Journal of Bone and Mineral Metabolism (2019) 37:987–995
https://doi.org/10.1007/s00774-019-01000-4

ORIGINAL ARTICLE

The association between CD31^{hi}Emcn^{hi} endothelial cells and bone mineral density in Chinese women

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Received: 10 January 2019 / Accepted: 11 March 2019 / Published online: 27 March 2019

Abstract

Osteoporosis is the most common bone disease in humans. During bone remodeling, specialized blood vessels influenced by the endothelial cells (CD31^{hi}Emcn^{hi}, also called type H cells) are formatted to supply nutrients. Reductions in vascular supply are associated with bone loss resulting in osteoporosis. Therefore, the objective of the present study was to explore the association between the CD31^{hi}Emcn^{hi} endothelial cells and bone mineral density (BMD). In this prospective study, 134 Chinese women were enrolled and examined. BMD was measured by DEXA method while the percentage of CD31^{hi}Emcn^{hi} endothelial cells in the intertrochanteric part was measured by flow cytometry. The percentage of CD31^{hi}Emcn^{hi} endothelial cells in postmenopausal subjects was significantly lower compared with premenopausal women ($8.7 \pm 4.0\%$ vs $13.2 \pm 5.6\%$, $P < 0.01$). Meanwhile, the CD31^{hi}Emcn^{hi} endothelial cell levels in osteopenia and osteoporosis were significantly lower compared with subjects with normal BMD ($9.84 \pm 4.2\%$ in osteopenia and $7.11 \pm 3.2\%$ in osteoporosis vs $12.7 \pm 5.6\%$ in subjects with normal T score, $P < 0.01$). Multiple regression analyses showed that the CD31^{hi}Emcn^{hi} endothelial cells level was positively associated with femur neck and total hip BMD, but not with lumbar BMD. Our study suggests a significantly positive association between CD31^{hi}Emcn^{hi} endothelial cells and local BMD in Chinese women. The proportion of CD31^{hi}Emcn^{hi} endothelial cells is a marker of bone quality and represents a potential target for treatment of bone loss.

Keywords CD31^{hi}Emch^{hi} endothelial cell · Type H · Bone mineral density · Osteoporosis

Introduction

Osteoporosis is one of the most common bone diseases in humans [1]. It is a kind of systemic metabolic bone disease which characterized as loss of bone mass, degeneration of bone tissue and augment of bone fragility [2]. A large number of studies have shown that the risk of osteoporosis increases with age, and in particular, postmenopausal women have a higher risk of osteoporosis than non-menopausal women [3–7]. Bone remodeling is in the dynamic transform process with new bone formation and old bone resorption, but the underlying relationship between bone metabolism and osteoporosis is incompletely understood [8–10]. As a highly vascularized tissue, bone has extensive network of blood vessels which play an important role in the process of bone growth, development, shaping, remodeling, and damage healing [11].

The importance of blood supply in bone was initially recognized by surgeons during bone repair and fracture healing in the nineteenth century [12]. Bone receives up to about

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10–15% of cardiac output [13, 14]. During organ and tissue development, specialized blood vessels influenced by the endothelial cells are formatted to supply nutrients. Vogt et al. found reduced blood flow to the extremities-affected bone remodeling, resulting in a decreased bone mineral density (BMD) [15]. So reductions in vascular supply are associated with bone loss resulting in osteoporosis [16].

Adams et al. found that there was a new type of vascular endothelial cell (also called type H vascular endothelial cell) in the mouse skeletal system which marked with high expression of CD31 and Endomucin ($CD31^{hi}Emcn^{hi}$) [17, 18]. They also found that type H endothelial cells mediated local growth of the vasculature and provided niche signals for perivascular osteoprogenitors. Notch and hypoxia-inducible factor are important positive regulators of type H endothelial cells. Type H vessel formation and the expression of potential angiocrine factors, for example noggin, promote osteogenesis [17, 18]. Previous studies confirmed that type H vascular endothelial cells decreased in osteoporotic patients [19], but no quantitative analysis was conducted and analyzed the association with BMD. In this study, we included a larger number of participants and measured the proportion of type H vascular endothelial cells in the intertrochanteric part by flow cytometry to explore the association of type H vascular endothelial cells and BMD.

