Author's response to reviews

Title: Comparison of postmenopausal endogenous sex hormones among Japanese, Japanese Brazilians, and non-Japanese Brazilians

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Author's response to reviews: see over
Dear Dr. Norton:

Thank you for your email of October 18, 2010, regarding our manuscript and the valuable comments of the referees. We wish to resubmit our revised manuscript here. We feel that the revised manuscript is a suitable response to the comments, and is significantly improved over the initial submission. We trust that it is now suitable for publication in *BMC Cancer*

Thank you again for your kind consideration of this paper.

Sincerely yours,

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This is an ambitious investigation aimed at evaluating whether sex hormone levels differ between postmenopausal Japanese Brazilians and Japanese. The authors undertook a cross-sectional study using control groups from two hospital-based breast cancer-case control studies conducted in Japan and Brazil. The authors observed higher levels of estrogens and androgens in Japanese Brazilians as compared with Japanese women and conclude that these differences in circulating hormone levels may contribute to the variation in breast cancer incidence rates among these populations.

The following comments fall under the category of “major compulsory revisions.”

Given the methodologic limitations of the study, the conclusions seem to be overstated. Further attention to the study limitations is needed, the most serious limitations being sample size (n=44 Japanese Brazilians) and heterogeneity between studies in the type of blood sample used (i.e., serum in Japan and plasma in Brazil). More specific comments follow.

Thank you very much for your review of our manuscript and comments. In particular, we agree with the addition of comments on the methodological limitations of the study in the Discussion to assist with interpretation of the findings. Considering the importance of these issues, we have mentioned them as an initial comment in the Discussion section, as follows:

(After: p.13, l.10- p.14, l.10 in the revised manuscript)

As an initial comment, several methodological limitations of this study should be considered. First, our findings might be subject to the difference in study methods between Japan and Brazil, albeit that the two studies were conducted under a similar protocol. For example, we used serum samples for Japanese and plasma samples for both Japanese Brazilians and non-Japanese Brazilians. In this regard, we measured estrone level in both serum and plasma from the same subject (n =38). Although both levels were highly correlated (correlation coefficient = 0.94) and the percentage difference was relatively small (mean = -4%, 95% confidence interval: -9%, 1%), we used corrected values for the present study because the kit for estrone was applicable to serum samples only. Concurrently, we compared estrone levels among the three populations using crude values and observed the same results. The difference in blood samples is therefore unlikely to have affected the difference in sex hormone levels between the two populations. Given that blood collection methods also differed between
Japan and Brazil, in addition to the type of blood sample used, we cannot exclude the possibility that our findings were affected by these differences. Another example is the difference in questionnaire data and their collection methods between Japan and Brazil. If such difference led to exposure misclassification, this might explain the observed absence of associations between sex hormone levels and known breast cancer risk factors or lifestyle factors. Second, although at least more than 96% of subjects had detectable levels of estradiol, estrone, bioavailable estradiol, SHBG, androstenedione and DHEAS, the proportion of subjects with levels below the LOD was relatively high for testosterone (24%) and free testosterone (69%). Our findings for testosterone and free testosterone should therefore be interpreted cautiously. Third, since our study included only a small number of Japanese Brazilians (n = 44), the findings might have been due to chance and should be interpreted with caution.

We have responded to each specific comment below.

Comment 1: How comparable were blood collection, processing (e.g., time between collection and freezing), and storage methods in Japan and Brazil?

Response to comment 1:
The protocols of the two studies in Nagano and São Paulo were designed to compare potential risk factors among Japanese living in Nagano, Japan, and Japanese Brazilians and non-Japanese Brazilians living in São Paulo, Brazil. However, as the reviewer pointed out, we cannot exclude the possibility that our findings were affected by the difference in blood collection methods, and have accordingly revised the corresponding parts, as follows:

(After: p.6, l.11-15 in the revised manuscript)
In addition to determining lifestyle factors and genetic susceptibility to the risk of breast cancer, the protocols of these studies were also designed to compare potential risk factors among Japanese living in Nagano, Japan, and Japanese Brazilians and non-Japanese Brazilians living in the state of São Paulo, Brazil.

