Author’s response to reviews

Title: Bone turnover in passive smoking female rat: relationships to change in bone mineral density

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Author’s response to reviews: see over
Dear Dr. Robert Layfield:

Appreciations to your kind consideration for our paper submission and quick review results. Although this manuscript is not considered for publication at the present edition, we are still encouraged by three kind peer reviewers, who all agreed that this paper was an article of importance in its field. So, we are glad to resubmit you our revised manuscript after a major revision according to the reviewer’s professional suggestions. Meanwhile, a detailed responses describing how we have responded to the specific points raised by the reviewers are also attached by the end of this letter. All changes made are highlighted in ‘green background’ of the revised text. For a more convenient review to you and the reviewers, I would like to upload our responses in another file separately, as we upload the revised manuscript.

If you have any other concern regarding to our revised manuscript, please feel free to contact me. At the same time, we would like to appreciate you and the nice reviewers who had given us professional comments to improve our manuscript.

Best wishes,

Sincerely yours,

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Response to Reviewer #1 (Hermizi Hapidin):

1) Materials & method section - the authors should mention the number of rats (n) in each groups (page 4)

Answer: Done accordingly (line109-111). The rats were randomly assigned to six groups with 8 rats in each.

2) Statistical analysis section (spelling error):
   a) correct "ManneWhitney U test" to "Mann-Whitney U test" (page 6, line 129)
   b) correct "KruskaleWallis test" to "Kruskal-Wallis test"

Answer: You are right. The statistical data analysis was conducted once again under the expert guidance of our statistics tutors. The section has been revised according to your professional comment (line150-158). In this study, results of all measurements are presented as mean ± S.D. for eight rats in each group. A one-way analysis of variance (ANOVA) was first performed to determine whether there were statistically significant (P<0.05) differences among the experimental groups. Further, the Duncan’s multiple range post-hoc test was used for comparisons between individual groups and to determine which means differed statistically significantly (P<0.05). Pearson’correlation was applied to determine the correlation between markers of bone turnover and BMD of the femur and lumbar vertebrae. Correlations were considered statistically significant at p < 0.05. Data analysis was performed with SPSS version 13.0 (Chicago, IL, USA). Because the populations from which the samples were normally or approximate normal distribution and the variances of the populations were equal, Manne-Whithey (for two groups) and Kruskal-Wallis analysis (for more than two groups) has not been employed to compare the means of groups. The redundant statistical methods have been deleted.

3) Results section - the authors need to discuss more on why there is no difference in weight between controls and smoke-exposed rats (page 6)

Answer: We have added a more detailed discussion in the revised manuscript (line
164-168). Because of identical conditions of setting and diet in laboratory animals, there was no difference in weight between the controls and the smoke-exposed female rats. All animals increased their weight during the experiment. The body weight gain of rats receiving passive smoking during the 2, 3, 4-month experimental period was similar to that noted in the control group. The smoke exposure had no effect on the body weight of rats.

4) Discussion section - the authors need to provide adequate and stronger justification as to why BMD of lumbar spine & femur was significantly lower in 4-month smoke exposed rats than in controls, but not in the 2-month and 3-month smoke exposed rats.

**Answer:** We have added a more detailed discussion in the revised manuscript (line206-234).

Our finding revealed a significant effect of smoking characteristic on BMD of lumbar spine and femur in rat, consistent with the results from previous studies[26-27]. We demonstrated that the BMD of lumbar spine and femur was lower in 4-month smoke exposed rats than in controls, but not in the 2-month and 3-month smoke exposed rats, which may be due to differences in time of passive smoking. César-Neto et al.[26] showed that 5-month cigarette smoke inhalation promoted a reduced bone density. Hapidin et al. [28] demonstrated that 4 months of nicotine treatment was associated with change of bone histomorphometric parameters and bone-resorbing cytokines. Tamaki et al.[29] reported the impact of smoking on bone status was mainly associated with the number of smoking years in elderly men. Furthermore, Sneve et al.[30] showed that smoking reduced BMD at the hip, distal and ultradistal forearm in males, and the effect appeared to be mainly time- but not dose-dependent.

