Author's response to reviews

Title: Association between low-dose pulsed intravenous cyclophosphamide therapy and amenorrhea in patients with systemic lupus erythematosus: A case-control study

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Author's response to reviews: see over
Association between low-dose pulsed intravenous cyclophosphamide therapy and amenorrhea in patients with systemic lupus erythematosus: A case-control study

We have revised our manuscript (1067683819515606) entitled, “Association between low-dose pulsed intravenous cyclophosphamide therapy and amenorrhea in patients with systemic lupus erythematosus: A case-control study” by Yasuhiro Katsumata et al. We believe that we have suitably and substantially revised the content in accordance with the comments we received, as described below. The following are point-by-point listings of the changes that have been made in the revised version. The changed sections are highlighted in the revised manuscript. In addition, although the original version of the manuscript was edited by a science manuscript editing service in the U.S.A., we have had another company (Nature Publishing Group Language Editing) edit the revised version of the manuscript, as recommended by the Referee 1.

Referee 1
Reviewer’s report: This manuscript is very interesting, however, several questions were raised below.

Major compulsory revisions
1) Aim of this study is to clarify the incidence of amenorrhea following treatment with low-dose IVCY and the association between amenorrhea and clinical parameters of SLE, and the authors concluded in this manuscript that patients who are < 40 years old have some risk for sustained amenorrhea with low-dose IVCY treatment, and it is better to consider a higher risk of sustained amenorrhea by treatment with IVCY for SLE patients who are ≥ 40 years old. However, based on results in this manuscript, age ≥ 40 years old is a risk factor of amenorrhea for SLE patients included in this study, irrespective of treatment arms.
>>> It is correct that “age ≥ 40 years old” is the strongest risk factor of amenorrhea for SLE patients included in this study, irrespective of treatment arms, as shown by the multivariate analysis (Table 5). However, as also shown by the same multivariate analysis, even low-dose IVCY may further increase the risk of transient amenorrhea. In addition, we performed ad hoc analysis answering the suggestions from another referee, and we found that the cumulative dose of IVCY may also be a risk factor (page 13, line 9).
2) In 2nd paragraph, Result section: Results of logistic regression analysis and those of univariate analysis are presented. In this paragraph, the authors mentioned there was no significant association between age and amenorrhea by logistic regression analysis which is a multivariate analysis, then back to an univariate analysis. This description might raise a question for the study design itself, since it is difficult to understand which analysis is more important for the authors.

>>> We are afraid that the referee misunderstood our meaning here, probably because the original description was elusive. Actually, in this section of the results, only univariate analyses were performed. Although a logistic regression analysis is usually used in multivariate analyses, it can also be applied for a univariate analysis where an association of 2 variables is evaluated. In statistics, logistic regression is used for prediction of the probability of occurrence of an event by fitting data to a logit function logistic curve. It is a generalized linear model used for binomial regression. Like many forms of regression analysis, it makes use of several predictor variables that may be either numerical or categorical. Logistic regression is a useful way of describing the relationship between ONE or MORE independent variables (e.g., age and sex) and a binary response variable, expressed as a probability, that has only two values, such as having cancer ("has cancer" or "doesn't have cancer"). We added this information in the revised manuscript as suggested by the reviewer (page 9, line 11).

Minor Essential revisions:
1) Percentages in Table 3 and 4 are difficult to understand. This is different from Table 1 and 2.
>>> We modified the tables in the revised manuscript as suggested by the reviewer (Tables 3 and 4). We appreciate the reviewer’s suggestions, which have given more readability to our data.

2) Some results in Table 2 are repeatedly appeared in Table 3.
>>> We eliminated the repetitions in the revised manuscript as indicated by the reviewer (Table 3).

3) In Table 4, a gap between Age ≥ 40 years old and IVCY group is not needed.
>>> We are afraid the “gap” was made when the original WORD file was converted to PDF. There was no such “gap” in the original WORD file.