(After: p.13, l.22-24 in the revised manuscript)
Given that blood collection methods also differed between Japan and Brazil, in addition to the type of blood sample used, we cannot exclude the possibility that our findings were affected by these differences.
Comment 2: A strength of the study is that the same laboratory was used to measure hormones for both studies. Please describe the batching scheme for the laboratory assays; were the samples from the two studies assayed within the same or different batches? Were the same quality control samples used for the serum and plasma assays? If so, then it may be possible to adjust values from each sample type (see Key T et al. CEBP 19(4) 2010).

Response to comment 2:
Thank you very much for this comment. In the present study, samples from the Nagano and São Paulo studies were assayed in the same laboratory but in different batches. However, inter-assay coefficients of variation were basically acceptable (5.3%-11.9%) (p.9, l.16-23 in the revised manuscript).

Unfortunately, since we did not use the same quality control samples for the serum and plasma assays, we cannot adjust values from each sample type.

Comment 3: Although the authors acknowledge in the discussion that one possibility for their observed findings is the different blood sample types, the authors need to provide additional evidence as to the comparability of blood collection methods and hormone assays for serum vs. plasma in order to assess whether the variation in hormone measurements is due to methods employed or to true differences in hormone levels between populations. The authors note that estrone levels were measured in both serum and plasma for 38 subjects; were these subjects postmenopausal women who were participants in the larger study analysis?

Response to comment 3:
We agree with the importance of the comparability of blood collection methods and hormone assays for serum vs. plasma, which is a major limitation of the present study. Accordingly, we have mentioned this as an initial comment in the Discussion section, as follows:

(After: p.13, l.10- p.14, l.4 in the revised manuscript)
As an initial comment, several methodological limitations of this study should be considered. First, our findings might be subject to the difference in study methods between Japan and Brazil, albeit that the two studies were conducted under a similar protocol. For example, we used serum samples for Japanese and plasma samples for
both Japanese Brazilians and non-Japanese Brazilians. In this regard, we measured estrone level in both serum and plasma from the same subject (n = 38). Although both levels were highly correlated (correlation coefficient = 0.94) and the percentage difference was relatively small (mean = -4%, 95% confidence interval: -9%, 1%), we used corrected values for the present study because the kit for estrone was applicable to serum samples only. Concurrently, we compared estrone levels among the three populations using crude values and observed the same results. The difference in blood samples is therefore unlikely to have affected the difference in sex hormone levels between the two populations. Given that blood collection methods also differed between Japan and Brazil, in addition to the type of blood sample used, we cannot exclude the possibility that our findings were affected by these differences. Another example is the difference in questionnaire data and their collection methods between Japan and Brazil.

If such difference led to exposure misclassification, this might explain the observed absence of associations between sex hormone levels and known breast cancer risk factors or lifestyle factors.

Regarding the 38 subjects for whom both serum and plasma samples were measured, the data were derived from our previous cross-sectional study among Japanese Brazilians in the city of São Paulo. These were women aged over 50 years but of unknown menopausal status. We have revised this part as follows:

(After: p.8, l.23 - p.9, l.2 in the revised manuscript)
We therefore measured estrone level in both serum and plasma from the same women aged over 50 years (n = 38)

Comment 4: How was menopausal status assessed? Did hormone levels differ by years since menopause?

Response to comment 4:
Menopausal status was determined by self-report in the present study. We have added this as follows:

(After: p.7, l.13-14 in the revised manuscript)
Menopausal status was determined by self-report and energy intake was assessed using a food frequency questionnaire (FFQ).
Regarding associations between sex hormone levels and years since menopause, we did not find a statistically significant association, although increasing age was significantly associated with lower levels of estrogens and androgens.

