Author's response to reviews

Title: Low-dose combined oral contraceptive use is associated with lower bone mineral content variation in adolescents over a 1-year period

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Author's response to reviews: see over
Dear Professor Arnaldi and Reviewers,

We thank you and the reviewers for your important suggestions that helped improve our manuscript. Some of the suggestions were accepted and others, although pertinent, were not included in the text and an explanation of our point of view is provided. Below please find our replies to the suggestions of the reviewers. We again thank the two reviewers for their important comments and the detailed appraisal of our manuscript.

We hope to have clarified all doubts and remain at your disposal for further clarifications.

Sincerely,

Prof. Tamara Goldberg and authors
Reviewer: Prof. Nihal Hatipoglu

What are the new contributions of this study considering the studies in the literature that evaluate more cases for longer durations on this topic?

We thank you for your comment. With respect to the contributions of this study compared to other already published studies, we would like to emphasize that questions on this topic still remain in the literature, favoring primary studies. We would like to stress that in our study all girls were adolescents with a median bone age of 16 years (range: 14 to 18 years), who were in late puberty according to the Tanner criteria (M4-M5), and that the group of contraceptive users always used the same formulation (20 µg EE and 150 µg desogestrel), in contrast to other studies in which the participants used different formulations. Doubts regarding this topic still exist since other studies did not use such strict inclusion criteria. Our approach differs from that of other articles, a fact permitting to emphasize the importance of our results.

We agree with the reviewer that the assessment period of only 12 months is a short time. However, longitudinal clinical trials are difficult to conduct, especially when the subjects are adolescents with changing psychosocial characteristics, which interfere with medication adherence over a prolonged period of time. We emphasize that a large portion of this group of contraceptive users is still under follow-up at our service, which will permit to obtain new results.

In this study, the number of cases is inadequate and also the duration is not long enough.

The sample size calculation and explanations provided by one of the authors, a statistician (HRCN), guarantee that the number of cases is sufficient for the differences observed, as described in the manuscript.

Why the authors didn’t choose femoral neck? This area is more valuable than the whole body with or without the cephalic region.

The femoral neck could also be used, as done for the evaluation of adults. However, the Official Pediatric Positions of the ISCD (2010, 2013) recommend the sites studied here for densitometric evaluation in children and adolescents.

In the exclusion criteria, you should also ask oligo/amenorrhea situation. We thank the reviewer for the suggestion and this information was added. None of the participants included presented oligo/amenorrhea. (manuscript - line 107)

In your groups, there are some cases who have advanced bone age. Is there any explanation for this?
The criterion of chronological age is known to be inaccurate in the age group studied here, since some girls of the same age may have started puberty, while others are in mid-puberty or late puberty. The use of bone age and pubertal events is therefore a sensitive alternative. In another study conducted by our group, we observed a correlation of 0.93 between bone age and pubertal events. As an inclusion criterion of the present study, the girls should have more than 2 years postmenarche, being in M4-M5 as described in the manuscript. It was expected that their bone ages were according to their pubertal events. We cite here the study published by our group in 2014: Relationship between chronological and bone ages and pubertal stage of breasts with bone biomarkers and bone mineral density in adolescents. http://ac.els-cdn.com/S0021755714000989/1-s2.0-S0021755714000989-main.pdf?_tid=80505e50-8c63-11e4-8ca5-00000aab0f01&acdnat=1419532151_048156ed1599cab0b8e102e42f0b4c80

In the COC user group, some patients have high BMI value (maximum BMI percentile is 97.38). As far as we know, BMI affects BMD and BMC, so, statistical outcomes may be affected.
Prof. Hatipoglu, we thank you for the excellent comment that points to an important aspect. We recently published a study in this regard: Mosca LN, Lederer Goldberg TB, Nóbrega da Silva V, da Silva CC, Kurokawa CS, Bisi Rizzo AC, Corrente JE. Excess body fat negatively affects bone mass in adolescents. Nutrition (Burbank, Los Angeles County, Calif.) 2013: 30; 847-52. Unfortunately, in the present study one of the oral contraceptive users exceeded our cut-off which would be the 95th percentile for BMI. However, as demonstrated in the previous study, fat percentage affects the increase in bone mass only in adolescent girls who are considered extremely obese, with BMI >99th percentile. In that study we report: Analyzing the variables according to CA and BA, we observed that the average results increased
progressively from eutrophic to extremely obese adolescents, with significant
differences between groups ($P < 0.01$). Conversely, average values of spine, whole-
body, and subtotal body BMD, and spine, femur, and whole-body BMC were lower
among extremely obese than obese female adolescents. In view of these arguments,
we can state that this possibility should not have interfered with our results since, as
the BMD and BMC values were not transformed, if there were interference, the
baseline values should have differed between the group of users (which included the
obese adolescent) and the group of non-users.

