**Title**

The effect of raloxifene on bone marrow adipose tissue and bone turnover in postmenopausal women with osteoporosis

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**Conflicts of interest**

**Abstract**

In patients with postmenopausal osteoporosis low bone volume is associated with high bone marrow adipose tissue (MAT). Moreover, high MAT is associated with increased fracture risk. This suggests an interaction between MAT and bone turnover, however literature remains equivocal. Estrogen treatment decreases MAT, however, the effect of raloxifene, a selective estrogen receptor modulator (SERM) registered for treatment of postmenopausal osteoporosis, on MAT is not known. The aim of this study is 1] to determine the effect of raloxifene on MAT and 2] to determine the relationship between MAT and bone turnover in patients with osteoporosis.

Bone biopsies from the MORE trial were used. The MORE trial investigated the effects of raloxifene 60 or 120 mg per day versus placebo on bone metabolism and fracture incidence in patients with postmenopausal osteoporosis. We quantified MAT in iliac crest biopsies obtained at baseline and after 2 years of treatment (n=53; age 68.2±6.2 years).

Raloxifene did not affect the change in marrow fat fraction after 2 years compared to baseline (placebo: 2.7±11.5%, raloxifene 60 mg: 9.1±13.1%, raloxifene 120 mg: -1.8±10.3%), but raloxifene dose-dependently attenuated or prevented the increase in adipocyte size (placebo: 2.2±4.7 µm, raloxifene 60 mg: 0.4±3.1 µm, raloxifene 120 mg: -2.0±3.9 µm, p<0.01 placebo vs raloxifene 120 mg). Adipocyte number decreased during placebo treatment (-7.7±25.6 cells/mm2) and increased during raloxifene treatment (60 mg: 35.2±58.9 cells/mm2; 120 mg 3.5±29.7 cells/mm2, p<0.01). MAT and adipocyte size were negatively correlated with osteoclast number (Baseline: r=-0.35, p<0.01 and r=-0.31, p=0.02 respectively). Finally, patients with vertebral fractures had higher MAT (55±10%) and larger adipocytes (59±4.7 µm) compared to patients without fractures (49±10% p=0.03, 55±5.1 µm p=<0.01 respectively).

In conclusion, raloxifene altered bone marrow adipocyte characteristics, it decreased adipocyte size and increased adipocyte number. MAT volume and adipocyte size were associated with bone resorption, but not with bone formation, suggesting a direct interaction between bone marrow adipocytes and bone resorption . In addition, we found that high MAT volume and larger adipocyte size are associated with prevalent vertebral fractures in postmenopausal women with osteoporosis, indicating that adipocyte size effects bone quality independent of bone volume.

**Highlights**

* Raloxifene treatment decreases the size and increases the number of marrow adipocytes in postmenopausal women with osteoporosis.
* Changes of marrow adipocyte characteristics are correlated with histomorphometric changes in bone resorption, but not with bone formation.
* Women with postmenopausal osteoporosis and vertebral fractures have high  marrow adipose tissue and larger marrow adipocytes.

**Keywords (6 in total)**

Marrow adipose tissue

Raloxifene

Postmenopausal osteoporosis

Vertebral fracture

Bone turnover

Clinical trial

**Abbreviations**

MAT marrow adipose tissue

BMD bone mineral density

MSC mesenchymal stem cell

SERM selective estrogen receptor modulator

MORE Multiple Outcomes of Raloxifene Evaluation

RANKL receptor activator of nuclear factor κ-B ligand

**1.** **Introduction**

High bone marrow adipose tissue (MAT) is associated with low bone mineral density (BMD) and MAT is increased in patients with osteoporosis [1–3]. MAT is also associated with vertebral fractures [4,5], independently of BMD in some studies [6].Two potential mechanisms for the association between MAT and bone have been proposed. First, mesenchymal stem cells (MSCs) may differentiate into either an adipocyte or an osteoblast [7]. Therefore, a shift in the lineage allocation of the MSCs towards the adipocyte would decrease the number of osteoblasts and thus bone formation. Secondly, in vitro studies have shown that adipocytes may directly influence osteoblast and osteoclast differentiation and function, through secretion of adipokines and free fatty acids [8–12], suggesting a direct effect of MAT on bone turnover. Indirect evidence for this hypothesis comes from animal studies showing a positive correlation of marrow adiposity with bone resorption [13], and a negative correlation with bone formation [14] in ovariectomized rats. Clinical studies have indicated a negative association between MAT and bone formation in healthy premenopausal women, but MAT was not associated with bone formation or resorption in premenopausal women with osteoporosis [15]. In contrast, in elderly men and women with hip fractures MAT was negatively associated with bone resorption but not with bone formation [16]. Altogether, clinical studies on the association between MAT and bone turnover are heterogeneous and have shown different results.