4) In Table 6, I do not find a suffix #1 in the table.
>>> We inserted the ‘*’ in the revised manuscript as indicated by the reviewer.
Discretionary Revisions

1) In this study, the authors mentioned that patients who are $\geq 40$ years old are risk factor for sustained amenorrhea irrespective of treatment arms. It seems to be quite natural that females who are $\geq 40$ years old have higher rate of sustained amenorrhea in general. Is there any difference when compared with healthy controls?

>>> Unfortunately, we do not have controls, such as healthy individuals in whom steroids or IVCY were administered or SLE patients without treatment. Although we agree that women $\geq 40$ years old have a higher rate of sustained amenorrhea in general, we believe sustained amenorrhea in women aged 40 to 44 is still not common.
Reviewer’s report: This retrospective study provides information on the rates of amenorrhea and sustained amenorrhea following treatment with low dose intravenous cyclophosphamide compared to steroid monotherapy in premenopausal women with systemic lupus erythematosus. The age ≥40 years was the only parameter that significantly associated with the development of amenorrhea in multivariate analysis. The use of IVCYC may also associate with amenorrhea but less significantly and mainly in women <40 years old. In contrast, IVCYC did not associate with sustained amenorrhea. However, the small number of patients did not allow applying multivariate models for sustained amenorrhea. A significant correlation between sustained amenorrhea and the age group of ≥40 years was demonstrated. It is of note that all women younger than 40 years old had a transient disturbance of the menstruation. The writing is adequate in general. The information given on the rates of amenorrhea with short-term IVCYC is not fresh knowledge; however, previous studies have compared short-term with long-term IVCYC and not with steroid monotherapy as the authors did.

Some major points should be discussed.

1. The number of patients ≥40 years old was very small in order to draw conclusions on the effect of IVCYC compared to steroids on the development of transient or sustained amenorrhea in this age group (N=8 in the IVCYC and N=3 in the GC group). Have other age groups been tested?

   >>> As shown in Table 3 in the original manuscript, patients < 40 years old were also evaluated. In fact, in this age group, the effect of IVCY on amenorrhea was more evident.

2. It is not clearly explained why the limit of 40 years was chosen to define age subgroups in this study. Previously, the age of 32 years was shown to be critical for the development of amenorrhea (Ioannidis et al, Ref 11). It would be interesting to give information on other age subgroups as well, such as <32 and ≥32 years of age.

   >>> We chose the age 40 based on previously published reports (e.g., Ref 4, 7, 8, and 16) as well as our preliminary statistical analyses. As described in the original manuscript, age at the initiation of treatment did not differ significantly between subjects with and without amenorrhea \((p = 0.19)\) (Table 2). The logistic regression analysis did not reveal significant correlation between age and amenorrhea \((p = 0.13; \text{OR} \, 1.06; \, 95\% \, \text{CI} \, 0.98-1.14)\). However, when all 62 study subjects were divided into two age groups, those < 40 years old and those ≥ 40 years old at the initiation of treatment, the univariate analyses revealed that the highest risk for developing amenorrhea was being ≥ 40 years old \((82 \, \% \, vs. \, 33\%; \, p = 0.005; \, \text{OR} \, 9.0; \, 95\% \, \text{CI} \, 1.7-46.5)\) (Tables 2 and 3). In comparison, when the patients were divided into the age groups of 32, 35, or 37
years, there was no association or only a weak statistical association (page 11, line 8). Thus, we speculate the age 40 is special for the patients in the present study for some reason. We discussed this issue in the revised manuscript as suggested by the reviewer (page 16, line 3). We appreciate the reviewer’s suggestions, which have given more strength to our data.

3. The authors mention that the minimum duration of follow up was 12 months. Though, no information is provided on the minimum follow up duration after the onset of amenorrhea. Were all patients with amenorrhea followed up for at least 12 months in order to conclude on the sustained, irreversible loss of menstruation?
>>> Actually, we followed up with the patients with amenorrhea for at least additional 12 months after the onset of amenorrhea. We added this information in the revised manuscript as suggested by the reviewer (page 8, line 8).

