Author's response to reviews

Title: Endothelin-1 plasma levels in patients with osteoporosis

Authors:

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Author’s response to reviews:

Peter Newmark,
The Editor-in-chief, BMC Musculoskeletal Disorders,
BioMed Central Ltd,
Middlesex House,
34-42 Cleveland Street,
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May, 07, 2005
Kindly Attention of Peter Newmark,
The Editor-in-chief, BMC Musculoskeletal Disorders,

We revised our manuscript entitled "Endothelin-1 plasma levels of patients with osteoporosis: a research article" according to reviewers' suggestions. The changes that we made and the answers to the reviewers' questions are explained in detail in the following part of this letter. The revised manuscript was read and approved by all authors.

Sincerely yours,

Hasan Hilmi Muratli
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Reviewer 1 (Miryoung L Lee)

Major compulsory revisions

Comment: This is an interesting manuscript that investigates whether there is any difference in endothelin-1(ET-1) levels among normal, low bone density (osteopenic), and osteoporotic study participants. In recent years, the ET-1 has been recognized as a physiologic regulator of bone remodeling as authors acknowledged in the text. However, there are a number of issues that the authors need to address. One of major concerns is a small number of study participants, because osteoporosis is complex disease, known to be affected by many factors, such as age, adiposity, menopause, and hormone replacement therapy.

Response: We agree with the reviewer therefore we add the same statement as "Our study has certain limitations. Firstly a number of study participants was relatively small because osteoporosis is a complex disease, known to be affected by many factors such as age, gender, adiposity, menopause etc." in the discussion part of the new text as an our study's important limitation.
Comment: The title should include the nature of current study design.
Response: According to reviewer's suggestion we changed the title as "Comparison of plasma endothelin levels between osteoporotic, osteopenic and normal subjects: a research article," in the new text.

Comment: Since authors only measured Endothelin-1 not other isomers (i.e., endothelin-2, endothelin-3), authors should state the endothelin-1 (ET-1), not endothelins, throughout the manuscript where the authors explained the study results. Also, once the abbreviation of endothelin-1 was introduced as ET-1, authors need to use it consistently.
Response: As we explained in the new text with detail in the method section although our kits name is Endothelin-1 EIA, this kit has 100% cross reaction for all ET subtypes. Therefore we prefer to use endothelins (ET) through the revised manuscript regarding our method and findings. We checked to use same words and abbreviations consistently in the new text.

Comment: Conclusion statement: The final sentence should be more conclusive, and reflect the characteristics of study design. Because authors performed the investigation using cross-sectional study design, the authors should not conclude or generalize that ET-1 does not have a role in the physiopathology of osteoporosis. Their negative findings may be results of small samples sizes, or measurement errors in estimating plasma ET-1 levels. It should read "We did not find any significant differences in plasma ET-1 levels among three groups of study participants."
Response: According to reviewer's suggestion we changed the conclusion statement as "No significant differences in plasma ET levels among three groups of study participants could be detected in this study." in the abstract and similar changes were performed in conclusion part of the main text.

Comment: What was the rational to study between selecting osteoporosis disease status in this manuscript, and not bone mineral density in the hip or lumbar spine in participants?
Response: As we explained in the last paragraph of the data analysis section and "other correlation studies part" of the results section we also performed correlation analysis with the T scores of bone mineral density measurement and ET levels. T score is defined as the difference between a measured bone density and the expected young normal value divided by the population standard deviation [1]. In our study "consistent data base regarding young normal value and the population standard deviation were used in order to calculate T score." (This statement was added the last paragraph of the group of method section of the method section into the revised manuscript). Therefore we believe that using bone mineral density values instead of T scores in our study would not cause any different results. Using of T scores for our analysis was just a preference. Because presently, the T score is established as the primary output obtained from a bone densitometry system and is most often used for diagnosis of osteoporosis and for making treatment decision [1]. In the background section and in the figures we only mentioned categorical evaluation although we also gave results of other evaluation (correlation study between the T scores and ET level) in the text. Because we mainly aimed to investigate if there was any difference between the plasma ET levels of osteoporotic patients and normals in the recent literature and our current practice, WHO criteria have been accepted largely to diagnosis osteoporosis [1].