However, previous investigations also have shown that the timing of BMD decrease may be associated with the dose level of smoke exposure. Epping-Jordan et al. [24] reported the mean blood nicotine concentration for smokers who smoked 30 cigarettes daily was 40-42 ng/ml. Ajiro et al. [27] found the BMD in the smoke-exposed rats was lower than that in controls at 8 weeks. The blood nicotine concentrations in 8-week smoke-exposed rats were 329.4 ng/ml, indicating that the
average blood nicotine concentration in the smoke-exposed rats was seven- and eight-fold greater than the average for heavy smokers[27]. Cornuz et al.[31] indicated that the risk of hip fracture increased linearly with greater cigarette consumption. It seems that in the case of high exposure, the effect of smoking develops very fastly and the exposure duration needed for developing of a measurable effect of smoking depends on its intensity. In this study, the blood nicotine concentrations in the 4-month smoke-exposed rats were 40.6 ng/ml, indicating that the average blood nicotine concentration was similar to the average for heavy smokers. The blood nicotine concentration did not differ among 2, 3 and 4 months, but there may be a harmful effect of longer smoke exposure for 4 months. Thus, we considered that the timing of BMD decrease may be associated with the dose level and the duration of smoke exposure. We could have shown a threshold dose if we examined an intermediate time point. It will be of interest to perform this analysis in a future study.

5) Discussion section - please provide Hapidin H et al. (2007), Mohamed N et al. (2010) as references.

Answer: Done accordingly (line211, 242; ref.28, 34).

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests.

Response to Reviewer #2 (Malgorzata Brzoska):

The subject and the findings of the study are interesting; however, the manuscript needs extensive attention. Several questions should be addressed. The main problems are related to this manuscript preparation, including the language and form of the results presentation and discussion. The main questions that should be addressed are listed below.
Abstract

- p.2, l.31; Wistar (not wistar)

**Answer:** Done accordingly (line 35, 105).

- l.37; ‘that were not exposed to smoke’ – this part of the sentence may be deleted because the fact that control rats were not exposed to smoke do not require explanation.

**Answer:** Done accordingly (line 41). ‘that were not exposed to smoke’ has been deleted.

- l.39; ‘In addition, passive smoking did not reveal….’ The previous sentence (l.38-39) suggests that this sentence should be revised.

**Answer:** The sentence has been revised as “However, there was no significant difference in serum osteocalcin levels between smoke-exposed rats and controls” (line 43-44).

- l.42 and throughout the manuscript; the Authors use the phrase ‘significantly correlated’, whereas the word ‘significantly’ is necessary. When the correlation occurs it has to be statistically significant (p < 0.05), in other cases (p > 0.05) there is no correlation.

**Answer:** You are exactly right. In the results section, it has to be statistically significant (p < 0.05) when the correlation occurs, ‘significantly’ is necessary. ‘significantly’ has been deleted in the other sections (line 42, 46, 48, 207, 211, 507, 509).

- l.47-50, the conclusions need revision because it repeats results, the activity of b-ALP (not concentration) were determined, the description ‘that smoking prevented bone formation’ is inappropriate (the word ‘inhibited’ is more appropriate); ‘…on BMD and bone status…’ – this description also needs correction (BMD is one of the parameters describing bone status).
Answer: The sentence has been revised (line 51-53). Our data suggest that smoke exposure can inhibit bone formation and increase bone resorption. The hazardous effects of passive smoking on bone status are associated with increased bone turnover in female rat.

Introduction.
- p.3, l.57; it should be ‘…in bone mass of smokers…’
Answer: Done accordingly. ‘…in bone mass of smokers…’ (line 62).

- 1.72; It is necessary to describe what other components of tobacco smoke may be responsible for bone damage (toxic heavy metals, PCB, dioxin, polycyclic aromatic hydrocarbons).
Answer: Done accordingly (line 77). Compared to nicotine treatment alone, cigarette smoke exposure has been found to be more detrimental to bone [Ref. 18], which suggests that cigarette smoke constituents (e.g., toxic heavy metals, PCB, dioxin, polycyclic aromatic hydrocarbons) other than nicotine might be responsible for the negative impact of smoking on bone.

- 1.74; Did you mean a direct effect of passive smoking whether an effect at all?
Answer: We considered that the effects of passive smoking on bone might be connected with its direct action as well as with its indirect action. As a result, the word of "direct" has been deleted in the revised sentence (line 95).

- 1.75; ‘have’ not ‘has’
Answer: Done accordingly (line 96).

- 1.79-81; needs correction
Answer: The sentence has been revised (line95-102). It is unclear whether passive smoking has an effect on BMD and bone turnover, and if such an effect could cause osteoporosis. We hypothesized that passive smoking may have a negative effect on
BMD in female rat by increasing bone turnover. To provide experimental proofs for this hypothesis and approach the possible mechanism of the association between smoking and osteoporosis, we examine the impact of smoking on bone turnover and BMD of the femur and lumbar vertebrae in a rat model of passive cigarette smoking.

- 1.96; The sentence ‘The impact of passive smoking was evaluated by measuring blood nicotine concentration’ needs correction. The blood concentration of nicotine reflects the intensity of the smoking habit.

**Answer:** The sentence has been revised as “The blood concentration of nicotine was measured to reflect the intensity of the smoking habit” (line 119-120).

1.123-133. Statistical analysis. Why Manne-Whithey and Kruskale-Wallis analysis was conducted ?. Why Anova with post-hock test has not been performed?

**Answer:** The statistical data analysis was conducted once again under the expert guidance of our statistics tutors. The section has been revised according to your professional comment (line 150-158).

In this study, results of all measurements are presented as mean ± S.D. for eight rats in each group. A one-way analysis of variance (ANOVA) was first performed to determine whether there were statistically significant (P<0.05) differences among the experimental groups. Further, the Duncan’s multiple range post-hoc test was used for comparisons between individual groups and to determine which means differed statistically significantly (P<0.05). Pearson’ correlation was applied to determine the correlation between markers of bone turnover and BMD of the femur and lumbar vertebrae. Correlations were considered statistically significant at p < 0.05. Data analysis was performed with SPSS version 13.0 (Chicago, IL, USA).

Because the populations from which the samples were normally or approximate normal distribution and the variances of the populations were equal, Manne-Whithey (for two groups) and Kruskale-Wallis analysis (for more than two groups) has not been employed to compare the means of groups. The redundant statistical methods have been deleted.
Results

- It seems necessary to separate subsections describing BMD, bone turnover and correlations between the parameters measured. Because the results on the BMD and markers of bone turnover are presented in figures there is no need to provide numerical values of particular parameters in the text. It is better to describe how were the changes (percentage of increase or decrease) compared to the control.

**Answer:** The section has been revised in accordance with your professional comments (line 177-203). The numerical values of particular parameters have been deleted. The percentage of increase or decrease has been described.

- 1.137-138; Why the authors conclude that there were no differences in food intake when they did not measure food consumption?

**Answer:** Done according to your professional comment (line167-168). Though individual food intake was not measured, total food supply in smoke-exposed group was similar to that in the control group.

- 1.143; ‘…BMD of the vertebrae and femur lumbar…’ !

**Answer:** The sentence has been revised (line178). ‘…BMD of the lumbar vertebrae and femur…’

- 1.153; ‘bone turnover’ should be used instead of ‘bone metabolism’ (BMD is also a measure of bone metabolism and it was presented in Fig 2).