Materials and methods

Subjects

A total of 134 Chinese women who admitted to Xiangya Hospital of Central South University for hip fracture between June 2016 and September 2018 were enrolled and signed a consent form to participate. The participants included 31 premenopausal women and 103 postmenopausal women. Participants with diabetes, chronic kidney disease and cardiovascular diseases which include coronary artery disease and cerebrovascular disease were diagnosed with criteria reported in literatures [20–23]. Inclusion criteria: (1) premenopausal women with regular menstrual cycle function and postmenopausal women aged 60 years old and over, with at least 6 years since menopause. (2) Patients with hip fracture who were performed hip replacement or intramedullary nail surgery within 48 h after the fracture. Exclusion criteria: (1) participants that have had or currently have medication to treat osteoporosis. (2) Women that have diseases, such as hyperparathyroidism, or treatment known to affect bone metabolism and quality.

Bone mineral density

The total lumbar (L1-L4), femur neck and total hip BMD and T-scores were measured for each participant by a dual-energy X-ray absorptiometry scanner (Hologic QDR-4500A). The healthy side of femur neck and hip was measured because fracture affects the accuracy of BMD. Daily quality control was carried out by measurement of a Hologic anthropomorphic spine phantom. The phantom precision expressed as the coefficient of variation was 0.97%. The BMD measurements were carried out by the same experienced technicians. Osteopenia and osteoporosis were diagnosed according to the WHO criteria [24, 25].

Flow cytometry

Bone samples were collected during hip replacement or intramedullary nail surgery. After cut by scissor, samples were crushed in ice-cold PBS using a mortar and pestle. To obtain a single-cell suspension, bone sample was digested with type II collagenase for 30 min at 37℃ and filtered with 40 μm filter. Subsequently, samples were treated with blocking solution (1% FBS) for 30 min, washed with PBS and immunostained with mouse anti-human CD31 coupled to PE-Cy^{tm}7 (563651, BD Biosciences, 1:50) for 30 min on ice. After washing, cells were stained with rat anti-endomucin antibody [V.7C7.1] (ab106100, Abcam, 1:100) for 30 min and then labeled by goat anti-rat IgG H&L Alexa Fluor 488 (ab150157, Abcam, 1:50) for another 30 min after washing. After thorough washing, cells were treated with DAPI to exclude dead cells. Finally, cells were acquired on a BD FACSCANTO II flow cytometer and analyzed using FlowJo Software (Version 10, FlowJo, LLC).

Statistical analysis

Mean ± standard deviation (SD) and the number and proportion were used to describe continuous variables and categorical variables separately. We used Chi-square tests for categorical variables, one-way ANOVA for normally distributed continuous variables, Kruskal–Wallis test for skewed continuous variables and Pearson rank correlation coefficient for correlation analysis. Regression coefficient using unadjusted and multivariate-adjusted logistic regression analyses were used to explore the association between BMD and the percentage of type H endothelial cells. Type H endothelial cells level was presented as median with interquartile range (Q1, Q2, Q3 and Q4) when comparing BMD. A comparison of the distributions of BMD between the groups was performed using an ANOVA (analysis of variance), and post hoc testing for the differences between two groups (Q1

vs Q4) was performed with Tukey's method. Models were used to control for potential confounders based on univariate analysis and stepwise methods. Statistical analyses were performed using STATA version 12 (StataCorp, College Station, TX, USA), SPSS version 21.0 (IBM Corporation, USA), R program version 3.5.2 and GraphPad Prism 6 computer program (GraphPad Software, Inc. USA). All *P* values were two-tailed with less than 0.05 being considered significant.