Methods
Comment: Please note how many patients (participants) were invited to the present study, and how many were excluded.
Response: According to reviewer's suggestion we added information about how many participants were invited to the present study and how many were excluded.

Comment: Did authors have chances to measure any of biochemical markers related to osteoporosis, which are mentioned in background and discussion (e.g., osteopontin, osteocalcin, or calcitropic hormones, 1,25
Response: We did not measure suggested biochemical markers, so we did not have a chance to look for any correlation.

Comment: Since authors mentioned that they had biochemical profiles or laboratory results to distinguish that none of the participants had any chronic diseases, it would be interesting to see whether there are any relationships between ET-1 and other biochemical profiles.
Response: As we mentioned in the group of patient part of the methods section we excluded patients with abnormally laboratory results. All our patients' routine hemogram parameters and routine biochemical tests results were in the normal limits. Thus we did not look for any correlation.

Comment: What was the coefficient of variation (CV) of the assay for ET-1?
Response: We gave the values of coefficient of variation of the assay for ET in the methods section of the new text as "The intra- and inter-assay coefficients of variation of the method were 5 and 6%, respectively."

Data analysis
Comment: The authors need to explain the statistical analyses more explicitly.
Response: According to reviewer's suggestion we tried to explain statistical analyses more explicitly.

Comment: S Were the endothelin-1 levels normally distributed to perform linear regression analysis (Analysis of Variance, T-tests)?
Response: We added the statement of "Prior to the analysis, all the data were examined for accuracy of data entry and fit between their distributions and the assumptions of univariate analysis. To improve pairwise linearity and to reduce the extreme skewness and kurtosis, the z score for all variables was computed. It was found that all dependent variables are normally distributed." in the first paragraph of the data analysis section.

Comment: S What variables/measures they used in the analysis? The authors need to clarify the dependent variable (e.g., adjusted ET-1 levels), independent variables, or covariates in the model.
Response: We described all dependent, independent variables and covariates in the data analysis section of the new text.

Comment: S Was there any analysis regarding power?
Response: Unfortunately, we did not performed any analysis regarding power.

Comment: S Which statistical program/software was used for analysis?
Response: We added the statement of "Data analyses were done by SPSS for Windows version 11.5." in the first sentence of the data analysis section.

Comment: S Replace length with weight on page 7 line 7 aEoeheight, length, T scores aEaEy
Response: We performed suggested changes in the new text.

Results
Comment: The authors need to specify whether plasma ET-1 levels are crude or adjusted values independent of sex (as explained in Method section), and its standard error (Figure 1 and Table 1). Statistical significance levels, as mentioned in the text, need to be added in the Table 1 (footnote).
Response: Figure 1 was changed according to reviewer's suggestion and in the new Figure 1 ET level of each group was presented with the graphic including both mean and SD values. Statistical significance levels are also presented in the new figure 1. Figure 1 legend was changed as "Plasma ET levels (mean+/−S.D.) before the adjustments for age, weight and height of each group are presented diagrammatically." So we clarify that given values in the Figure 1 are not adjusted values. The word of "unadjusted" was added in the "Endothelin fasting plasma levels" and "Levels according to gender" sections of the results sections in the revised manuscript.
Statistical significance levels, as mentioned in the text were added in the Table 1 as a footnote.

Discussion
Comment: The authors discussed that the estrogens may explain the sex difference, especially in men, among three groups. In addition, the authors need to discuss the influence of menopausal status among female participants since the hormone profiles and bone remodeling differ in pre- and post-menopausal women.
Response: After we received reviewer comments we reviewed the literature again about postmenopausal hormone profiles of the woman and man in the same ages period in order to better discuss our findings. As we mentioned third paragraph of the discussion part of the revised text it is generally accepted that
significant decrease in the circulating estrogen levels are observed in woman in the postmenopausal period. Therefore considering our findings in which we obtained significantly higher plasma ET level in the osteoporotic men in comparison to normal man and osteoporotic woman we believed that it is better to discuss our results through the hormonal profiles changes of men in the older age. So we changed our discussion and added the statement of "Studies about the men osteoporosis demonstrated us that although total estradiol levels do not change substantially over life in men, bioavailable estradiol levels decrease to 50% of the levels in young men in the older ages. It is thought that this decline in bioavailable estradiol levels may be the major cause of bone loss in elderly osteoporotic men [28, 29]. In previous laboratory studies it was shown that estrogens down regulated ET-1 both through the secretion from the vascular endothelial cells and m-RNA expression levels [30, 31]. Upon these observations we believed that possible reason for higher plasma ET levels of osteoporotic men then the normal men in our study may be because of the lower bioavailable estrogen concentration of the males in these ages and as a result possible decrease of estrogens effect in down regulation in ET and consequently increase of ET amount and effects. In fact bone loss is more accelerated in women after menopause as a result of a decline in circulating estrogens levels then the men in the same ages period [32]. However considering presence of no difference in the plasma ET levels between the osteoporotics, osteopenics and normals in the women population of our study participants it is not possible to say same mechanism is true for women regarding the ET and estrogens interaction."

Comment: On second paragraph on page 11, the authors mentioned of significant correlation between T-scores and ET-1 levels. Can authors give any information regarding the correlation between bone mineral density and ET-1 levels in all participants? Did authors try to analyze bone mineral density as a continuous variable, not as a categorical variable (i.e., osteoporosis status by WHO criteria) in association with ET-1 levels?
Response: As we mentioned previously in this letter (2nd response for background part) in this study we analyzed bone mineral density both as a continuous and a categorical variable through the calculated T scores.

Comment: The authors provided detailed information regarding the relationships between ET-1 and hormones and cytokines from in vivo and in vitro studies. However, the discussion and conclusions was not adequately supported by the provided data since the authors need to provide more information to conclude. In addition, some of the sentences are not clear in the context and need to be re-written. For example, on page 12, line 4, "Matching of the parathyroid hormone at first leaded to decreasing of ET-A and ET-B mRNA levels. Overall, the authors need to organize and shorten the discussion to be more relevant to study hypothesis and results.
Response: We tried to reorganize and shorten the discussion. However because other reviewers wanted and asked another questions we had to add new statements in different topics. We shortened and deleted some sentences especially about the methods of referred invitro or in vivo studies as we already gave information regarding ET's important effects in the bone tissue in the introduction. According to reviewer suggestion we only mentioned ET's effects which may cause osteoporosis with shortened and conclusive statements. We think this time it is better.

Comment: Please state any study limitations, such as selection bias of study participants, and generalization of study findings in other populations.
Response: According to reviewer's suggestion we mentioned our study's important limitations in the discussion part of the new text with the statements of "Our study has certain limitations. Firstly a number of study participants were relatively small because osteoporosis is a complex disease, known to be affected by many factors such as age, gender, adiposity, menopause etc. We believe that subsequent studies should be performed with larger number of participants especially for men. In addition although we only included patients with no known disease and with normal laboratory findings in the routine evaluation tests and normal blood pressure, it was not possible to know with this limited evaluations if these patients had any disease which are not possible to diagnose with our screening tests for this study and which may also cause changes of plasma ET level as mentioned and referenced below. Although it is known by many studies that [27, 33, 34] increased level of ET concentrations can be detected in the plasma as a result of overproduction of ET released from pathologic tissues and/or due to hypervascularization associated with the lesion in osseous or non-osseous pathologies, it can be thought that systemic circulation may thus not entirely reflect local changes in the bones. So in the substantial studies ET concentrations at the site of osteoporotic bone tissue should also be evaluated by biopsies."

References
Comment: Reference No. 26 was not referred in manuscript.
Response: We reviewed the references. Because we added new statements and removed some
statements 5 new references were added (reference number: 28, 29, 32, 33, 34) and 5 references were removed (reference number: 26, 29, 30, 31, 32). Necessary changes were made in the text regarding this changes.

List of abbreviations
Comment: The authors need to define the following abbreviations in the text where these are first used, or a list of abbreviations needs to be provided. IL-6: interleukin 6, NO: nitric oxide, PGE-1: prostaglandin E1. Response: According to reviewer's suggestion we defined the abbreviations in the text where these are first used.

Quality of written English
Comment: Need some language corrections and spell check before being published. Response: We tried to correct language mistakes. We checked to spelling errors and corrected them. We think that this time it is better.

Comment: The authors need to check standard scientific notations throughout the manuscript. Examples: Consistent notation for ET-1 levels: pg/ml (page 1), pg/ml (page 6), pg/ml (page 8), pg/ml (Figure 1) Response: We used consistent notation for ET levels as pg/ml in the revised manuscript and figure 1.

Comment: Spell check: 1,25 dihidroksivitamin D3 (page 3, 4) A 1,25 dihydroxyvitamin D3, Prostasicline (page 11) A prostacyclin, Endoperoksid G/H synthaz (page 12) A endoperoxide G/H synthase Response: We corrected spelling errors according to reviewer's suggestion in the revised manuscript.

Statistical review
Comment: Reviewer's decision: Yes. Reason: The authors need to be more explicit in explaining and writing the statistical methods and models. For example, the rationale of Student's t-test was not clear since the comparison between sexes can be made in the analysis of variance. Response: According to reviewer's suggestion we tried to explain statistical analyses more explicitly as we mentioned in the data analysis section of our responses in this letter. In addition according to reviewer's suggestion we reperformed analysis with one-way ANOVA to confirm differences of endothelin level between the males and females for each group. And the previous statement was changes as "One-way ANOVA test was used to confirm the difference of endothelin level between the males and females for each group separately after controlling for covariates." in the data analysis section of the new text. Because we did not detect any changes in the results with ANOVA there was no need for a correction in the results section.

Reviewer 2 (Peter Vestergaard)
Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached) bone mineral. They find no overall difference in endothelin levels in patients with osteoporosis, osteopenia, and normal bone mineral.
I have a number of reservations for this study:
Comment: More detail should be provided for the inclusion procedure: How many patients were screened, were any patients excluded, and how many, and for what reasons? Response: According to reviewer's suggestion we explained our inclusion and exclusion criteria with detail and we stated how many patients were screened, invited and excluded from the study with reasons in the groups of patients part of the methods section of the new text.

Comment: The potential aspects of selection bias in relation to what kind of patients was referred, and the patients excluded need to be discussed. Response: We described our exclusion criteria in the groups of patients section of method section. Patients with systemic diseases (diabetes, hypertension, renal disease, or clinical manifestation of atherosclerosis or known another diseases) and patients with abnormal laboratory results (regarding routine hemogram parameters and routine biochemical test) were excluded from the study. There are many reports which
confirm that ETs have significant roles in the physiopathology of certain diseases about different systems including cardiovascular, gastrointestinal, urogenital etc. (some of them are referred through the revised manuscript. Ref. number: 27, 33, 34 ) and plasma ET levels were found to be increased in these entities. Therefore by asking their known disease and limited biochemical and hemogram parameters we tried to exclude these cases.

However in order not to more lengthen discussion part (according to other reviewers' suggestion) we just addressed our limitation regarding this screenings with the statement of "In addition although we only included patients with no known disease and with normal laboratory findings in the routine evaluation tests and normal blood pressure, it was not possible to know with this limited evaluations if these patients had any disease which are not possible to diagnose with our screening tests for this study and which may also cause changes of plasma ET level as mentioned and referenced below." (following these statements we mentioned and referenced some conditions some of which may be impossible to exclude with our exclusion criteria).

Comment: More detail should be provided for the assay used: coefficients of variation should be provided.
Response: We gave the values of coefficient of variation of the assay for ET in the methods section of the new text as "The intra- and inter-assay coefficients of variation of the method were 5 and 6%, respectively."

Comment: Regarding the point raised above, a potentially severe problem arises when one compares the results of the study with the description of the assay used by the manufacturer. On their website (http://www.caymanchem.com/pdfs/583151.pdf), the manufacturers state that the assay cannot measure endothelin within the normal range seen in humans (<1 pg/ml), and that measurements in the range <50 pg/ml needs special care with respect to purification. The manufacturers state that only elevated levels of endothelin can be reliably measured by the assay. Yes the authors present values from supposedly normal subjects in the range of 100 pg/ml. This needs clarification and discussion.
Response: According to reviewer's comment we tried to clarify and discuss this point with the statement of "According to manufacturer's instructions normal levels of ET-1 in human plasma are below the detection limit of the kit which we used; therefore purification and concentration of the sample is necessary for accurate measurement of ET-1 levels. Considering 100% cross reactions of this kit for all ET subtypes including ET-1, ET-2 and ET-3 and performing our analysis without prior purification process it should be addressed that our measurements reflects total endothelin measurements, not ET-1 alone. We did not perform purification because manufacturer states that samples can be assayed with no prior purification in general and they suggest performing purification process only for samples containing low concentration of endothelin (0-50 pg/ml). They also state that samples must be 50 pg/ml in order to be assayed accurately with this kit and all average ET levels of our groups was already in this range. In addition all samples obtained from both normals and pathologics regarding bone densitometric evaluation were evaluated with the same method, without prior purification, and all obtained measurements were within the detection range of this kit (0-250 pg/ml). So we believe that in the evaluation of our results and comparison of these findings with other studies these points should be taken into consideration." in the discussion part of the new text.