**Answer:** Done accordingly (line 31, 95, 188). ‘bone turnover’ has been used instead of ‘bone metabolism’

- 1.154; ‘reveal’ whether ‘several’?

**Answer:** The sentence has been revised (line 189-190). Passive smoking had no effect on serum osteocalcin concentrations.
- 1.157; IU (not ‘iu’)

**Answer:** The sentence has been revised (line 191).

**Discussion**

- The discussion section needs extensive attention. Numerous repetitions of the results have been done. All repetitions of the results should be deleted and the authors should focus on explaining the possible causes of the observed changes in the skeleton metabolism due to passive smoking as well as on importance and implications of their results.

**Answer:** The discussion section has been revised extensively (line 205-320). All results repetitions have been deleted. The possible causes of the observed changes in the skeleton metabolism due to passive smoking as well as on the importance and implications of their results have been explained and discussed.

- 1.181; This sentence needs clarification.

**Answer:** The sentence has been revised as “Previous investigations also have shown that the timing of BMD decrease may be associated with the dose level of smoke exposure” (line 217-218).

- 1.208-210 need revision; What do you mean writing BaP/DMBA (BaP or DMBA?).

**Answer:** Based on our exact meaning, it has been classified as “BaP and DMBA” (line 294).

- L.224; the word ‘inhibited’ in more appropriate than ‘prevented’

**Answer:** Done accordingly (line 268).

- L.226; ‘negatively correlated’ (not ‘negative correlated’)

**Answer:** Done accordingly (line 275).

- L.235; ‘disease causing effects of tobacco’ – what did you mean?
Answer: The sentence has been revised as “disease caused by tobacco” (line 303).

- L.234; Why the Authors considered that the fact that females’ skeleton may be more susceptible than that of males? is a limitation of the study. It is well established that the bone susceptibility to damage is to a high extent determined by a gender, and if we want to conclude regarding the bone effect in men and women the study should involve both male and female rats. Thus, the Authors findings allow for the conclusion that passive smoking may increase the risk of bone damage in women.

Answer: The section has been revised according to your comments (line 297-307).

Previous studies found that passive smoking caused some eight percent of 18,000 female deaths and one percent of 49,000 male deaths from lung cancer, as well as nine percent of 34,000 female deaths and four percent of 42,000 male deaths from ischemic heart disease, which suggests that women are the main victims of passive smoking. We confirmed that passive smoking increase the risk of bone damage in female rat. Thus, our findings allow for the conclusion that passive smoking may increase the risk of bone damage in women.

Female may be more susceptible than male to the disease caused by tobacco. It is well established that the bone susceptibility to damage is to a high extent determined by a gender, and if regarding the bone effect in men and women the future study should involve both male and female rats. Furthermore, comparison of impact of passive smoking on the bones in female rat with in male rat was also necessary.

- 1.239-240. Why the Authors have recognized the fact that ‘the model used in the present study reflected the effects of passive cigarette smoking, but not those of active smoking’ is the next limitation of the study whereas the study was aimed to investigate the impact of passive smoking?

Answer: The section has been revised according to your comment (line 308-313).

Though the study was aimed to investigate the impact of passive smoking, examining the effects of active smoking on bone was also valuable. Difficulties existed in drawing a direct comparison to smoking in humans, since humans inhale tobacco
directly from cigarettes. Thus, the model used in the present study reflected the effects of passive cigarette smoking, but not those of active smoking. Humans are active smokers, and we cannot comment on the effects of active smoking using our model.

- Figures
- Fig. 1-3. Captions under bars presenting particular parameters in the groups exposed to smoke should indicate that these groups were exposed to smoke.
**Answer:** Done according to your comment (Fig. 1-3).

- All figures; Authors are asked to check and correct units of measured parameters.
**Answer:** Done according to your comment (Fig. 3-4).