4. It is not mentioned if any woman had been treated with CYC (intravenous or oral) in the past. If so, these women should be excluded from the study.
>>> In fact, as described in the original manuscript, no woman enrolled in the present study had been treated with CYC (intravenous or oral) in the past (page 7, line 7). Patients who were treated with cyclophosphamide before this study were excluded.

5. Page 9, line 7. The fact that the SLEDAI score was higher in the IVCYC compared to the steroid group is a limitation of this study since high disease activity has been previously associated with the development of amenorrhea in lupus (Shabanova SS et al. Ovarian function and disease activity in patients with systemic lupus erythematosus. Clin Exp Rheumatol 2008). This is not discussed by the authors.
>>> We have discussed this issue in the revised manuscript as suggested by the reviewer (page 18, line 4). However, in the present study, there was no association between the SLEDAI-2K score and amenorrhea.

6. Page 9, line 8. The median IVCYC dose was very low. There were patients receiving only one pulse of IVCYC of 500 mg. Moreover, the range of the cumulative CYC dose was very broad (400-6500 mg). Has the association between the cumulative IVCYC dose and the development of amenorrhea and/or sustained amenorrhea been tested?
>>> As shown in Table 2 in the original manuscript, the total dose of cyclophosphamide was not significantly different among the patients who received IVCY with or without amenorrhea. However, when the total dose of cyclophosphamide was considered as a numerical predictor variable and a univariate logistic regression analysis was applied for all of the patients, including
patients from both the IVCY and steroid groups, the ORs were 1.0 ($p = 0.08$) for amenorrhea and 1.0 ($p = 0.09$) for sustained amenorrhea, respectively (although the incidence of sustained amenorrhea was probably too low to analyze in this way). Thus, treatment with IVCY may display a trend to be weakly associated with amenorrhea and sustained amenorrhea. With multivariate logistic regression analyses, the total dose of cyclophosphamide was also weakly associated with amenorrhea ($p = 0.11$) but not with sustained amenorrhea ($p = 0.98$). These data were similar to those from the analyses when the IVCY was analyzed as a categorical valuable, as described in the original manuscript. We discuss this issue in the revised manuscript as suggested by the reviewer (page 13, line 9).

7. The treatment of severe lupus with such a low dose of CYC (median dose was 1g) is not widely applied in clinical practice. Besides, no woman received maintenance therapy following IVCYC. On the other hand, the second group of patients was indeed treated only with steroids for lupus nephritis, cns lupus, vasculitis and cytopenias?

>>> Because cyclophosphamide (and azathioprine) had not been officially approved for treatments of SLE in Japan until recently, we tended to use glucocorticoid steroids. In my opinion, that is probably why many SLE patients in the present study, some with severe manifestations, can be treated only with steroids. In addition, partly thanks to the Japanese public health insurance system, Japanese SLE patients tend to see rheumatologists early in their disease courses (i.e., because they do not need referrals to see specialists) and, thus, can be treated with weaker regimens. In this context, we stated in the original manuscript that, considering that the patients that could not respond to the questionnaire because of severe or fatal disease were excluded from the study and that some of the study subjects needed additional therapy, our low-dose IVCY regimen would not be necessarily suitable for all severe manifestations of SLE.

8. Page 10, lines 6-8. The authors have not recorded the SLE duration as well as the presence of autoantibodies such as anti-U1RNP and anti-Ro, parameters shown to associate with the development of amenorrhea in previous studies (Ioannidis et al, Ref 11).

>>> We performed additional analyses for these parameters in the revised manuscript. However, there was no association between these parameters and amenorrhea in the present study (Table 2).

9. Page 10, line 6 from top and lines 1-3 from bottom. The information on sustained amenorrhea is also provided in the next paragraph “Frequency of resumption of menses” on page 11. Thus, it could be skipped at these points to avoid repetitions.