Comment: The authors measure serum levels of endothelin. Normally endothelins work locally, in this case in the skeleton. The systemic circulation may thus not entirely or reliably reflect local changes in the bones. The authors need to discuss this limitation, and whether systemic measurements do in fact reflect local changes in the bones. E.g. is the half-life of the endothelins so short, that they may not enter systemic circulation.
Response: According to reviewer suggestion we discuss this limitation with the statement of "Although it is known by many studies that [27, 33, 34] increased level of ET concentrations can be detected in the plasma as a result of overproduction of ET released from pathologic tissues and/or due to hypervascularization associated with the lesion in osseous or non-osseous pathologies, it can be thought that systemic circulation may thus not entirely reflect local changes in the bones. So in the substantial studies ET concentrations at the site of osteoporotic bone tissue should also be evaluated by biopsies." in the discussion part of the new text.

Comment: Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
Response: We tried to make this necessary revisions. We reorganised the figure 1. And we changed figure 1 legend as "Plasma ET levels (mean+/S.D.) before the adjustments for age, weight and height of each group are presented diagrammatically."

Comment: Needs some language corrections before being published
Response: We tried to correct language mistakes. We checked to spelling errors and corrected them. We think that this time it is better.
Reviewer 3 (R Swaminathan)
Comment: This is a simple study in which the authors have measured plasma endothelin 1 levels in normals and osteoporotic subjects. Authors fail to acknowledge that circulating concentrations may have no relevance to tissue levels of endothelin-1. Furthermore the authors have only taken into account height, weight and BMI as possible factors influencing endothelin 1. There may be other factors which may be important.
Response: We agree with the reviewer and his/her concerns therefore we added the statements of "Although it is known by many studies that [27, 33, 34] increased level of ET concentrations can be detected in the plasma as a result of overproduction of ET released from pathologic tissues and/or due to hypervascularization associated with the lesion in osseous or non-osseous pathologies, it can be thought that systemic circulation may thus not entirely reflect local changes in the bones. So in the substantial studies ET concentrations at the site of osteoporotic bone tissue should also be evaluated by biopsies." and "Our study has certain limitations. Firstly a number of study participants were relatively small because osteoporosis is a complex disease, known to be affected by many factors such as age, gender, adiposity, menopause etc. We believe that subsequent studies should be performed with larger number of participants especially for men. In addition although we only included patients with no known disease and with normal laboratory findings in the routine evaluation tests and normal blood pressure, it was not possible to know with this limited evaluations if these patients had any disease which are not possible to diagnose with our screening tests for this study and which may also cause changes of plasma ET level as mentioned and referenced below." in the discussion part of the revised manuscript in order to address to these limitations.

Comment: Even though the subjects are not hypertensive BP should be factored into the analysis.
Response: Average systemic blood pressure of our patient was 126+/-8 (Mean+/-S.D.) mmHg and 75+/-9 mmHg for systolic and diastolic levels. This infromation was mentioned in the revised manuscript.

Comment: As the number of males in this study is far too small I suggest only include females in this report or include more male subjects.
Response: We prefered to not exclude male subjects from this study. Because one of the other reviewers ask a question about the possible reason of detected differences of ET levels regarding gender. In addition we clearly stated that "Although plasma ET levels of osteoporotic men were found significantly higher than normal men we believed that it could be speculative to make conclusion with these findings because there were only 4 osteoporotic men in the series." and we did not make any conclusion with this finding. We also believe that these findings may encourage other investigator to make substantial studies in order to clarify these findings.

Quality of written English:
Comment: Needs some language corrections before being published
Response: We tried to correct language mistakes. We checked to spelling errors and corrected them. We think that this time it is better.