**In the statistical analysis, you should make adjustments for age and BMI or
use Z-score or SD values for BMD and BMC.**
The simultaneous adjustment for age and BMI requires a parametric model. Parametric models, in turn, require knowledge of the probability distribution of the outcome.

During the exploratory phase, the probability distribution of the outcome was not
recognized by the Shapiro-Wilk test. Therefore, if performed, simultaneous
adjustment would be technically incorrect. Correction variable by variable would
require stratification of the base, reducing the power of the test.

With respect to Z-score, variation in lumbar spine BMD ($p=0.864$) and total body
BMD ($p=0.602$) Z-scores was observed among non-users of COC.

Among contraceptive users, variation was observed in total body BMD Z-scores
($p=0.003$), but not in lumbar spine BMD Z-scores ($p=0.120$).

**Are there any patients with osteoporosis in COC group, at the end of the
study?**
Our objective was to analyze a possible increase or not in bone mass between the
beginning and end of the study. However, continuing the evaluation of our patients,
we may find some girls with low bone mass.

**Reviewer: Prof. Jan Stepan**

We thank you for your valuable comments and some considerations are reported
below.
The short period of treatment (12 months) seems not sufficient to observe any relevant change in bone density even in growing subjects, and does not allow for impact of longer-term effects of a change in the bone physiology. Especially, rate of bone gain is very different in very young subjects (12 years old) and 20 year old women.

The arguments of the reviewer are correct; however, we used the age group called adolescents considering the criteria of the WHO (1995), which encompasses the second decade of life, ending at 20 incomplete years. (manuscript line 34)

As explained in the reply to Prof. Hatipoglu, our study is based on the evaluation of bone age and pubertal events, which were carefully evaluated, and not only on chronological age. For inclusion in the study, these girls needed to have menstruated for the first time (menarche) at least 2 years ago and to be in Tanner stage M4-M5, even if the chronological age was lower. According to a study published by our group last year (Fortes CMT, Goldberg TBL, Kurokawa CS, Silva CC, Moretto MR, Biason TP, Teixeira AS, Nunes HRC. Relationship between chronological and bone ages and pubertal stage of breasts with bone biomarkers and bone mineral density in adolescents. J Ped (Rio de Janeiro) 2014; 90(6): 624-31) and numerous studies reported in the literature, in adolescent girls the peak increase in bone mass occurs when they are in M3 (mid-puberty); however, an increase in bone mass is still observed after this period. Our intention was to evaluate how much the introduction of COC interferes with this increase.

With respect to the long-term effect of change in bone physiology, we currently cannot offer anything concrete, only possible inferences.

Minor comments


2. Methods. A sentence on EE and desogestrel administration is missing in the Methods.

We agree with the suggestions of the reviewer and the text was modified accordingly. (manuscript - line 65 to 77)
3. Please, state the short-term in-vivo precision errors for lumbar spine and total body BMC; state the long-term precision error using the Hologic phantom. Did daily scanning of a phantom show absence of machine drift during the study?

All DXA norms were used following the recommendations stated in the Official Position of the International Society for Clinical Densitometry (ISCD) (http://www.iscd.org/documents/2013/03/facility-accreditation-glossary.pdf).

The CV was estimated from repeated measurements for all regions mentioned (lumbar spine and total body). With the results in hand, CVs of 0.6 % and 1.3 % were obtained for the lumbar spine and for the whole body, respectively. (manuscript - line 158 to161)

4. Was the sampling and storage done for the measurement of biochemical markers?

Yes, they were stored for the measurement of bone biomarkers (OC, BAP and S-Ctx).

5. I don't think the assay for estradiol detects the ethinyl estradiol concentrations. This should be stated in the methods section because it is easy to get confused with serum measurements of 17-beta estradiol vs ethinyl estradiol.