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:** 'I declare that I have no competing interests'

**Response to Reviewer #3** (Cortino Sukotjo):

1. Please introduce the bone turnover marker used in the study in the introduction.

**Answer:** We have added a more detailed introduction in the revised manuscript (line 81-94). There has been recent interest in the use of bone-turnover markers to evaluate osteoporosis. Bone turnover markers can be categorized as bone formation markers, measured in the serum, or bone resorption markers, measured in the urine. Compston [19] showed that higher levels of bone resorption in studies with older smokers and lower levels of bone formation in studies with early postmenopausal women have been reported in smokers, though the mechanisms have not been clearly established. At present, the most sensitive markers for bone formation are serum
osteocalcin, Bone-specific alkaline phosphatase (B-ALP) and procollagen type I N-terminal propeptide (PINP). Bone resorption can be assessed by several biochemical markers, N-terminal and C-terminal crosslinking telopeptides of type-I collagen (NTX-I and CTX-I), deoxypyridinoline (DPD) and TRAP-5b. Serum TRAP-5b reflects the number and activity of osteoclasts on bone surface. Serum TRACP 5b levels are elevated in patients with bone diseases and decreased in subjects on antiresorptive treatment, suggesting that serum TRACP 5b is a specific and sensitive marker of bone resorption[20-22].

2. You hypothesize that passive smoking may has a negative effect on BMD in female rat by "increasing" bone turnover. What do you mean by increasing bone turnover? **Answer:** We mean "increasing bone turnover", the bone turnover is included as a part of bone metabolism (line 97).

3. Page 7, line 174: Significantly lower BMD was found at the femur and lumbar spine in 8-week smoke-exposed rats compared to controls. You also mentioned about this statement on page 8, line 182. It's redundant, so please modify your discussion. **Answer:** The previous sentence (line 174) has been deleted. The discussion has been revised (line 226-232). Ajiro et al. [27] found the BMD in the smoke-exposed rats was lower than that in controls at 8 weeks. The blood nicotine concentrations in 8-week smoke-exposed rats were 329.4 ng/ml, indicating that the average blood nicotine concentration in the smoke-exposed rats was seven- and eight-fold greater than the average for heavy smokers[27]. It seems that in the case of high exposure, the effect of smoking develops very fastly and the exposure duration needed for developing of a measurable effect of smoking depends on its intensity.

4. Some numbers on your result section was mentioned again in the discussion. It's redundant, so please modify your discussion. **Answer:** The sentence has been revised (line 221-228). The redundant and unnecessary results have been deleted. To compare our result with previous report,
part numbers was reserved in the discussion (e.g., the blood nicotine concentrations, line 202, 208).

5. Page 8, line 189: The mean blood nicotine concentration in our study for smokers who smoked 30 cigarettes daily was 40-42 ng/mL[20]. Were you referring to ref #20? Please clarify.

Answer: The sentence has been revised (line 219). To compare the mean blood nicotine concentration in our study with previous report, we cite the paper (ref #20) as the reference. 'in our study' has been deleted.

6. Please discuss why osteocalcin was not affected in the discussion.

Answer: We have added a more detailed discussion in the revised manuscript (line 248-261). Previous studies of the effects of smoking on osteocalcin have also produced conflicting results, possibly because the number of subjects studied has been relatively small [35-37]. Nielsen et al. [35] and Tamaki et al. [29] reported that there was no effect of smoking on osteocalcin in humans. Ortego-Centeno et al. [36] and Supervia et al. [37] found that there were no different values of serum osteocalcin between smokers and non-smokers in studies with young men. Gürlek et al. [38] indicated that osteocalcin levels in smoker patients were lower than the controls. Reduced serum osteocalcin levels have also been reported in early postmenopausal women who smoked [39]. The data on effect of smoke exposure on osteocalcin in rats are completely lacking. In this study, smoking did not seem to decrease osteocalcin level in rats, consistent with the results of previous report [29, 35-37]. It remains unclear why the osteocalcin level was not decreased during the whole smoke exposure, in spite of decreased activity of b-ALP in serum. Because of lower osteocalcin values, rats may occur at smaller percentage of changes in the osteocalcin levels than in humans.

7. Your Standard deviation for BMD is very small. Is it real?

Answer: In this study, the standard deviation for BMD is very small.
First, these resulted from the fact that in laboratory animals raised under identical conditions of setting and diet, a same batch and being a single strain, the BMD values are in narrower range of values and thus the SD values are lower compared to the much broader genetic and environmental experience base of the human population.

Secondly, our measurements was repeated for three times. The coefficients of variations (CV) for repeated measurements on the same bone was <1.0% for BMD.

Thirdly, the results of the BMD in femur are expressed as the mean value of both femurs. Similarly to the femur, the results of the BMD in lumbar spine are expressed as the mean value of L4-L6.

The final data, which were applied in all analysis, consisted therefore of a mean of three independent measurements and different bone. Thus, the BMD values are in narrower range of values and the SD values are lower.

The original data of lumbar spine BMD and statistical results are listed below.

<table>
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<th>Bone mineral density of the lumbar vertebrae in the controls, 2-month, 3-month and 4-month smoke-exposed rats.</th>
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LSBMD

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ANOVA

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<td>Within Groups</td>
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<td>.000</td>
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<tr>
<td>Total</td>
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Post Hoc Tests

Homogeneous Subsets

LSBMD

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<td>Sig. 1.000</td>
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Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 8.000.

Means Plots
8. Probably it's a little premature to conclude that the bone turnover rate was increased in the study when your data was only supported by 2 markers (ALP and Trap). Please clarify.

**Answer:** This is a very professional point.

We do agree that the more markers are, the better it will be. Bone turnover markers can be categorized as bone formation markers or bone resorption markers. At present, the most sensitive markers for bone formation are serum osteocalcin, Bone-specific alkaline phosphatase (B-ALP) and procollagen type I N-terminal propeptide (PINP). Bone resorption can be assessed by several biochemical markers, N-terminal and C-terminal crosslinking telopeptides of type-I collagen (NTX-I and CTX-I), deoxypyridinoline (DPD) and TRAP-5b. Serum TRAP-5b reflects the number and activity of osteoclasts on bone surface and is a specific and sensitive marker of bone resorption.

In many reports similar to the present study, many authors chose 2-3 markers to describe bone turnover rate. [eg. (1)Rector RS, Loethen J, Ruebel M, Thomas TR, Hinton PS:Serum markers of bone turnover are increased by modest weight loss with or without weight-bearing exercise in overweight premenopausal women.Appl Physiol Nutr Metab 2009,34(5):933-41.(2)Waltman NL, Twiss JJ, Ott CD, Gross GJ, Lindsey AM, Moore TE, Berg K, Kupzyk K:The effect of weight training on bone mineral density and bone turnover in postmenopausal breast cancer survivors with]

In this study, bone turnover was assessed by determining serum osteocalcin and b-ALP for bone formation and TRACP 5b levels for bone resorption. We demonstrated that smoke exposure increased the level of TRAP 5b, a systemic bone resorption marker, and decreased activity of b-ALP, a bone formation marker, suggesting that smoking increased bone resorption and inhibited bone formation. The rate of bone resorption exceeding that of bone formation seems to increase rate of bone turnover in rats which leads to bone loss. Because the purpose and emphasis of this observational study were to investigate the effect of passive smoking on BMD and bone turnover and the relationship between BMD and bone turnover in female rat, but not to identify which markers causing the change of bone status, we didn’t examine more biomarkers of bone turnover. However, we are also very interested this important point, and that is exactly what we are planning to do in the near future to further investigate the detailed biomarkers of bone turnover and molecular mechanisms of smoke exposure on bone in rats. As a result, we are pleased to add more details in the discussion (line 262-271).

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:** I declare that I have no competing interests