**Comment 5:** Were exogenous hormone users excluded from the study? If so, how many were excluded?

**Response to comment 5:**
We excluded subjects (n = 19) who were suspected of using exogenous hormones based on levels of estrone, estradiol, or testosterone, because we did not collect information on exogenous hormone use in either study. We have mentioned this point in the initial manuscript (p.10, l.4-6 in the revised manuscript).

**Comment 6:** Risk factor data were collected via a self-administered questionnaire in Japan and by interview in Brazil. As one of the authors’ aims is to examine associations between circulating hormone levels and breast cancer risk factors, the difference in data collection methods is an important limitation. The potential for exposure misclassification should be acknowledged and implications discussed.

**Response to comment 6:**
Thank you very much for this comment. We have added comments on this issue in the Discussion section, as follows:

(After: p.13, l.24- p.14, l.4 in the revised manuscript)

Another example is the difference in questionnaire data and their collection methods between Japan and Brazil. If such difference led to exposure misclassification, this might explain the observed absence of associations between sex hormone levels and known breast cancer risk factors or lifestyle factors.

**Comment 7:** Please explain what is meant by “bioavailable” estradiol (e.g., non-SHBG or non-albumin bound?). Did the authors consider estimating free estradiol using the SHBG measurements?

**Response to comment 7:**
In accordance with this comment, we have revised the corresponding parts below. Unfortunately, our data do not allow the estimation of free estradiol level.

(After: p.8, l.19-20 in the revised manuscript)
Bioavailable estradiol (free and albumin-bound estradiol) was measured by the ammonium sulfate precipitation method.

Comment 8: In the first paragraph of the results section, the authors should additionally compare and contrast characteristics of Japanese with those of Japanese Brazilians.

Response to comment 8:
In accordance with this comment, we have described these findings in Table 1, as follows:

(After: p.11, l.4-8 in the revised manuscript)
Japanese Brazilians had an earlier menarche, more births, greater BMI, smoked more, drank less, and were physically less active than Japanese, but had a later age at menopause and age at first birth, fewer births, lower BMI, smoked less, and were shorter and physically more active than non-Japanese Brazilians.

Comment 9: Did the authors assess whether the results in Table 4 differed by sample type (i.e., serum vs. plasma)?

Response to comment 9:
In accordance with this comment, we performed stratified analyses by study site (i.e. the study in Nagano, Japan vs. the study in São Paulo, Brazil) and have added the findings as follows:

(After: p.12, l.13-14 in the revised manuscript)
Stratified analyses by study site (i.e. the study in Nagano, Japan vs. the study in São Paulo, Brazil) showed similar results for the two study sites.

(After: p.12, l.22-23 in the revised manuscript)
In stratified analyses by study site, however, we did not observe any findings which were consistent between the sites.
Comment 10: *In the last paragraph of the results and discussion sections, the direction of the associations should be described.*

Response to comment 10:
In accordance with this comment, we have revised the corresponding parts as follows:

(After: p.12, l.14-22 in the revised manuscript)
No statistically significant associations were observed between sex hormone levels and family history of breast cancer, past history of benign breast disease, age at menarche, age at menopause, parity, number of births, age at first birth, breast feeding, height, smoking, alcohol drinking, or physical activity in the past five years *except for the following*. We found a significantly higher level of SHBG among women who had a later age at menopause and shorter women. We also observed a significantly higher level of DHEAS among women who had more births and a significantly lower level of testosterone among physically more active women.

(After: p.15, l.21- p.16, l.3 in the revised manuscript)
*In the present study, we found a higher level of SHBG among women who had a later age at menopause and shorter women. We also observed a higher level of DHEAS among women who had more births and lower level of testosterone among physically more active women. In addition to the lack of consistency in these findings between the two study sites (i.e. the study in Nagano, Japan vs. the study in São Paulo, Brazil), our findings are inconsistent with those of previous studies.*
Reviewer: Andiara Schwingel

Thank you very much for your review of our manuscript and positive comments. We have responded to each comment below.

Comment 1: abstract - conclusion: add findings that compare Japanese Brazilians with non-Japanese Brazilians, both living in Brazil

Response to comment 1:
In accordance with this comment and comment 6, we have added the findings for the comparison of sex hormone levels between Japanese Brazilians and non-Japanese Brazilians as follows:

(After: p.3, l.17-22 in the revised manuscript)
Japanese Brazilians had significantly higher levels of estradiol, bioavailable estradiol, estrone, testosterone, and free testosterone, and lower SHBG levels than Japanese. Japanese Brazilians also had significantly higher levels of bioavailable estradiol, estrone, and DHEAS, and lower levels of SHBG and androstenedione than non-Japanese Brazilians. Levels of estradiol, testosterone, and free testosterone, however, did not differ between Japanese Brazilians and non-Japanese Brazilians.

(After: p.4 l.2-3 in the revised manuscript)
We found higher levels of estrogens and androgens in Japanese Brazilians than in Japanese, and similar to or higher levels than in non-Japanese Brazilians.

(After: p.10 l.16-17 in the revised manuscript)
For comparisons among the three populations, Japanese Brazilians living in São Paulo were used as the reference group.

(After: p.11 l.16- p.12 l.8 in the revised manuscript)
Japanese Brazilians had significantly higher levels of estradiol, bioavailable estradiol, estrone, testosterone, and free testosterone, and lower SHBG levels than Japanese, whereas levels of androstenedione and DHEAS did not differ between the two populations (Table 2). Similar results were seen for analyses stratified by BMI (under and over 25), except for androstenedione level, which did not differ between Japanese Brazilians and Japanese whose BMI was under 25 but was significantly lower among Japanese Brazilians than Japanese whose BMI was over 25 (Table 3).
Japanese Brazilians had significantly higher levels of bioavailable estradiol, estrone, and DHEAS, and lower levels of SHBG and androstenedione than non-Japanese Brazilians. Levels of estradiol, testosterone, and free testosterone, however, did not differ between Japanese Brazilians and non-Japanese Brazilians (Table 2). Similar results were obtained when analyses were stratified by BMI (under and over 25), except for estrone and DHEAS: levels of estrone were significantly higher among Japanese Brazilians than non-Japanese Brazilians in those with a BMI under 25 but did not differ between the two populations in those whose BMI was over 25, while DHEAS level did not differ regardless of BMI (under and over 25) (Table 3).

(After: p.13 l.5-6 in the revised manuscript)
In particular, levels of estrogens and androgens in Japanese Brazilians were higher than in Japanese and similar to or higher than in non-Japanese Brazilians.

(After: p.14 l.20-22 in the revised manuscript)
We also found higher circulating levels of bioavailable estradiol and estrone in Japanese Brazilians than non-Japanese Brazilians, although levels of estradiol, testosterone, and free testosterone did not significantly differ between two populations.

(After: p.16 l.11-12 in the revised manuscript)
We found that levels of estrogens and androgens in Japanese Brazilians were higher than in Japanese and similar to or higher than levels in non-Japanese Brazilians.

Comment 2: Methods: describe in more detail the Japanese Brazilian group, more specifically, their generation. If living in Sao Paulo city or coming for treatment only.

Response to comment 2:
In accordance with this comment and comment 3, we have added information on the definition of subjects as follows:

(After: p.6, l.20-23 in the revised manuscript)
Inhabitants of the state of São Paulo were recruited and asked their ethnicity. Japanese and their descendants were defined as Japanese Brazilians, and white, black and mixed populations were defined as non-Japanese Brazilians.
**Comment 3:** Methods: Explain who non-Japanese Brazilians are - e.g. their ethnicity

**Response to comment 3:**
In accordance with this comment and comment 2, we have added information on the definition of subjects. Please see the above response.

**Comment 4:** Methods: explain how energy intake was assessed.

**Response to comment 4:**
In accordance with this comment, we have revised the corresponding parts as follows:

(After: p.7, l.13-14 in the revised manuscript)
Menopausal status was determined by self-report and energy intake was assessed using a food frequency questionnaire (FFQ).