Reassessing the manual of the Architect Estradiol kit (Abbott), page 7 states:

Table B*

The ARCHITECT Estradiol assay recovery in the presence of the following compounds is 100 ± 40% at the concentrations listed below: A study was performed in which synthetic specimens containing estradiol were supplemented with potential interferents at the concentrations listed and tested for estradiol. The percent recovery is shown below:

<table>
<thead>
<tr>
<th>Interferent</th>
<th>Concentration</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilin 1.2 ng/mL</td>
<td>98.4</td>
<td></td>
</tr>
<tr>
<td>Equilin Sulfate 10 ng/mL</td>
<td>92.6</td>
<td></td>
</tr>
<tr>
<td>Ethynylestradiol 0.8 ng/mL</td>
<td>88.6</td>
<td></td>
</tr>
<tr>
<td>Mestranol 0.8 ng/mL</td>
<td>100.5</td>
<td></td>
</tr>
<tr>
<td>Norethindrone 32 ng/mL</td>
<td>76.9</td>
<td></td>
</tr>
<tr>
<td>Norethindrone acetate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Thus, so that there is not doubt, the following information was added in the Methods section of the manuscript. (manuscript - line 128 to 133)

Blood samples were taken from COC users six months after entering the study to measure estradiol levels by Chemiluminescent Microparticle Immunoassay (CMIA) using the ARCHITECT® Estradiol Kit (Abbott Laboratories, USA) in order to indirectly determine the effect of EE use. The percent recovery of estradiol in the presence of ethynylestradiol (interferent content) with this method is reported to be 88.6%

6. Results. Change in BMD compared with baseline should be provided instead of Variation.

Our hypothesis was that non-users differ from COC users in terms of the evolution of bone mass increase. Hence, as the outcome is the evolution, baseline and final (after 12 months) results were combined and the outcome that expresses evolution was thus created. Mathematically, evolution is expressed as the difference (in percent) between BMD values obtained at 12 months and baseline values. Comparison of time points within each group (baseline x final) does not answer whether the groups differ in terms of evolution. The approach suggested permits to know whether a change occurred in each group, but does not permit to answer whether the change differed between groups, which is exactly the hypothesis of the study.

7. Was BMC change significant after adjustment for age and body size?
8. Was change in BMD significant when normalized using Z-scores?

The same questions were raised by Prof. Hatipoglu and our reply was:
The simultaneous adjustment for age and BMI requires a parametric model. Parametric models requires knowledge of the probability distribution of the outcome. During the exploratory phase, the probability distribution of the outcome was not recognized by the Shapiro-Wilk test. Therefore, if performed, simultaneous adjustment would be technically incorrect. Correction variable by variable would require stratification of the base, reducing the power of the test.
With respect to Z-score, variation in lumbar spine BMD (p=0.864) and total body BMD (p=0.602) Z-scores was observed among non-users of COC. Among contraceptive users, variation was observed in total body BMD Z-scores (p=0.003), but not in lumbar spine BMD Z-scores (p=0.120).

9. Discussion. The authors should note that the ethinyl estradiol is much more potent at activating the estrogen receptors than estradiol, so that with COC's the overall activation of estrogen receptors is not measured by serum levels of 17-beta estradiol. Furthermore, the COC's have major effects on SHBG so the bioavailable estrogen should be considered.

Your comments and suggestions are very pertinent and information to clarify this point was added in the Discussion. In a study using a similar method to evaluate the binding of EE and E2 to ER, Churchwell et al. (2014) demonstrated this binding to be 1 for E2 and 1.2 for EE.

Comparison of life-stage-dependent internal dosimetry for bisphenol A, ethinyl estradiol, a reference estrogen, and endogenous estradiol to test an estrogenic mode of action in Sprague Dawley rats. Churchwell MI¹, Camacho L, Vanlandingham MM, Twaddle NC, Sepehr E, Delclos KB, Fisher JW, Doerge DR.

Regarding the effect on sex hormone binding globulin (SHBG), Scholes et al. (2010) showed that COCs cause 2- to 6-fold increases in SHBG, decreasing free androgen levels and probably reducing the incorporation of bone mass.

(Manuscript- line 252 to 255 and line 262 to 277)

10. The discussion correctly notes that estrogens are important in growth hormone metabolism, but it does not discuss the difference between endogenous effects and exogenous effects (J Clin Endocrinol Metab. 2004 Dec;89(12):6185-92).

We thank the reviewer for the comment and the differences between the effects of endogenous and exogenous estrogen and possible resulting hypotheses were addressed in the Discussion. (Manuscript- line 262 to 277)