Results

Basic characteristics

The patient characteristics of current study including age, BMI, BMD and so on are presented in Table 1. This study included 134 participants with 31 premenopausal women and 103 postmenopausal women. The mean age was 37.2 ± 8.3 and 73.4 ± 8.9 years while the body mass index (BMI) was 26.4 ± 11.5 and 25.4 ± 4.9 kg/m^2 in premenopausal women and postmenopausal women, respectively. Osteopenia was diagnosed in 9 premenopausal women and 46 postmenopausal women. Osteoporosis was diagnosed in 0 premenopausal women and 43 postmenopausal women. Cardiovascular disease, osteoporosis and osteopenia were more likely to be observed in postmenopausal group. The percentage of type H levels in postmenopausal subjects was significantly lower compared with premenopausal women (8.7 ± 4.0% vs 13.2 ± 5.6%, $P < 0.01$, Table 1).

Association between type H endothelial cells and BMD

The percentage of type H levels in osteopenia and osteoporosis were significantly lower compared with subjects with normal BMD (9.84 ± 4.2% in osteopenia and 7.11 ± 3.2% in osteoporosis vs 12.7 ± 5.6% in subjects with normal T score, $P < 0.01$, Fig. 1a–d).

In the univariate regression model, age, weight, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) and type H levels were significantly associated with femur neck BMD (Table 2, Fig. 2). Furthermore, after controlling for potential confounders with three models (Model 1: age, weight and type H; Model 2: age, BMI, type H; Model 3: age, weight, BMI, SBP, DBP and type H), multivariate regression analysis was conducted and the result showed that ages and type H levels were still associated with femur neck BMD. Age was significantly negatively associated with femur neck BMD while the type H levels were significantly positively associated with femur neck BMD (Table 3, Fig. 3a).

Table 1 General characteristics of the subjects (Mean ± SD or *N* %)

	Total ($n = 134$)	Premenopausal women ($n = 31$)	Postmenopausal women ($n = 103$)	*P* values
Age (year)	65.1 ± 17.7	37.2 ± 8.3	73.4 ± 8.9	**0.01**
Height	163.8 ± 7.9	164.6 ± 7.7	163.6 ± 8.0	0.61
Weight	68.9 ± 13.8	71.9 ± 15.3	67.9 ± 13.3	0.54
BMI	25.7 ± 4.8	26.4 ± 11.5	25.4 ± 4.9	0.74
Diabetes	12	2	15	0.38
Kidney disease	9	1	8	0.63
Smoking	21	5	16	0.84
Drinking	18	4	14	0.85
Cardiovascular disease	33	2	31	**0.01**
SBP	130.8 ± 16.1	112.0 ± 1.5	136.5 ± 12.6	**0.01**
DBP	82.7 ± 11.2	75.9 ± 8.4	84.8 ± 11.1	**0.01**
Total Lumber BMD	0.872 ± 0.168	1.008 ± 0.126	0.831 ± 0.157	**0.01**
Femur neck BMD	0.677 ± 0.151	0.814 ± 0.158	0.635 ± 0.122	**0.01**
Total Hip BMD	0.783 ± 0.183	0.936 ± 0.184	0.737 ± 0.156	**0.01**
T score	− 1.8 ± 1.3	− 0.5 ± 1.2	− 2.2 ± 1.1	**0.01**
Normal	36	22	14	**0.01**
Osteopenia	55	9	46	0.12
Osteoporosis	43	0	43	**0.01**
Type H%	9.7 ± 4.8	13.2 ± 5.6	8.7 ± 4.0	**0.01**

Bold indicates a statistically significant difference with a *p*-value less than 0.05

