Author's response to reviews

Title: Reference Intervals for Serum Osteocalcin Concentrations in Adult Men and Women from the Study of Health in Pomerania

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Author's response to reviews: see over
Dear Ms Nolasco,

We would like to thank you and the reviewers of *BMC Endocrine Disorders* for their time to evaluate our research and providing constructive comments. We have modified the manuscript according to the reviewers’ suggestions and are confident that our manuscript has now substantially improved. All authors involved helped revising the manuscript and consented to the changes introduced. If there are further comments and suggestions, we are happy to address them. Enclosed you will find the revised version of the manuscript as well as our responses to the reviewers’ suggestions. Changes to the manuscript are marked in red.

Yours sincerely,

Anke Hannemann
Below follows our response to the **REVIEWER COMMENTS** and related revisions that have been made. All comments have been carefully worked through and changes have been applied and/or discussed below. Reviewer comments have been repeated in 10pt Arial font in boxes, our own comments follow in 12pt Times.

**REVIEWER #1**

**Major Compulsory Revisions:**

1- The European guidance for the diagnosis and management of osteoporosis in postmenopausal women was updated in 2012 and markers of bone turnover are currently not sufficiently validated for fracture risk prediction. Some statements in abstract and introduction need to be revised according to recent guidelines that do not recommend OC anymore to assess bone remodeling (“Serum OC concentrations are used to assess fracture risk and monitor treatment of osteoporosis and other disorders of bone metabolism”; “Together with procollagen I N-terminal extension peptide and type I collagen and C-telopeptide breakdown products, OC was recommended as one of the most informative markers to reflect bone turnover in the 2008 European guidance for the diagnosis and management of osteoporosis in postmenopausal women [6]”). The authors are encouraged to update the introduction of their manuscript and state their work according to this current context. The following references are of particular interest for that:

- Kanis, Osteoporos Int (2013) 24:23–57
- Vasikaran, Osteoporos Int 22:391–420
- Lee, Ann Lab Med 2012;32:105-112

The main issue highlighted in these position papers is the need of international reference standards. This latest point should serve as a good background for the authors study.

We thank the reviewer for the careful reading and the suggestion of additional relevant and most recent literature. The article by Kanis and colleagues [1] was published online only two weeks before we submitted our manuscript to BMC Endocrine Disorders. We apologize for having missed this publication prior to submission of our manuscript. As requested by the reviewer, we updated our manuscript thereby incorporating the suggested literature (Abstract lines 3-4, Introduction page 4, lines 5-16)

Currently, several different laboratory methods to determine serum OC concentrations are in use. These methods differ in important aspects including the recognition of OC fragments (intact and/or mid-fragment) [2]. In all three articles mentioned by the reviewer [1, 3, 4] the authors conclude, that a standardisation of laboratory methods to determine BTM concentrations is required in order to make study results more directly comparable. We fully agree on this point. In fact, in our manuscript we propose to establish method-specific reference intervals for the serum OC concentration. Our reference intervals fill an important gap, as until now, there are no published reference intervals for serum OC concentrations measured with the IDS-iSYS N-Mid Osteocalcin assay. We slightly modified our introduction in order to emphasize this important aspect of our study (Introduction page 5, lines 8-11).
The Study of Health in Pomerania is described as a population-based cohort. 2150 on 3300 patients were used as the reference population after excluding subjects with exclusion criteria. How did the authors choose their exclusion criteria, in other words why did they not exclude patients with diabetes (whereas associations between osteocalcin and metabolic syndrome/diabetes were previously reported), or women taking sex hormones for contraception or hormone replacement (possible effect on bone metabolism, as vit D deficiency and medications…) while they excluded patients with renal disease, hyperparathyroidism/hyperthyroidism, cancer, osteoporosis, liver diseases with serum 25-hydroxy vitamin D deficiency or medications affecting bone metabolism? Can the authors provide some additional comments on their reference population (healthy? representative of a community dwelling population…?)