>>> We eliminated this part in the revised manuscript as indicated by the reviewer.
10. In the discussion section, in the second paragraph, the authors compare their results to the sustained amenorrhea rates following long-term IVCYC treatment reported in literature. It would be advisable to comment and compare as well with the rates of amenorrhea following short-term IVCYC reported in previous studies (Laskari et al, Ref 10# Boumpas et al, Ref 5# Mok et al, Ref 7# Huong et al, Risk of ovarian failure and fertility after intravenous cyclophosphamide. A study in 84 patients. J Rheumatol 2002# Appenzeller et al, Ref 9# Park et al, Ref 8).

>>> Although only long-term IV CY was evaluated in most of the previous reports listed above, we discuss this issue in the revised manuscript as suggested by the reviewer (page 15, line 7).

The rates of sustained amenorrhea in the previous reports:
- #10: 4%, 5-7 months (short), + MMF; 51%, “prolonged” (long), without MMF
- #5: 12%, 6 months (short); 39%, 2 years (long); 0%, steroids only
- #7: 26%, 2 years (long) + oral CY (+AZA)
- #8: 0%, < 5g (short); 22%, 2 > 5g (long)
- #9: 18%, long
- Huong et al: 68%, mostly long

11. The rates of amenorrhea given in this study are very high. Amenorrhea develops in approximately 20-50% of women treated with long-term IVCYC, whereas in 6-20% of women treated with short-term low dose IVCYC. In this study, the rates of amenorrhea were 65% in women treated with low-dose IVCYC and 33% in women treated only with steroids. Given that the mean age of Japanese women at menopause is 50 years, as mentioned on page 7, lines 5-7, the difference in the observed amenorrhea rates between this and other studies is remarkable.

>>> In most of the previous reports listed above (#10), only long-term IV CY was evaluated, and sustained but not transient amenorrhea was the main focus. Thus, we are afraid that the rates of overall amenorrhea were not clearly shown in those studies. In addition, in the present study, the rates of transient amenorrhea in the IVCY and steroids groups were actually 59% (17/29) and 27% (9/33), respectively, which are lower than mentioned by the referee. However, these figures are still probably higher than “estimated” rates of transient amenorrhea in other studies. We speculate that previous studies may have underestimated the real incidence of amenorrhea and that these conflicting results might be due to reporting biases derived by their retrospective designs. To support this view, the incidence of chemotherapy-induced amenorrhea resulting from doxorubicin and cyclophosphamide (4 cycles) in premenopausal women with breast cancer is usually reported to be approximately 50-60%. We discuss this issue in the revised manuscript as suggested by the reviewer (page 17, line 2).

The rates of amenorrhea in the previous reports:
#10: 14%, 5-7 months (short), + MMF; 56%, “prolonged” (long), without MMF
#5: 13-33% (?), 6 months (short); 39-52% (?), 2 years (long); 0%, steroids only
#7: 30%, 2 years (long) + oral CY (+AZA)
#8: 37%, 2 years (long)
#9: 20%, long

# Huong et al: 39% (including patients with SLE and other diseases), mostly long; This report was consisted of the data of 84 women: 56 with SLE, 28 with other diseases, mainly Wegener's granulomatosis and systemic vasculitides.

12. Finally, in Table 3 the number of patients with amenorrhea in the steroid group should not be 9 instead of 6 according to the data given in Table 4?

>>> Actually, it is correct as it is. The number of patients with amenorrhea in the steroid group is 9 = 6+0+2+1, from Table 4.
Reviewer’s report: Baba et al. studied how IVCY affect ovary function in 45 year-old or younger female Japanese patients with SLE, and clarified clinical parameters, which is associated with amenorrhea after IVCY treatment by a single-center case-control retrospective study.

General comments
Although there are some limitations such as selection bias and insufficiency of subject number, the incidence for sustained amenorrhea by IVCY in Japanese patient was described by retrospective study. Authors also clarified that even low-dose IVCY caused transient amenorrhea, although it resumed in all 40 year-old or younger patients after IVCY, which might be interpreted as the one of the benefits in IVCY for Japanese patients younger than 40 with SLE. It was found in this study that 40 and older might be the risk factor for sustained amenorrhea. Complying with the review guideline by the editorial team, comments are categorized as follows:

Major Compulsory Revisions
1) Time between treatment and amenorrhea, duration of amenorrhea, and time to resumption of menses were raised in “Data Collection” part. However, the data were not presented anywhere. They are also important and helpful for physician and patients to plan the treatment, and especially, time to resumption would be one of the concerns for patients. Please consider describing these data.

>>> We added the data in the revised manuscript as suggested by the reviewer (page 12, line 11). We are afraid we do not have appropriate data about time between treatment and amenorrhea, and thus we eliminated this part from the revised manuscript.

Minor Essential Revisions
1) In Table 2 and 3, some parts show P value and OR comparing longitudinally and the other parts showed them comparing transversally, and that makes readers confusing. Please specify which data was subject to each univariate analysis in an appropriate way.
One of the examples are as follows: In “Associations between amenorrhea and clinical parameters” part, P value, odds ratio and 95% CI (0.02, 3.5 (1.3-11.0) for the difference of amenorrhea incidence between the IVCY group and the steroid group described in the text are all same as what is described in the table 2 for amenorrhea incidence in the IVCY group. When readers see table 2, they might think that 0.02 and 3.5(1.3-11.0) are the data comparing 17/26 and 12/36 within the IVCY group. Regarding the data comparing amenorrhea cases between subjects <40 and subjects 40 and older, it might be acceptable, since we could tell from the “raw” data – 17 and 9, described both in table 2 and 3.
>>> We modified the tables in the revised manuscript as suggested by the reviewer (Tables 3 and 4). We appreciate the reviewer’s suggestions, which have given more readability to our data.

2) In the third paragraph of “Discussion” part, we see the sentence “The strongest risk factor for developing…”, and from the context, it could be known that the sentence is describing your study, but it might puzzle some readers. Some words or phrases might be added to this sentence to make readers understand that it describes your data, not data previously reported.
>>> We inserted the phrase “in the present study” in the sentence of the revised manuscript as suggested by the reviewer (page 15, line 18).

3) “in a retrospective study” might be changed to “in a single center retrospective study” in the beginning of the fifth paragraph of “Discussion” part.
>>> We inserted the phrase “single center” in the sentence of the revised manuscript as suggested by the reviewer (page 17, line 12).

4) “reported previously” might be added at the last of the sentence, “However, the size of our study population was comparable to similar studies” in the fifth paragraph of “Discussion” part.
>>> We inserted the phrase “reported previously” in the sentence of the revised manuscript as suggested by the reviewer (page 17, line 17).

Discretionary Revisions
1) If the incidence of pregnancy would be included in your database for this study, that would be more helpful for clinician to offer IVCY to patients with SLE. However, pregnancy itself is a risk factor for recurrence of lupus, and it might be difficult to find such cases.
>>> There is a patient who experienced pregnancies and delivers twice in the IVCY group. However, we feel it is difficult to discuss the effect of IVCY on pregnancy because there are many other factors involved, as the reviewer suggested.

2) In “Discussion” part, comparison of your data and the previous data in high-dose IVCY is described, and this might be presented as Table 7, since this is also one of the most important parts, which makes your data valuable and appealing to the readers.
>>> This study was not designed to perform comparisons between low-dose and high-dose IVCY. Thus, we are afraid that it would cause other problems to present a table that shows direct and detailed comparisons. In addition, authors are encouraged to be concise in this journal. Because we already have 6 tables, we are afraid that it would be redundant to add another table describing other investigators’ data.