls without amenorrhoea.

## Materials and methods

From June 2009 to June 2011, 65 female JSLE patients were followed at our University Hospital. Of them, 38 were excluded due to: absence of menarche (n=21), pregnancy (n=0), hormonal contraceptive use (n=6) or did not agree to participate in this study (n=11). Therefore, 27 JSLE female patients (American College of Rheumatology classification criteria) (6) followed at the Paediatric Rheumatology Unit and Rheumatology Division and 13 healthy controls followed at the educational and preventive Adolescent Unit of our University Hospital were prospectively evaluated for at least 6 consecutive months. All controls were not under hormonal contraceptive agents and none of them had current pregnancy or amenorrhoea (cessation of menstruation for more than three cycles after menarche) (3, 7). The Local Ethics Committee approved the study and an informed consent was obtained from all participants and parents.

Ages at menarche of patients and controls, as well as of their mothers were registered based on recollection. Menstrual flow duration and cycle length were evaluated prospectively for 6 consecutive months. Normal cycle was defined as flow duration of 3 to 7 days and length of 25 to 35 days (3, 7). Secondary sexual characteristics were classified according to Tanner pubertal changes (8) and body mass index (BMI) was defined by weight in kilograms/height in metres$^2$ (kg/m$^2$).

Complete ovarian function was concomitantly assessed by serum levels of hormones on the follicular phase (third to fifth day) of the menstrual cycle and evaluated according to pubertal changes and age. Follicle stimulating hormone (FSH) (reference levels: 2.4–9.3 IU/L), luteinising hormone (LH) (reference levels: 2.2–6.8 IU/L) and estradiol (reference levels: 22–215 IU/L) were measured by fluoroimmunoassay using kits from DELPHIA$^R$ time-resolved fluoroimmunoassay (WALLAC Ou, Turku, Finland). Intra- and inter-assay coefficients of variation were limited to 3.5% and 2.1%, respectively. Inhibin A, inhibin B and AMH were measured by enzyme-linked immunosorbent assay (DSL, Webster, Texas, USA) in duplicated samples. Intra- and inter-assay coefficients of variation were limited to 6.0% and 8.0%, respectively. Ovarian volume and total antral follicle count (AFC) >4mm were also measured by abdominal ultrasonography (9).

Disease activity was assessed at study entry using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2K) (10) and disease damage using the Systemic Lupus International Collaborating Clinics/ACR Damage Index (SLICC/ACR-DI) (11). Data of treatment before and after menarche were also determined.

### *Statistical analysis*

Results were presented as the median (range) for continuous variables and number (%) for categorical variables. Continuous variables were compared using the Mann-Whitney test to evaluate differences between JSLE patients and control group. For categorical variables differences were assessed by Fisher's exact test. In all statistical tests significance was set at a $p$-value<0.05.

## Results

*JSLE patients and healthy controls*

The median of current age and body mass index were similar in JSLE patients and controls (16.2 *vs*. 15 years, $p$=0.452; 23 *vs*. 22.4 kg/m$^2$, $p$=0.479; respectively). Nine patients had menarche before disease onset. The age at menarche was significantly higher in JSLE patients that presented their first menstruation after disease onset *versus* controls (13 *vs*. 12 years, $p$=0.03), although similar menarche age was reported between their mothers ($p$=0.288). The menstrual cycles parameters were alike in both groups ($p$>0.05). A trend of lower frequency of Tanner pattern 4 or 5 was observed in JSLE patients compared to controls (63% *vs*. 92%, $p$=0.068). Also, a trend of lower median AFC was evidenced in JSLE patients compared to controls (9 *vs*. 14.5, $p$=0.062), whereas with similar ovarian volumes ($p$=0.461). The median levels of serum FSH ($p$=0.516), LH ($p$=0.718), estradiol ($p$=0.581), inhibin A ($p$=0.951), inhibin B ($p$=0.564) and AMH ($p$=0.613) levels were comparable in patients and controls.

*JSLE patients*

The median age at JSLE diagnosis and disease duration were 12.2 (5.9–14.6) years and 4.2 (0.2–13.3) years, respectively. The median of SLEDAI-2K was 2 (0-16) and SLICC/ACR-DI was 0 (0-1). Twenty-four (88.9%) JSLE patients were under current prednisone use (median dose of 11.3 mg, range 1–40 mg). Fourteen (51.9%) patients were receiving azathioprine, five (18.5%) mycophenolate mofetil and 26 (96.3%) chloroquine diphosphate. Eleven patients underwent IVCYC with median duration time between the last dose and study entry of 2.5 years (range 0-7.7). The median IVCYC cumulative dose was 16.7 (0.6–22.3) g with 2.8 (0.1–3.4) years median duration of this therapy.