As the reviewer rightly points out, we did not exclude subjects with diabetes mellitus or women taking sex hormones from our primary analyses. We would like to point out, however, that we performed additional analyses in which we excluded these subgroups of our initial sample. We further performed sensitivity analyses in which we excluded subjects with high or low BMI as this parameter has also been reported to potentially affect serum osteocalcin concentrations (Results p. 10, lines 4-7). Our decision not to exclude subjects with diabetes mellitus, low or high BMI, or women taking sex hormones in our primary analyses was based mainly on two reasons. First, in clinical practice serum OC concentrations have to be evaluated in women using estrogens, in overweight individuals and in patients with diabetes mellitus. As these are very common conditions in the general population we aimed to provide clinicians with reference intervals that account for these specific conditions. In fact, our reference population comprises a large proportion of individuals with diabetes mellitus (8.5%) or low or high BMI (28.3%) as well as of premenopausal women taking oral contraceptives (23.1%) or postmenopausal women taking hormone therapy (9.6%). None of these individuals had any other condition affecting bone metabolism. Second, performing multiple analyses with and without the alleged subgroups allowed us to quantify the impact of diabetes mellitus, low or high BMI, and estrogen intake on the reference intervals. Previous studies reported an influence of these conditions on mean serum OC concentrations, e.g. postmenopausal women with type 2 diabetes had lower OC concentrations than postmenopausal women without type 2 diabetes [5]. Our data show that the exclusion of subjects with diabetes mellitus or low or high BMI, or of women using estrogens had only small effects on the lower and moderate effects on the upper serum OC reference limits (Results, p. 10 lines 22 through p. 6 line 4).

The following are some additional comments on our reference population as ask for by the reviewer.

Our reference population was selected from the participants of the first follow-up of the Study of Health in Pomerania (SHIP). SHIP is a population-based cohort study in northeast Germany. For the baseline study (SHIP-0) a representative sample of the adult population between 20-79 years (N=158864) living in the region of West Pomerania was drawn. The
study region comprised the three cities Greifswald, Stralsund and Anklam and the surrounding 29 communities. The sample was restricted to German citizens but included institutionalized individuals. From the sample of 7008 subjects, 4308 consented to take part in the baseline examinations and 3300 were re-examined in the first five-year follow-up examination. Due to drop-out between baseline and follow-up examination, the SHIP-1 study population is not truly representative for the study region. As requested by the reviewer we added a paragraph regarding the study population in the methods section (Methods p. 5 lines 20-25; p. 6 line 2-3).

3- About 10% of the patients were excluded because of vitamin D deficiency (25OHD < 10ng/ml). Vitamin D insufficiency was described as interfering with bone remodeling until levels of 25OHvitD of 20-30ng/ml. May the authors provide data regarding osteocalcin levels according to 25OHvitD levels.

There was no correlation between serum 25OHD concentrations and serum OC concentrations in our reference population (Spearman correlation coefficient: 0.01; p-value 0.64). In case the reviewer or the editor request to include this information in the manuscript, we will be happy to perform all necessary editing.

4- Any data regarding osteocalcin levels and creatinine/GFR (knowing that all patients had GFR>30ml/min)?

There was a very weak correlation between the eGFR and the serum OC concentrations in our reference population (Spearman correlation coefficient: -0.08; p-value <0.01). This relation is illustrated in Figure I below. We assume that the weak correlation between the eGFR and the serum OC concentration had no influence on our calculations. In case the reviewer or the editor request to include this information in the manuscript, we will be happy to perform all necessary editing.

**Figure I.** Scatterplot illustrating the relation between the estimated glomerular filtration rate (eGFR) and the serum osteocalcin (OC) concentration in the reference population (n=2150). eGFR values >210 mL/min were set to 210 mL/min (n=13), serum OC concentrations <60 ng/mL were set to 60 ng/mL (n=4)
Minor Essential Revisions:

1- The authors found that "In premenopausal women the upper and lower reference limits for serum OC concentrations decreased markedly between 25-34 years of age and remained stable after an age of 34 years » (page 10 and fig 1). Any hypothetical comment explaining the marked decrease of OC in this age class?