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *BMD* bone mineral density

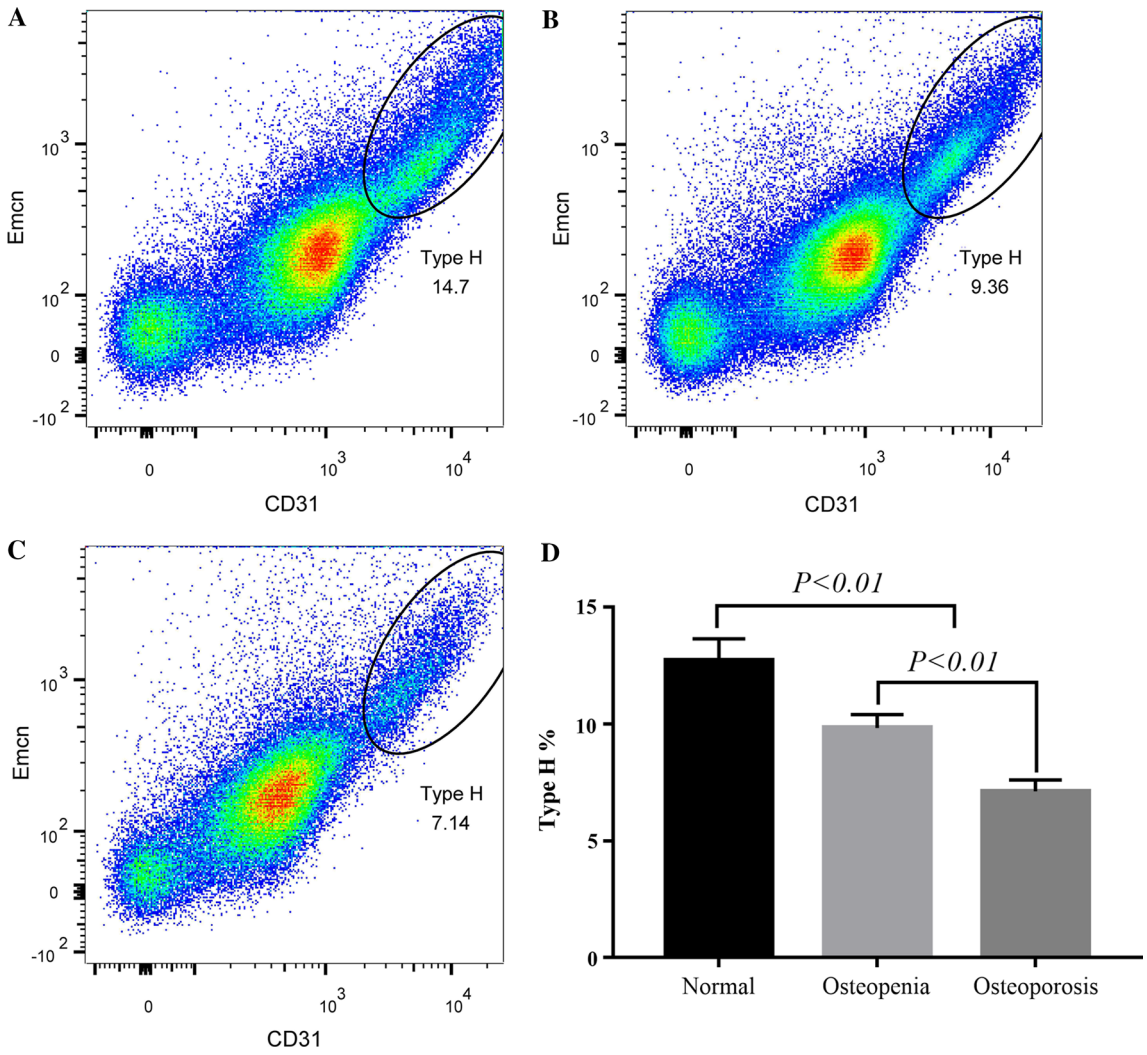

Fig. 1 **a** Representative case from the current study of a participant with normal T score. **b** Representative case of participant with osteopenia. **c** Representative case of patient with osteoporosis. **d** The percentage of type H endothelial cells in normal, osteopenia and osteoporosis group ($P < 0.01$)

Similarity, univariate regression model analyses showed that age, weight, BMI, SBP, DBP and type H levels were associated with total hip BMD while age, weight, SBP and type H levels were associated with total lumbar BMD(Table 2). After multiple regression for adjustment, the results demonstrated that age was negatively while type H levels were positively associated with total hip BMD, respectively(Model 1: age, weight and type H; Model 2: age, BMI, type H; Model 3: age, weight, BMI, SBP, DBP and type H). Only age, not type H level, was found to be associated with total lumbar BMD negatively (Model 1: age and type H; Model 2: age, weight, type H; Model 3: age, weight, SBP, and type H) (Table 3, Fig. 3b, c). In addition, as shown in Fig. 3d, comparing to the highest quartile (Q4) with type H level, femur neck and total hip BMD statistically decreased in the lowest quartile (Q1) of type H level ($P < 0.01$).

Discussion

The importance of the vascular supply for bone is well-known but is still rather overlooked. Bone requires a substantial blood flow to supply oxygen, nutrients and growth factors, as well as to eliminate metabolic waste products. The blood flow reflects not only the requirements of the bone cells, such as osteoblasts, osteoclasts and osteocytes, but also the bone marrow and endothelial cells [16]. A substantial vascular supply could provide requisite oxygen and nutrients to bone growth and repair while impairment of the blood

Table 2 Univariate regression analyses for effect on femur neck, total hip and total lumbar BMD

	Femur neck BMD			Total hip BMD			Total lumbar BMD		
	β	95% CI	*P* value	*β*	95% CI	*P* value	*β*	95% CI	*P* value
Age	−0.0040	−0.0053, −0.0027	<**0.01**	−0.0045	−0.0061, −0.0029	<**0.01**	−0.0039	−0.0054, −0.0023	<**0.01**
Height	0.0031	−0.0002, 0.0063	0.06	0.0031	−0.0008, 0.0071	0.12	0.0020	−0.0017, 0.0056	0.29
Weight	0.0031	0.0013, 0.0049	<**0.01**	0.0036	0.0014, 0.0058	<**0.01**	0.0024	0.0004, 0.0045	**0.02**
BMI	0.0067	0.0015, 0.0120	**0.01**	0.0081	0.0017, 0.0145	**0.01**	0.0055	−0.0004, 0.0115	0.07
Diabetes	0.1261	−0.0780, 0.1032	0.78	−0.0206	−0.1304, 0.0894	0.71	−0.0304	−0.1314, 0.0705	0.55
Kidney disease	−0.0572	−0.1601, 0.4575	0.27	−0.0681	−0.19304, 0.0567	0.28	−0.0583	−0.1731, 0.0566	0.32
Smoking	−0.0062	−0.0774, 0.0650	0.17	−0.0237	−0.1100, 0.0625	0.59	−0.0330	−0.1122, 0.0462	0.41
Drinking	−0.0262	−0.0987, 0.0463	0.48	−0.0475	−0.1352, 0.0402	0.29	−0.0278	−0.1057, 0.0561	0.55
Cardiovascular diseases	−0.0303	−0.0890, 0.0284	0.31	−0.0096	−0.0811, 0.6179	0.79	−0.0135	−0.0792, 0.0522	0.69
SBP	−0.0028	−0.0043, −0.0013	<**0.01**	−0.0029	−0.0048, −0.0011	<**0.01**	−0.0025	−0.0042, −0.0007	<**0.01**
DBP	−0.0028	−0.0051, −0.0006	**0.01**	−0.0029	−0.0057, −0.0002	**0.04**	−0.0018	−0.0043, 0.0008	0.18
Type H	0.0145	0.0098, 0.0193	<**0.01**	0.0145	0.0084, 0.0205	<**0.01**	0.0081	0.0023, 0.0140	<**0.01**

Bold indicates a statistically significant difference with a *p*-value less than 0.05

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure

flow is recognized to reduce growth and repair, ultimately bone loss. Osteoporosis is characterized by decreased bone strength which increases the risk of fracture, especially in postmenopausal women [26, 27].

In postmenopausal women, cardiovascular disease and osteoporosis are two common diseases that result in significant mortality and morbidity. More and more evidence suggests that these two conditions have an association but the mechanism is still unclear [28]. Several research groups found that the risk of cardiovascular disease and osteoporosis increases in postmenopausal women due to estrogen deficiency, which could cause endothelial dysfunction [29, 30]. Their work demonstrated patients with endothelial dysfunction have impaired bone metabolism, leading to osteoporosis. So endothelial cells play an important role in bone remodeling. Potente et al. showed that specialized blood vessels were formatted because of the different structural and metabolic requirements of organs and tissues during development [31]. Those specialized new blood vessels, which are influenced by the endothelial cells, deliver molecules that affect cell differentiation in the organs [32]. Adams and colleagues found that specialized endothelial cells, which are strongly positive for CD31 and Emcn, support bone maturation and regeneration [17, 18]. Their work suggested a link between osteogenesis and angiogenesis due to close spatial and temporal association. These special endothelial cells provide nutrients to osteoprogenitors cells which are close to special subtype vessels. This process maintains osteoprogenitors cell growth and proliferation, and sustains bone development [17, 18, 33]. They also found that the number of $CD31^{hi}Emcn^{hi}$ endothelial cells and the surrounding osteoprogenitors cells significantly decreased with age, especially in elderly mice. Wang et al. investigated the distribution of $CD31^{hi}Emcn^{hi}$ endothelial cells in rat tibiae during the bone defect repair process. Their work suggested that $CD31^{hi}Emcn^{hi}$ endothelial cells proliferated and were extensively distributed across the entire repair area during the early stage [34]. Another Chinese research group demonstrated that the abundance of $CD31^{hi}Emcn^{hi}$ endothelial cells was obviously lower in the proximal and distal metaphysis of the aged mouse compared with that of the juvenile and adult mice by immunofluorescence staining [19]. Similar results were obtained in human subject. Meanwhile, they explored the expression level of $CD31^{hi}Emcn^{hi}$ endothelial cells in osteopenic and osteoporotic groups and the results showed that the abundance of the $CD31^{hi}Emcn^{hi}$ endothelial cells was significantly reduced in the combination group of osteopenia and osteoporosis compared with that of the control group. However, no significant difference was observed between the osteopenic and osteoporotic groups [19]. Due to limited human subjects and imprecise quantification of $CD31^{hi}Emcn^{hi}$ endothelial cells by immunofluorescence staining, current study which enrolled more participant, using exact quantification method, such as flow cytometry, was needed.

Our study reported a correlation between type H endothelial cell and BMD in Chinese women. After multivariate-adjusted analyses, we observed a significantly positive association between type H endothelial cells and BMD at the femur neck and total hip. No association between type H endothelial cells and lumbar BMD was found after multivariate-analysis in current study. It is probable that bone samples were taken from hip not from vertebral body of

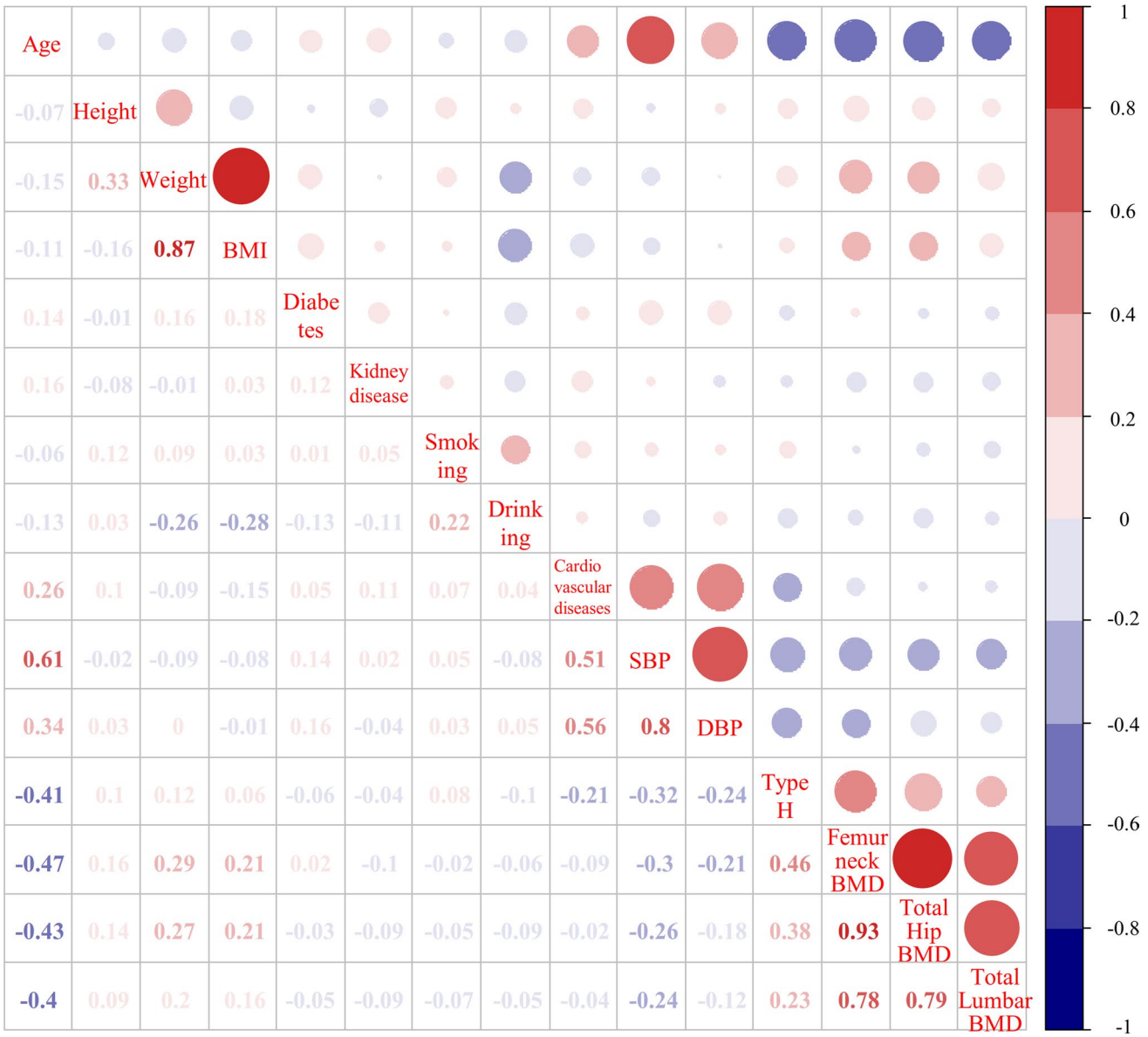

Fig. 2 The correlation matrix for the age, height, weight, BMI, diabetes, kidney disease, smoking, drinking, cardiovascular diseases, SBP, DBP, type H endothelial cells, femur neck, total hip and total lumbar BMD. Every correlation coefficient which matches two variables was calculated with spearman method in R. Numbers range from −1 to 1 are Spearman's rank correlation coefficients of variables on horizontal and vertical axes. Color depth and size of the circles indicate the correlation strength

Table 3 Multivariate regression analyses with type H and femur neck, total hip and total lumbar BMD

	Femur neck BMD			Total hip BMD			Total lumbar BMD		
	β	Adjusted R^2	P value	β	Adjusted R^2	P value	β	Adjusted R^2	P value
Model 1	0.0098	0.33	**< 0.01**	0.0088	0.26	**< 0.01**	0.0028	0.16	0.36
Model 2	0.0101	0.32	**< 0.01**	0.0091	0.25	**< 0.01**	0.0027	0.16	0.37
Model 3	0.0096	0.33	**< 0.01**	0.0086	0.25	**< 0.01**	0.0028	0.16	0.36

Bold indicates a statistically significant difference with a p-value less than 0.05