JSLE patients treated with IVCYC had a trend of higher age at menarche compared to healthy controls (13 *vs*. 12, $p$=0.052).

Further evaluation of patients treated with IVCYC showed that there was no difference in FSH ($p$=0.416), LH ($p$=1.0), estradiol ($p$=0.338), inhibin A ($p$=0.718), inhibin B ($p$=0.164) and AMH ($p$=0.27) levels compared to healthy controls. Hormones levels and

**Table I.** Demographic features, menstrual cycles, ovarian reserve hormonal profile and ovarian ultrasound in juvenile systemic lupus erythematosus (JSLE) patients and controls without amenorrhea.

| Variables | JSLE with IVCYC (n=11) | JSLE without IVCYC (n=16) | Controls (n=13) | P[1] | P[2] | P[3] |
|---|---|---|---|---|---|---|
| Demographic features | | | | | | |
| Current age, years | 16.2 (11.3-20) | 15 (12-21) | 15 (12-21) | 0.282 | 0.759 | 0.452 |
| Age at disease onset, years | 11.5 (6.2-13.3) | 12.6 (5.9-14.6) | – | – | – | 0.208 |
| Disease duration, years | 5.6 (1.4-9.1) | 3.3 (0.2-13.3) | – | – | – | 0.089 |
| BMI, kg/m$^2$ | 23 (15.5-34.2) | 22.4 (17.1-25.8) | 22.4 (17.1-25.8) | 0.885 | 0.228 | 0.479 |
| Tanner 4 or 5 | 6 (60) | 4 (80) | 12 (92.3) | 0.142 | 0.093 | 0.6 |
| Menstrual cycles | | | | | | |
| Age at menarche, years | 13 (10-16) | 12.8 (11-15) | 12 (10-13) | 0.052 | 0.181 | 0.804 |
| Age at maternal menarche, years | 12.5 (11-16) | 12 (11-14) | 13 (11-16) | 0.767 | 0.056 | 0.704 |
| Gynecological age*, years | 3 (0.3-7.3) | 3.3 (1.3-6.9) | 4 (1-9) | 0.684 | 0.472 | 0.839 |
| Flow duration, days | 5 (3.5-6) | 5 (4-7) | 5 (5-8) | 0.256 | 0.518 | 0.665 |
| >7 days | 0 | 0 | 1 (7.7) | 1.0 | 0.448 | 1.0 |
| Cycle length, days | 28 (17.5-36) | 29 (24-38) | 29 (27-42) | 0.442 | 0.795 | 0.299 |
| >35 days | 1 (9.1) | 2 (12.5) | 1 (7.7) | 1.0 | 1.0 | 1.0 |
| Ovarian reserve | | | | | | |
| FSH, UI/L | | | | | | |
| Median (range) | 6.4 (2.9-21.5) | 4.6 (2.8-6.5) | 5.6 (2.5-9.7) | 0.416 | 0.104 | 0.023 |
| Elevated levels, n (%) | 1 (9.1) | 0 (0) | 1 (7.7) | 1.0 | 0.448 | 0.407 |
| LH, UI/L | | | | | | |
| Median (range) | 2.7 (1.1-8.1) | 2.9 (1.2-9.9) | 3 (1-8.3) | 1.0 | 0.599 | 0.43 |
| Elevated levels, n (%) | 1 (9.1) | 2 (12.5) | 1 (7.7) | 1.0 | 1.0 | 1.0 |
| Estradiol, pg/mL | | | | | | |
| Median (range) | 50 (14-67) | 38 (18-133) | 34 (25-70) | 0.338 | 0.945 | 0.337 |
| Decreased levels, n (%) | 2 (18.2) | 2 (12.5) | 0 (0) | 0.199 | 0.448 | 1.0 |
| Inhibin A, pg/mL | | | | | | |
| Median (range) | 1.1 (0-8.4) | 0 (0-18.4) | 0.1 (0-19.1) | 0.718 | 0.683 | 0.489 |
| Inhibin B, pg/mL | | | | | | |
| Median (range) | 10.8 (0-51) | 27.6 (2.4-112.5) | 29.3 (0-65.2) | 0.164 | 0.878 | 0.175 |
| AMH, ng/mL | | | | | | |
| Median (range) | 0.6 (0-10) | 1.5 (0-4.4) | 1.4 (0.2-4) | 0.27 | 0.965 | 0.276 |
| Ovarian volume, cm$^3$ | 3.4 (2.2-6) | 5 (2.1-19.6) | 4.7 (3.5-11) | 0.142 | 0.878 | 0.133 |
| Total antral follicle count, >4mm | 9 (4-27) | 9 (1-28) | 14.5 (2-30) | 0.111 | 0.121 | 0.826 |