Hormone replacement therapy in postmenopausal women effectively improves bone mass by decreasing bone turnover and decreasing MAT [17]. However, estradiol is no longer recommended as a treatment for postmenopausal osteoporosis due to the increased risk of breast cancer and venous thromboembolism [18,19]. Raloxifene, a selective estrogen receptor modulator (SERM), has been developed as an osteoporosis therapy to specifically relay the beneficial effects of estrogen on bone, without the adverse side effects [20]. In vitro raloxifene increased lipid deposition in differentiating 3T3-L1 cells [21] and in vivo, in rats, raloxifene decreased MAT [22]. Clinical data on the effect of raloxifene on MAT are lacking.

The first aim of this study is to determine the effect of raloxifene on MAT in patients with postmenopausal osteoporosis. We hypothesized that raloxifene decreases MAT, similar to estradiol [17]. The second aim is to explore the association between MAT, bone volume, bone turnover and vertebral fracture in postmenopausal women with osteoporosis and the interaction with raloxifene treatment. We hypothesized that MAT is inversely associated with histomorphometric measures of bone volume and bone turnover and that MAT is increased in vertebral fracture patients.

**2. Materials and Methods**

2.1 Study design and subjects

The present study analyzed marrow adiposity in bone biopsies previously obtained for an ancillary histomorphometry study [23] of the MORE study, conducted between 1994 and 1999 to examine the effect of raloxifene on bone mineral density and fracture risk [20]. The MORE study was a multi-center, double-blind, randomized, placebo-controlled clinical trial. In the main study, 7705 postmenopausal women with osteoporosis were randomly assigned to treatment with placebo, 60 mg or 120 mg of raloxifene hydrochloride daily in addition to daily supplements of 500 mg of calcium and 400-600 IE of vitamin D during 36 months. Of the total, 88 participants were included in the ancillary bone histomorphometry study which was conducted at two centers in the US and two centers in Europe and included bone biopsies before start of treatment and after 24 months of treatment.

Eligible women were at least two years postmenopausal and had osteoporosis, defined by either femoral neck or lumbar spine bone mineral density (BMD) T-score < -2.5 (study group 1) or a] one or more moderate to severe (25-40% reduction from expected vertebral height) or two or more mild (20-25% reduction from expected vertebral height) vertebral fractures and low BMD or b] two or more moderate to severe vertebral fractures regardless of BMD (study group 2). Exclusion criteria were severe or long-term disabling conditions; metabolic bone diseases; endocrine conditions requiring hormonal therapy (except stable hypothyroidism and type 2 diabetes mellitus); use of systemic estrogen, progestogen, or androgen during the previous 6 months; a known, suspected, or history of breast cancer, endometrial cancer, or abnormal uterine bleeding; thromboembolic events or stroke during the past 10 years; any type of cancer besides superficial skin cancer in the previous 5 years; active renal lithiasis; abnormal hepatic function or consumption of more than four alcoholic drinks per day.

All subjects provided written informed consent. Approval was obtained from all local institutional review boards. The trial was registered (clinicaltrials.gov NCT 00670319).

2.2 Measurements

*2.2.1 Histomorphometry*

Bone biopsies were obtained at baseline and after 24 months of treatment by a transverse biopsy from the anterior iliac crest following double tetracycline fluorescent labeling. Biopsies were cut with a Jung microtome into 5-8 µm sections and stained with Goldner’s stain or tartrate-resistant acid phosphatase stain. The biopsy procedure and handling of the samples and bone histomorphometric measurements have been described in more detail previously [23]. All assessments of the slides were performed blinded to the treatment assignment and to the timing of the biopsies (baseline or post-treatment).

*2.2.2 Marrow adiposity parameters*

For the adipocyte parameters, we used sections stained with the Goldner’s stain.