(After: p.7, l.20-23 in the revised manuscript)
The two questionnaires contained closely similar questions concerning demographic characteristics, medical history, family history of cancer, menstrual and reproductive history, anthropometric factors, physical activity, smoking habits, and dietary factors assessed by FFQ.

**Comment 5:** The number of participants differ significantly between JB and the other groups. Confirm homogeneity of variance for main information assessed.

**Response to comment 5:**
As you mentioned, the number of Japanese Brazilians were smaller than of the other two populations. Since we recognized this as one of the methodological limitations of the study, we have added a comment on this point in the Discussion section below. We compared sex hormone levels of Japanese Brazilians with Japanese and non-Japanese Brazilians and have added the findings. Please see our response to comments 1 and 6.

(After: p.14, l.8-10 in the revised manuscript)
Third, since our study included only a small number of Japanese Brazilians (n = 44), the findings might have been due to chance and should be interpreted with caution.
Comment 6: Conclusion: add finding comparing JB and non-JB.

Response to comment 6:
In accordance with this comment and comment 1, we have added findings of the comparison of sex hormone levels between Japanese Brazilians and non-Japanese Brazilians. Please see the above response.

Comment 7: Table 1: identify where p was significant.

Response to comment 7:
We compared factors in Table 1 across the three populations using Japanese Brazilians as the reference group and have added the p values in Table 1. We have also revised the corresponding parts in the Results section as follows:

(After: p.10, l.24- p.11, l.4 in the revised manuscript)
Japanese had a later menarche, fewer births, lower BMI, smoked less, drank more and were physically more active than the other two populations. On the other hand, non-Japanese Brazilians had an earlier age at menopause and age at first birth, more births, greater BMI, smoked more, and were taller and physically less active than the other two populations.

Comment 8: if more information about diet was available (e.g. isoflavones), the authors should add in discussion. Page 13 sounds out of the scope of the study as it reads, and decreases the value of the rich data assessed in the study.

Response to comment 8:
We assessed dietary intake by a food frequency questionnaire (FFQ) in both the Nagano and São Paulo study. However, we did not use these data for the present analyses because the FFQs used in the two studies were different. We mentioned this point in the initial manuscript (p.15, l.9-11 in the revised manuscript). We agree that it would be interesting to use the information on dietary intake. Considering the other reviewer’s comments which mentioned misclassification due to differences in questionnaire data (comments 6), however, we did not take into account the information on dietary intake in the present study.
In the present study, we aimed to investigate the association between endogenous sex hormones levels and known breast cancer risk factors or lifestyle factors in addition to comparing sex hormone levels across the three populations. We presented the corresponding findings in Table 4 and discussed them on Page 13 in the initial manuscript. We therefore consider that the comments on Page 13 in the initial manuscript are necessary for the present study.

Comment 9: I would suggest that the authors name the Japanese group as "Native Japanese"

Response to comment 9:
Thank you very much for your suggestion. This might be easier for readers to understand than ‘Japanese living in Japan’ but we would like to use the current word in view of the desire to retain consistency with our previous studies.

Comment 10: abstract - background: i suggest using "Brazil" instead of Sao Paulo

Response to comment 10:
Considering the comment 2, we have specified the area and revised the corresponding part as follows:

(After: p.3, l.5-8 in the revised manuscript)
we compared postmenopausal endogenous sex hormone levels among Japanese living in Japan, Japanese Brazilians living in the state of São Paulo, and non-Japanese Brazilians living in the state of São Paulo.

Comment 11: the authors could revise the title and add a direction of the study results. For example: "Japanese immigrants have higher risk of developing breast cancer than Native Japanese"

Response to comment 11:
Thank you very much for your suggestion. Considering the methodological limitations of this study, which were also pointed out by the other reviewer (comments 1, 3 and 6), we would like to use the present title, which we consider to be suitably conservative.