BMD bone mineral density

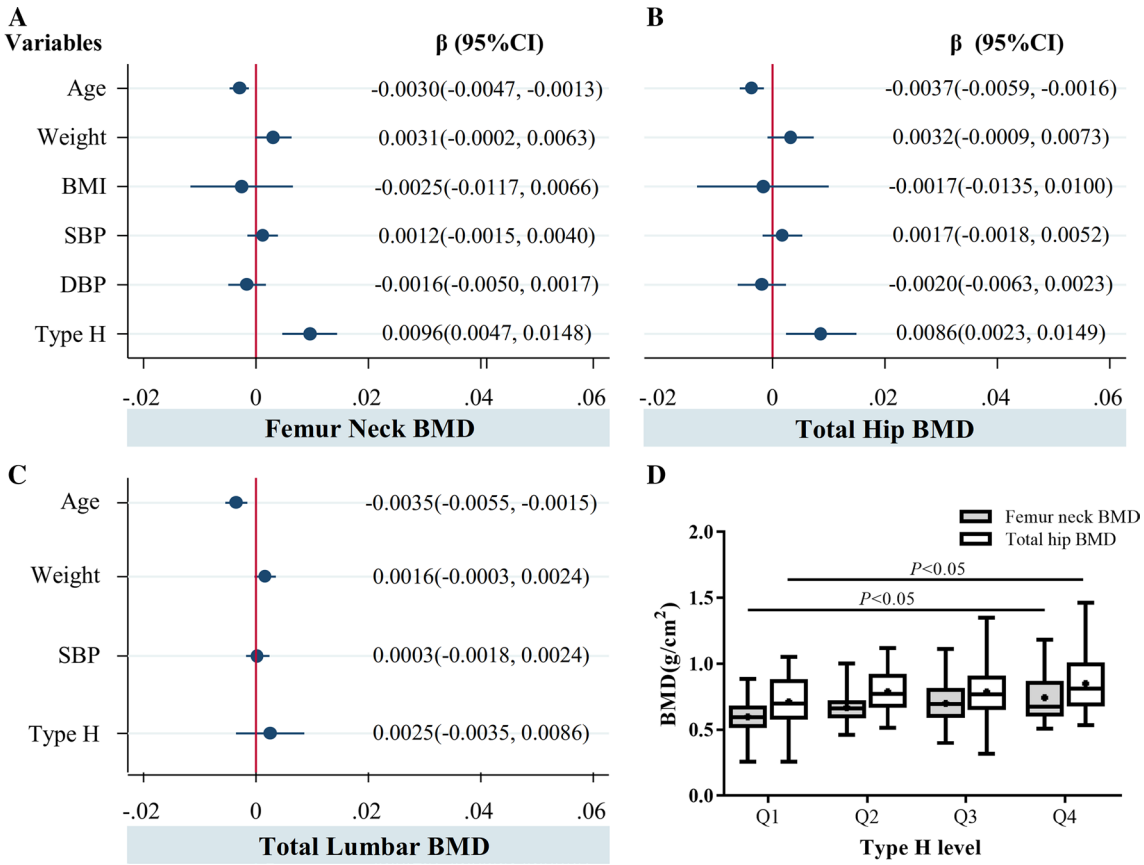

Fig. 3 **a** Multivariate regression analysis for variables effect on femur neck BMD (age and type H, $P<0.01$). **b** Multivariate regression analysis for variables effect on total hip BMD (age and type H, $P<0.01$). **c** Multivariate regression analysis for variables effect on lumbar BMD (age, $P<0.01$). **d** Difference in femur neck and total hip BMD among type H endothelial levels ($P<0.01$)

lumbar. So type H endothelial cells are positively correlated with local BMD.

There are several advantages in our study. First, this is the first study to report a strong association between type H endothelial cells measured by quantitative flow cytometry and local BMD in an Asian population. Second, our sample size was lager enough. Third, we collected and adjusted for various possible confounding factors. However, this current study still has several limitations which may affect the conclusion. First, perimenopause women were not enrolled in this study which may have selection bias. Second, although reliable standard method of flow cytometry was established, measurement errors may occur due to flow cytometry conducted separately. In addition, BMD measured by dual-energy X-ray absorptiometry may also cause measurement errors [35]. Finally, some confounding variables which could affect BMD might be omitted.

In conclusion, our study suggests a significantly positive association between $CD31^{hi}Emcn^{hi}$ endothelial cells and local BMD in Chinese women. The proportion of $CD31^{hi}Emcn^{hi}$ endothelial cells is a marker of bone quality and represents a potential target for treatment of bone loss.

Acknowledgements This work was supported by Hunan Provincial Natural Science Foundation (2016JJ6165).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the Institutional Research and Ethical Committee of Xiangya Hospital and with the 1964 Helsinki Declaration and its later amendments.

Informed consent All the participants signed a written informed consent and the work was approved by the Ethics and Research Committee of the Xiangya Hospital.

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