Values expressed in n (%) or median (range). JSLE: juvenile systemic lupus erythematosus; IVCYC: intravenous cyclophosphamide treatment; BMI: body mass index, *time between menarche and current age, FSH: follicle stimulating hormone; LH: luteinizing hormone; AMH: anti-Müllerian hormone. P[1]: JSLE with IVCYC *vs*. controls; P[2]: JSLE without IVCYC *vs*. controls; P[3]: JSLE with IVCYC *vs*. JSLE without IVCYC.

ovarian ultrasound were also alike in JSLE patients not treated with IVCYC and healthy subjects ($p>0.05$) (Table I). The comparison of patients treated with cyclophosphamide and those without this drug revealed higher median FSH levels (6.4 *vs*. 4.6 IU/L, $p=0.023$). Inhibin B (10.8 *vs*. 27.6 pg/mL, $p=0.175$), AMH levels (0.6 *vs*. 1.5 ng/mL, $p=0.276$) and ovarian volume (3.4 *vs*. 5 $cm^3$, $p=0.133$) were also lower but did not reach statistical significance. LH (2.7 *vs*. 2.9 IU/L, $p=0.43$), estradiol (50 *vs*. 38 pg/mL, $p=0.337$) and inhibin A (1.1 *vs*. 0 pg/mL, $p=0.489$) levels were comparable in both groups (Table I).

No correlation was observed between the time since last dose of IVCYC and FSH levels ($p=0.297$), as well as between FSH levels and IVCYC cumulative dose ($p=0.708$).

## Discussion

To our knowledge this was the first study that performed a complete hormonal profile concomitantly with ovarian ultrasound in JSLE adolescent patients without amenorrhoea and identified a subclinical impaired ovarian reserve after cyclophosphamide treatment.

The advantage of the present study is the complete evaluation of ovarian reserve during early follicular phase that provides a more accurate estimation of ovarian function (1), in view of the fact that hormone concentrations may fluctuate depending on the day of sampling (12). In addition, the restricted selection criteria of JSLE patients and controls with menstrual cycles and without hormonal treatment or current pregnancy are relevant, since these alterations may affect gonadal evaluation (5). However, the prospective six month design associated with the strict exclusion criteria resulted in a limited number of patients receiving IVCYC and low number of controls hampering the interpretation of FSH levels in healthy controls intermediate between JSLE patients with or without IVCYC treatment. In addition, the potential impact of disease duration on the results and possible recollection bias between patients and controls in describing the age of menarche cannot be completely excluded.

We have confirmed our previous observation of late occurrence of the first period in JSLE (3, 7, 13) and juvenile dermatomyositis (14) patients compared with normal Brazilian adolescents. This delay of menarche was not attributed to the genetic background given that the menarche age of their mothers was similar in groups.

The novel finding of elevated FSH in spite of the presence of menstrual cycles in JSLE treated with IVCYC suggest a subclinical impairment of ovarian reserve. Reinforcing this observation inhibin B and AMH levels were lower in these patients even though not statistically significant. Likewise, in adult SLE including those with amenorrhoea, reduction of follicular ovarian population was reported according to AMH evaluation (15, 16). This hormone is considered a valuable parameter of ovarian reserve, with a good correlation with chronological age and AFC (12). The term ovarian reserve is defined, however, as the number or quality of oocytes in the female gonad and no single test is precise to predict ovulation. Ovarian evaluation includes therefore measurement of various parameters, including FSH, LH, estradiol (3, 14), inhibin B, AMH and ultrasound (12).

In fact, the FSH is a marker of ovarian function (17) and we previously demonstrated that menstrual alterations are associated with high levels of FSH in JSLE, regardless of IVCYC therapy (3). On the contrary, we showed elevated levels of FSH with severe sperm abnormalities (18) and testicular Sertoli cell dysfunction according to inhibin B levels in male SLE under IVCYC (19). Indeed, IVCYC reacts with DNA bases and damages the DNA repair mechanisms with consequent inhibition of cell replication and cell death. In the immature rat ovary, CYC reduced the number of ovarian granulosa cells and caused ovarian fibrosis (20). In humans, oral and IVCYC impairs reproductive function due to ovarian primordial follicle damage, impairment of follicle maturation, follicle depletion and eventual exhaustion. This damage is generally cumulative and irreversible causing POF (4). Of note, the median cumulative IVCYC dose was very high in our patients, contrasting with the current recommendation of low doses of this gonadotoxic agent in the treatment of JSLE.

In conclusion, our study suggests that ovarian reserve after cyclophosphamide treatment may be hampered in spite of the presence of menstrual cycles emphasizing the relevance of patient information and of possible alternatives to high-dose IVCYC in female JSLE. Further studies on the effectiveness of gonadal protection during the use of this alkylating agent are necessary.

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