The following MAT outcome parameters were measured and calculated: 1] total adipose tissue volume as a percentage of the tissue volume (Ad.V/TV; %), 2] total adipose tissue volume as a percentage of the marrow volume (Ad.V/Ma.V; %), 3] mean adipocyte diameter (Ad.Dm; μm), calculated using the formula: $2\*\sqrt{\frac{Ad.Ar/N}{π}} $$2\*\sqrt{2\*\sqrt{\frac{Ad.Ar/N}{π}}\frac{Ad.Ar/N}{π}}$, assuming that all adipocytes are essentially circular, representing adipocyte size 4] adipocyte density (Ad.Dn; cells/mm2 marrow area) representing adipocyte number. These measurements were performed by tracing out individual adipocytes ‘ghosts’ in all the fields analyzed. Adipocyte ghosts appear as distinct, translucent, yellow ellipsoids in the marrow space. A standardized area in the secondary spongiosa was measured in 1-4 slides per biopsy including a mean marrow area of 15.24 mm2 (range 2.88 - 62.83 mm2; SD ± 6.82 mm2) per biopsy. A watershed algorithm was used to separate the individual adipocytes. Fields were captured using a Nikon Microscope (Eclipse E 800), a DS-U1 camera (Nikon) and NIS Elements software (version 2.34, Nikon) at 40x magnification. Adipocyte analysis was performed using a semi-automated measurement program based on ImageJ [24]. All assessments of the slides were performed by examiners who were blinded to the treatment assignment and to the timing of the biopsies (baseline or post-treatment).

*2.2.3 Bone turnover parameters*

Cancellous bone volume as percentage of tissue volume (BV/TV; %), osteoid surface as a percentage of the total bone surface (OS/BS; %), number of osteoclasts along the bone surface (N.Oc/BS, in number of cells/mm2) and bone formation rate (BFR/BV; referenced to bone volume, in %/year) were measured as secondary outcomes according to the guidelines of the ASBMR nomenclature committee [25] and were published previously [23].

*2.2.4 Bone mineral density and vertebral fracture status*

Lumbar spine and femoral neck BMD were measured by dual-energy x-ray absorptiometry (DXA) (Hologic, Bedford, MA, USA or Norland, White Plains, NY USA). A conventional radiography of the spine was obtained at baseline, and assessed for vertebral fractures by two radiologists [20].

2.3 Statistical Analysis

The statistical analysis was performed with IBM SPSS Statistics for Windows (version 24; SPSS Inc., Chicago, IL, USA). The mean and standard deviation (SD) or the median and interquartile ranges (IQRs) are reported depending on the distribution of the data.

To compare the treatment groups at baseline ANOVA or Kruskall Wallis tests were used depending on the distribution of the data. To assess the association of adipocyte parameters with bone parameters at baseline, Spearman or Pearson correlations were used depending on the distribution. To assess the effect of treatment on changes in adipocyte parameters, ANOVA or Kruskall Wallis tests were used depending on the distribution of the data. To compare treatment groups (post hoc) and correct for multiple testing, we used Tukey tests. Linear regression analysis was performed to test for effect modification by interaction due to treatment on changes in adipocyte and bone parameters. Assumptions underlying the linear regression model were met. All statistical tests were two-sided and a p-value of 0.05 was considered significant.

**3. Results**

3.1 Subjects

In the MORE trial ancillary histomorphometry study, 88 subjects were included at baseline and 65 paired biopsies were available for bone parameter analysis at 24 months. In the present study, 59 biopsies at baseline and 53 paired biopsies after 24 months were of sufficient quality to analyze the bone marrow adipocyte parameters.

As expected from randomization, no differences were observed between the treatment groups at baseline. Table 1 shows the baseline characteristics of the subjects. BMD and bone histomorphometric changes have been previously reported [23], Table 2 shows the changes in bone variables in the subset of patients specifically analyzed in this study.

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| **Table 1 Baseline characteristics** |  |  |  |
|  | **Placebo** | **Raloxifene 60 mg** | **Raloxifene 120 mg** |
| N | 26 | 17 | 16 |
| biopsy at 24 months (N) | 22 | 16 | 15 |
| age (years)  | 67 ± 5.7 | 70 ± 6 | 69 ± 7 |
| years postmenopausal (years)  | 18 (7) | 23 (8) | 21 (10) |
| BMD lumbar spine (g/cm2)  | 0.815 ± 0.120 | 0.821 ± 0.130 | 0.826 ± 0.099 |
| BMD femoral neck (g/cm2)  | 0.605 ± 0.111 | 0.634 ± 0.070 | 0.547 ± 0.052 |
| vertebral fracture (N) | 9 | 6 | 4 |
| Bone volume (BV/TV, %)  | 18 ± 6 | 19 ± 4 | 16 ± 4 |
| Osteoid surface (OS/BS, %)  | 11 ± 7 | 10 ± 5 | 14 ± 7 |
| Osteoclast number (N.Oc/BS, cells/mm2)  | 0.65 ± 0.33 | 0.88 ± 0.60 | 0.92 ± 0.54 |
| Bone formation rate (BVR/BV, %/y)  | 27 ± 18 | 28 ± 18 | 31 ± 18 |
| Marrow adipose volume (Ad.V/TV, %) | 44 ± 8 | 40 ± 8 | 40 ± 9 |
| Marrow adipose volume (Ad.V/Ma.V ,%)  | 54 ± 11 | 49 ± 10 | 48 ± 9 |
| Adipocyte size (Ad.Dm, um)  | 57 ± 6 | 56 ± 5 | 55 ± 4 |
| Adipocyte number (Ad.Dn, cells/mm2)  | 211 ± 34 | 197 ± 25 | 202 ± 21 |

**Table 1** Baseline characteristics of study patients per treatment group. Mean ± standard deviation or Median (IQR).

N=number of subjects; BMD=bone mineral density.

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| **Table 2 Changes in bone parameters**  |  |  |
|  | **Placebo** | **Raloxifene 60 mg** | **Raloxifene 120 mg** |
| N | 22 | 16 | 15 |
| ΔBMD Lumbar spine (g/cm2) (SD) | 0.007 ± 0.044 | 0.023 ± 0.038 | 0.013 ± 0.033 |
| ΔBV/TV (%) (SD) | 0 ± 7 | -2 ± 6 | -1 ± 4 |
| ΔBFR/BV (%/y) (SD) | -7 ± 17 | -8 ± 16 | -13 ± 21 |
| ΔN.Oc/BS (cells/mm2) (SD) | 0.18 ± 0.43 | -0.06 ± 0.49 | -0.02 ± 0.66 |

**Table 2** Mean changes ± standard deviation in bone parameters in the subset of patients analyzed in this study. N=number of subjects, BMD=bone mineral density.

3.2 Raloxifene effect on marrow adiposity

2 years of raloxifene treatment did not affect the change in MAT volume compared to baseline, neither when expressed as a percentage of the marrow volume (Ad.V/Ma.V; change per group: placebo 2.7±11.5%, raloxifene 60 mg 9.1±13.5%, raloxifene 120 mg -1.8±10.3%); nor when expressed as percentage of the total volume (Ad.V/TV; change per group: placebo 2.2±11.4%, raloxifene 60 mg 8.5±10.3%, raloxifene 120 mg -0.9±8.2%) compared to placebo (Figure 1A).

The adipocyte size (Ad.Dm) dose-dependently decreased during treatment (change during placebo 2.2±4.7 µm, raloxifene 60 mg 0.4±3.1 µm and raloxifene 120 mg -2.0±3.9 µm treated groups) (placebo vs raloxifene 60 mg p=0.37, placebo vs raloxifene 120 mg p<0.01, raloxifene 60 vs 120 mg p=0.24) (Figure 1B).

The adipocyte number (Ad.Dn) decreased in the placebo group (-7.7±25.6 cells/mm2) whereas it increased in the raloxifene 60 mg (35.2±58.9 cells/mm2) and in the raloxifene 120 mg (3.5±29.7 cells/mm2) treated groups (placebo vs raloxifene 60 mg p<0.01, placebo vs raloxifene 120 mg p=0.68, raloxifene 60 vs 120 mg p=0.08) (Figure 1C).



**Figure 1** Changes in marrow adipose tissue volume (Ad.V/Ma.V; %), adipocyte size (Ad.Dm; micrometer) and adipocyte number (Ad.Dn; cells/mm2) during the 24 months study period.

3.3 Marrow adiposity and bone turnover

At baseline, MAT volume (Ad.V/Ma.V) and adipocyte size (Ad.Dm) were negatively correlated with osteoclast number (N.Oc/BS) (r=-0.35, p<0.01 and r=-0.31, p=0.02 respectively) but not with bone formation (osteoid surface; OS/BS and bone formation rate; BFR/BV).

The changes in MAT volume and adipocyte number during the 2 year treatment with raloxifene 60mg were associated with the changes in osteoclast number (regression functions in table 3). In addition raloxifene 60mg significantly modified the interaction between the changes in adipocyte number and osteoclast number (interaction: p<0.001). Changes in MAT were not associated with changes in bone formation (osteoid surface; OS/BS and bone formation rate; BFR/BV).

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| **Table 3 Regression functions interaction treatment with change in MAT and bone parameters** |
|  | ΔAd.V/Ma.V | R2 | p |
| placebo | 2.51+1.15\*ΔN.Oc/BS  | 0.314 |  |
| raloxifene 60 mg | 10.23+19.38\*ΔN.Oc/BS |  | 0.022 |
| raloxifene 120 mg | -1.67+5.62\*ΔN.Oc/BS |  | 0.288  |
|  | ΔAd.Dn | R2 | p |
| placebo | -5.64-11.27\*ΔN.Oc/BS | 0.486 |  |
| raloxifene 60 mg | 40.35+86.73\*ΔN.Oc/BS |  | <0.001 |
| raloxifene 120 mg | 3.86+15.52\*ΔN.Oc/BS |  | 0.331  |

**Table 3** Regression functions for the change in adipocyte parameters and bone parameters.

ΔAd.V/Ma.V: change in MAT volume; ΔN.Oc/BS: change in osteoclast number; ΔAd.Dn: change in adipocyte number.

3.4 Vertebral fracture and marrow adiposity

At baseline, marrow adipose tissue volumes (both Ad.V/Ma.V and Ad.V/TV) were higher (55±10% and 46%±8%) and adipocyte size was larger (59±4.7 µm) in the patients with vertebral fractures and low or normal BMD, compared to the patients without fractures with BMD T-scores <2.5 (49±10%, p=0.02 and 40±9%, p=0.02 and 55±5.1 µm, p<0.01 respectively) (Figure 2).



**Figure 2** Marrow adipose tissue volume (Ad.V/Ma.V), adipocyte size (Ad.Dm) and adipocyte number (Ad.Dn) in patients with BMD T-score<-2.5 without vertebral fracture and patients with vertebral fracture.

**4. Discussion**

The present study showed that raloxifene treatment decreases the size and increases the number of marrow adipocytes. At baseline, marrow fat content was inversely related to osteoclast number, but not to parameters of bone formation. Patients with prevalent vertebral fractures had higher MAT and larger marrow adipocytes compared to postmenopausal women with osteoporosis without vertebral fractures.

4.1 Effects of Raloxifene on MAT in postmenopausal osteoporosis

In the current study, raloxifene did not decrease MAT volume, but it did change adipocyte characteristics; it increased adipocyte number and decreased adipocyte size. Previous research by Syed et al. showed that estradiol decreases MAT volume by both a decrease in adipocyte size and number in postmenopausal women with osteoporosis[17]. It is known that raloxifene does not have the same effect as estradiol on bone and our results indicate that also the effect on MAT is partly different. A possible explanation for this different effect could come from the complex interactions between raloxifene, estrogen receptors, coactivators and corepressor proteins [26–28] within the heterogeneous cell types in the bone microenvironment. The results of previous preclinical research on the effects of raloxifene on MAT are inconsistent. Murase et al. showed that, in vitro, raloxifene dose dependently increased lipid deposition in differentiating 3T3-L1 cells [21]. But since MAT appears to be a distinct adipose tissue[29,30], it is unknown if the results also apply on MAT. In vivo research by Somjen et al showed that MAT decreased after raloxifene treatment in ovariectomized rats [22]. Since these rats were ovariectomized during growth and MAT was quantified directly under the growth plate, thus including the primary spongiosa, the results could be related to modeling activity rather than remodeling activity, which makes extrapolation to the clinical situation of postmenopausal osteoporosis difficult.

Changes in marrow adipocyte size and number are interesting since previous research showed that large subcutaneous adipocytes secrete more adipokines with a shift towards pro-inflammatory adipokines than smaller adipocytes [31]. It is unknown if large bone marrow adipocytes also secrete more pro-inflammatory adipokines than smaller ones. Nevertheless, this could indicate that the raloxifene induced decrease of the adipocyte size, leads to a more favorable profile of adipokines secreted locally and possibly exerting a positive influence on bone metabolism. [8–11]. Accordingly, other anti-osteoporosis therapies such as estrogen, teriparatide and bisphosphonates also decrease marrow adipocyte size, which is accompanied by an increase in BMD and a decrease in fracture risk [17,32,33]. Thus it seems that decreasing marrow adipocyte size has a bone-favorable effect. This notion is further supported by our observation that patients with vertebral fracture had larger marrow adipocytes than patients without fracture. Since the size of an adipocyte is mainly determined by its lipid content, these lipids might exert the negative effect on bone cells. Interestingly, a recent study on the interaction of marrow adipocytes with leukemia cells in the bone marrow, showed that adipocytes can transfer their lipids to leukemic cells [34] supporting their maintenance. Whether marrow adipocytes are also capable of transferring lipids to bone cells and what their role is, remains to be determined.

4.2 MAT and bone turnover before and after raloxifene treatment

Both at baseline and during treatment, MAT and bone resorption were associated. Several reports have indicated that bone marrow pre-adipocytes secrete receptor activator of nuclear factor κ-B ligand (RANKL) which stimulates osteoclast differentiation and activation (Fan et al., 2017; Takeshita, Fumoto, Naoe, & Ikeda, 2014). RANKL could therefore be a mediator between marrow adipocytes and bone resorption. At baseline marrow adipocyte size was associated with bone resorption, and during treatment changes in marrow adipocyte number were associated with changes in osteoclast number. These results suggest a possible role for adipocyte characteristics in the association between MAT and bone resorption.

Raloxifene 60mg interacted significantly in the association between adipocyte number and bone resorption. Different effects of 60 and 120 mg of raloxifene were also observed on bone parameters [23]. A possible explanation for this difference could be receptor kinetics, for example desensitization or saturation, but this has never been investigated so far. We measured osteoclast number (N.Oc/BS) as a measure of bone resorption, although osteoclast number does not provide information on osteoclast function, which is a limitation of histomorphometric studies in general. Another histomorphometric measure of bone resorption is eroded surface, but we did not choose to use this parameter in this study due to greater inter-observer variability compared to osteoclast number [38]. Moreover, resorption pits were scarce in these biopsies and therefore were not representative of the overall resorption rate [23].

4.3 MAT and vertebral fracture

We showed, for the first time, that besides MAT volume, adipocyte size is associated with vertebral fractures; vertebral fracture patients have larger marrow adipocytes compared to patients without vertebral fracture. Previous studies showed that high MAT is associated with vertebral fractures (Schellinger et al., 2001; Schwartz et al., 2013; Wehrli et al., 2000). These studies used magnetic resonance imaging (MRI) techniques to detect MAT, consequently marrow adipocyte size and number could not be determined. [4–6]. Our study confirms the association of increased MAT with vertebral fractures. Interestingly, there was no difference in bone volume or BMD between the patients with and without fracture, possibly indicating that the negative effect of the larger marrow adipocytes could be independent of bone volume and BMD.

 **Conclusions**

This study shows that raloxifene modifies marrow adipocyte characteristics in postmenopausal women with osteoporosis. Furthermore, MAT volume and adipocyte size were associated with bone resorption, but not with bone formation which may point to a direct effect of the marrow adipocytes on osteoclasts. Finally, we showed for the first time that the higher MAT volume in patients with vertebral fractures compared to patients without fracture was caused by larger marrow adipocytes, which supported the tenet that larger adipocytes indeed have a negative effect on bone cells, possibly by secreting adipokines or fatty acids. This will be an important question for further research, since targeting adipocyte size might represent a future therapy for bone disease and fracture prevention.

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**Author contributions**

KB, AV, PB and NB designed the study; KB, AO, SO, RH and PL acquired the data; KB, AV and MT analyzed the data; KB, AV, PB and NB interpreted the data; KB, AV, PB and NB drafted the manuscript; all authors critically revised the manuscript and approved the final version of the manuscript to be submitted.

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