The reviewer raises an interesting question addressing the decrease in serum OC concentrations in 25-34 year-old premenopausal women. Glover and colleagues [6] reported similar observations in 153 premenopausal women aged 30-45 years. They [6] reported that premenopausal women younger than 35 years of age had elevated serum OC concentrations, compared to women between 35-45 years of age. The authors [6] hypothesize, that higher serum OC concentrations in young women indicate that peak bone mass is not yet reached. We share this idea, which is supported by a recent study [7] demonstrating that in white premenopausal women bone mineral density in the spine peaks at the age of about 30 years. By the age of 35 skeletal maturity is reached in all women and bone turnover reaches a phase of stability.

We added a respective explanation in the discussion section of the manuscript (Discussion p. 11, lines 15-19).

2- Table 4: Could the authors precise the number of patients in each columns of the table (how many patients after exclusion of diabetes, hormone intake…?)

As requested by the reviewer we added the number of men, pre- and postmenopausal women to the respective columns in Table 4 of the manuscript.

3- Discussion: OC was supplanted by P1NP in recent guidelines for bone formation assessment, in part because of too many different assays for OC. May the authors provide comments on how they integrate their findings in the current place accorded to OC in clinical practice or for studies, in particular versus P1NP. Do these reference intervals fill a gap to boost back OC in bone remodelling assessment, or highlight that there are too many variability factors interfering with OC levels to provide reference intervals for clinical practice in an individual patient?

P1NP has been recommended as reference parameter for bone formation [1]. We understand that P1NP is given preference over OC mainly because fewer laboratory methods are in use to measure this parameter. Yet, apart from the laboratory methods, we do not think that OC is influenced by substantially more factors than P1NP. Several studies demonstrated that P1NP and OC are both influenced by several highly prevalent factors, such as intake of oral contraceptives [8], hormone replacement therapy [9], or diabetes mellitus [5]. Thus, when using method-specific reference intervals, serum OC concentrations may be as useful as serum P1NP concentrations for the assessment of bone turnover.
Moreover, we think that OC will be of future clinical importance due to its relations with energy metabolism and male fertility [10, 11]. Recent studies postulated a feedback-loop between the skeleton and energy metabolism, and suggested that OC may be a biomarker for insulin resistance in humans [10]. Normative data on serum OC concentrations from a healthy adult population may thus be useful, for further observational studies investigating the relation between OC and energy metabolism. This aspect was included in the discussion section in a new paragraph (Discussion, p. 14, lines 3-8).

**REVIEWER #2**

**Minor Comments:**

In table 3 the estimate for intercept should be removed.

The quadratic transformation for age does not make sense since authors have decided to use a median/quartile regression model.

We agree with the reviewer, that the intercept is not necessary to illustrate the association between age and serum OC concentrations. On the other hand, the intercept is indispensable to calculate the 2.5\textsuperscript{th} and the 97.5\textsuperscript{th} reference limits. While we provide reference intervals for 5-year age groups (Table 4), we also wanted the reader to be able to calculate single age reference intervals from our data, if he/she wants to do so. The intervals can be calculated from the parameter estimates for the intercept and age-square given in Table 3. We therefore prefer not to delete these two parameters from the table. In case the reviewer or the editor prefer us to move the Table 3 to the supplemental material, we will be happy to perform the necessary editing.

In addition to providing appropriate data for the calculation of single age reference intervals, there is yet another reason for retaining the parameter estimates for age square in the manuscript. As the reviewer rightly points out, the median (quantile) regression model is not sensitive to the distribution of the error term [12, 13]. Thus, the dependent variable osteocalcin was not required to show normal distribution. We tested in the quantile regression model whether the median serum OC concentration was age-dependent. Likelihood ratio tests indicated that age and age-square were significantly associated with serum OC concentrations in men and premenopausal women. Hence, we fitted the 2.5\textsuperscript{th} and 97.5\textsuperscript{th} quantiles in the regression models with age and age-square and provide the respective parameter estimates in Table 3.
References:


