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Long-term use of fluoxetine accelerates bone loss through the disruption of sphingolipids metabolism in bone marrow adipose tissue

Huili Zhang¹, Kefeng Li², Yanna Zhao², Yilan Zhang¹, Jiawen Sun³, Shihong Li⁴ and Guangwu Lin⁴

Abstract

Fluoxetine is a commonly prescribed antidepressant, and the mechanisms of increased bone fragility with its long-term use remain largely unknown. Here, we show that long-term administration of fluoxetine induces the disruption of sphingolipids metabolism in bone marrow adipose tissue (BMAT) through the inhibition of acid sphingomyelinase (ASM). Similarly, a significant reduction of the bone volume was observed in mice with ASM knockout (*Smpd1*^{−/−}). In detail, inhibition of ASM by fluoxetine reduces the sphingosine-1-phosphate (S1P) level in bone marrow adipocytes, leading to the increase of receptor activator of nuclear factor- κ B ligand (RANKL) secretion, a key regulator for the activation of osteoclastogenesis and bone loss, through the upregulation of cyclooxygenase-2 and its enzymatic product prostaglandin E2 (COX-2/PGE2). In contrast, overexpression of ASM by cisplatin normalizes fluoxetine-induced RANKL overproduction. Furthermore, we conducted a clinical trial with L-serine, a precursor of sphingolipids biosynthesis. The results show that oral supplementation of L-serine (250 mg/kg/d) prevents the acceleration of bone loss caused by long-term fluoxetine (12 months) in postmenopausal women with major depressive disorder (mean total hip bone mineral density reduction: −2.0% vs −1.1%, $P = 0.006$). Our study provides new insights and potential treatment strategy on the bone loss caused by long-term use of fluoxetine.

Introduction

Major depression is one of the most common psychological disorders in older people and has become the leading cause of disease burden around the world¹. Many more women than men are affected, and the incidence of depression is even higher following menopause^{2,3}. A study conducted by Timur and Sahin reported that out of 685 women sampled, 56.9% of postmenopausal women suffered from depression⁴. In another study reported recently, the depression frequency was shown to be as high as 59.3% in postmenopausal women⁵.

Antidepressants have become one of the most commonly prescribed drug classes in the United States^{6,7}.

Common side effects of antidepressants include noradrenergic effects (orthostatic hypotension), histaminergic effects (sedation and weight gain), and anticholinergic effects (dry mouth, impaired vision, and constipation)⁸. Recently, antidepressant use was reported to have negative impacts on bone, especially in older people⁹. Among postmenopausal women, several previous studies had found a significant inverse association between antidepressant use and bone mineral density (BMD)^{10–12}. In addition, two longitudinal studies showed that the use of antidepressants accelerates bone loss in postmenopausal women^{11,13}.

The mechanisms of bone loss with chronic use of certain antidepressants remain largely unknown. It was proposed that the bone loss might be due to the inhibition of serotonin receptors and transporters in bone cells by selective serotonin reuptake inhibitors (SSRIs)^{14,15}. In addition, both in vitro cell culture and in vivo animal studies showed that SSRIs like fluoxetine directly

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inhibited human osteoclasts and osteoblasts differentiation^{16,17}. However, a large cohort cross-sectional study (73,072 subjects) found that the differences in fracture risk across specific antidepressant medications were not dependent on the affinity for serotonin transport receptors⁹. The above study indicated that other mechanisms might be present other than the inhibition of serotonin transport receptors by SSRIs.

Bone marrow adipose tissue (BMAT) accounts for up to 70% of bone marrow volume. BMAT is a unique fat depot that is distinct from peripheral white adipose tissue, both developmentally and functionally. BMAT has been considered as a functional organ and the regulator of bone metabolism due to its unique secretory and metabolic functions in osteoclastogenesis and hematopoiesis¹⁸. BMAT had been reported to be regulated by a range of metabolic diseases and treatment such as diabetes, obesity, glucocorticoids as well as aging-associated bone loss, and osteoporosis^{19,20}. There is no information available regarding the effects of antidepressants on BMAT.

In this study, we first investigated the influence of long-term fluoxetine treatment on bone loss and the metabolism of BMAT using two OVX animal models (rabbits and rats) followed by an in-depth mechanistic analysis of its effects on bone marrow adipocytes (BMAs). We showed that fluoxetine-induced bone loss is associated with the disruption of sphingolipids metabolism in BMAT by inhibiting acid sphingomyelins (ASM). In addition, we evaluated whether oral supplementation of L-serine, a precursor of sphingolipids biosynthesis, could prevent the accelerated bone loss caused by fluoxetine through both animal models, and a small clinical trial.

Materials and methods

The brief materials and procedures were summarized here, and the additional details were described in the Supplemental materials.

Animals, materials, and reagents

Animals: Female New Zealand rabbits (5-month-old, 2.95 ± 0.42 kg) and 7-week-old female Sprague Dawley (SD) rats (190 ± 20 g) were purchased from Guangdong Medical Laboratory Animal Center. Four-week-old female C57BL/6 (wild type) and ASM knockout (*Smpd1*^{-/-}) mice were purchased from the Nanjing Biomedical Research Institute of Nanjing University. The animals were housed under standard conditions (12:12 light-dark cycle; food and water ad libitum; temperature: 22–24 °C and humidity: 40–60%). All the animal protocols in this study were approved by the Institutional Animal Care and Use Committee of Jiangsu Vocational College of Medicine (YCZYX-2015-1327) and carried out in accordance with the National Institutes of Health (NIH)

Guidelines for the use of animals in research. All animals were acclimatized for 1 week before the experiments.

Materials and reagents: commercial stable isotope standards including ceramide (d18:1/18:1)-¹³C₁₈ and ceramide (d18:1/16:0)-¹³C₁₆ were obtained from Cambridge Isotope Laboratories Inc (MA, USA). SM(d18:1/17:0) and sphingosine-1-phosphate-d7 used for sphingolipids quantification was obtained from Avanti Polar Lipids (AL, USA). All other stable isotope internal standards were prepared according to the previous report²¹. The antibodies, including Anti-ASA3 (Catalog no. ab113916), Anti-Acid sphingomyelinase (Catalog no. ab83354), Anti-COX2 (Catalog no. ab169782), Goat Anti-Rabbit IgG (Catalog no. ab6721) were all purchased from Abcam (CA, USA). Pierce™ ECL Western Blotting Substrate (Catalog no. 32106) was obtained from ThermoFisher (MA, USA). Fluoxetine tablets (Prozac) were made by Eli Lilly and Company (IN, USA). L-serine powder and the placebo dextrose powder were provided by Jiangsu Metabo Biotech (Jiangsu, China). Other reagents were purchased from Sigma Aldrich.

Rabbit and rat animal models

For bilateral ovariectomy (OVX) model of rabbit: the bilateral ovaries were surgically removed under the aseptic condition to mimic the postmenopausal changes according to the previous procedures²². The sham group underwent the same surgical procedures except for the removal of ovaries. Gentamicin (40,000 U) was given intramuscularly for 5 days to prevent infection after surgery. The wound healing started at about 5 days after surgery. Rabbits then received either fluoxetine (10 mg/kg/d) or saline for 5 months.

For OVX SD rats with chronic unpredictable mild stress (OVX-CUMS): we created a rat model of postmenopausal depression as described²³. The bilateral ovaries were surgically removed under the aseptic condition. The sham group underwent the same surgical procedures except for the removal of ovaries. After 2 weeks of recovery, the rats were exposed to different and unpredictable stressors that were randomly altered during the experiment (Fig. S1). At week 4, rats were treated with either fluoxetine (10 mg/kg/d) or saline for another 6 weeks. Behavior tests including tail suspension tests (TSTs) and forced swim tests (FSTs) were conducted before fluoxetine treatment and 6 weeks after treatment. The duration of immobility in both TSTs and FSTs was calculated using the last 4 min of the testing period. The experimenters were blind to the group conditions for all behavior tests.

BMD analysis was performed using the dual-energy X-ray absorptiometry (DEXA) (PIXImus 2, GE Healthcare, WI, USA). The BMD values of the distal right femora for rabbits and rats were obtained using the manufacturer-supplied software Lunar Piximus 2 (V2.0).

Metabolomic analysis of BMAT

After overnight fasting (about 12 h), BMAT was isolated from the femurs based on the protocol of Tencerova and the co-authors with modifications²⁴. Briefly, the femurs were longitudinally bisected using a Dremel rotary tool with a 409 cutoff wheel, and the BMAT was then removed using a stainless-steel spatula. The BMAT was snap-frozen for further metabolomic analysis.

The extraction of metabolites was performed using the prechilled (-20°C) extraction buffer containing MeOH-ACN- H_2O (43:43:16, v/v/v) and stable isotope standards as described before^{25,26}. The metabolomic analysis was performed on an Agilent 1290 HPLC coupled with a SCIEX QTRAP 5500 triple quadrupole mass spectrometer (LC-MS/MS)²⁶. The metabolites were separated on a Luna NH_2 HPLC column (250 mm \times 2.0 mm, 5 μm , Phenomenex, USA). The MS was performed by multiple reaction monitoring (MRM) in both negative and positive mode with rapid polarity switching (50 ms). A total of 420 metabolites covering all the major metabolic pathways were targeted²¹. Data were log 2 transformed before statistical analysis. Multivariate analysis (Partial least squares discriminant analysis, PLS-DA), pathway analysis, and ANOVA simultaneous component analysis were performed using Metaboanalyst 4.0 (www.metaboanalyst.ca). The details were described in the Supplementary method.

Quantification of total ceramides, sphingomyelins (SMs), and sphingosine-1-phosphate (S1P)

BMAT was collected after overnight fasting of the animals. The extraction and quantification of sphingolipids, including ceramides, SMs, and S1P in BMAT were performed by LC-MS/MS (Agilent 1290 HPLC coupled with SCIEX QTRAP 5500)^{27,28}. Total lipids were extracted from 50 mg of BMAT tissue using 300 μL of extraction buffer containing chloroform/methanol (1:2, v/v) and stable isotope standards. The lipids were separated on an ACQUITY UPLC BEH C_{18} column (2.1 \times 150 mm, 1.7 mm) and quantified on a QTRAP 5500 MS/MS (SCIEX, USA) in both negative and positive mode. A total of 36 sphingomyelins and 38 ceramides were targeted. The MRM transition for S1P was 380.3 and 264.3 (Q1 and Q3). The details were described in the Supplementary method.

Western blot analysis

We subjected protein extracts from BMAT and bone marrow adipocytes (BMAs) to western blotting using the following primary antibodies: Anti-ASAH3 (1:5000), Anti-SPHK1 (1:2000), Anti-ASM (1:5000) and Anti-COX2 (1:5000). We visualized immunocomplexes with a horse-radish peroxidase (HRP)-conjugated anti-rabbit- or anti-mouse-IgG antibody (1:5000), followed by incubation with the enhanced chemiluminescence solution (ThermoFisher). Signals were detected using the ChemiDoc

XRS+ Imaging System (BioRad) and the imaged blots were quantified using the ImageJ software. The details were described in the Supplementary method.

μCT analysis

Bone skeletal microarchitecture of the right femora for rats and the mice (wild type and ASM knockout) was determined using a high-resolution μCT machine (inspeXio, SMX-100CT, Shimadzu). The settings for the X-ray tube were voltage 90 kV and the current 100 μA . The samples were placed in a 20-mm diameter micro-centrifuge tube and scanned at a voxel size of 15.0 μm . The trabecular bone region of the right distal femora was selected for the analysis of bone microarchitecture parameters, including bone volume/total volume (BV/TV, %), trabecular number (Tb.N) and trabecular spacing (Tb.Sp) using the manufacturer's software TRI/3D-BON. Eight animals per group were used.

Cell culture, in vitro treatments and imaging

BMAs were isolated from 3-month-old wild-type rabbits and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin at 37°C with 5% CO_2 as described before²⁹. The cells were treated with various doses of fluoxetine (0.005, 0.01, 0.05, 0.1, 0.5, and 1 μM), cisplatin (CDDP, 2.5 μM), S1P (0.05, 0.5, 5 μM) and L-serine (1 μM) for 24 h. All the chemicals were dissolved in DMEM/F12 medium before treatment. After 24 h, the culture was then centrifuged, and both the cells and the media were collected and snap-frozen in liquid nitrogen for further analysis. Six replicates were set up for each group.

Osteoclast precursor RAW 264.7 cells (American Type Culture Collection) were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin). The cell line was tested for mycoplasma contamination before experiment (Biotool Mycoplasma Removal Kit, Bimake). To investigate the effects of fluoxetine-treated BMAT on osteoclastogenesis, RAW 264.7 cells were seeded in 96-well plates and rat BMAT supernatant from OVX-CUMS-Flx (OVX-CUMS rats treated with fluoxetine for 6 weeks) and OVX-CUMS-Flx-Ser (OVX-CUMS rats with fluoxetine and L-serine) was added. Cultures were maintained for 5 days. TRAP staining was performed using a TRAP staining kit (B-Bridge, Tokyo, Japan). Briefly, the primary osteoclast cultures were fixed in 10% formalin, washed and incubated with TRAP staining reagent according to the manufacture's instruction. The cultures were visualized with a Leica DM400B microscope (Wetzlar, Germany). ImageJ software was used to quantify the number of cells and nuclei for each osteoclast. Stained osteoclasts with ≥ 3 nuclei were classified as being TRAP-positive.

Receptor activator of nuclear factor-kappa-B ligand (RANKL) and PGE2 ELISA assay

The levels of RANKL in BMAs and the supernatant were measured using a RANKL ELISA kit (Abcam, Shanghai, China). The concentration of prostaglandin E2 (PGE2) in BMA was determined using a PGE2 ELISA kit (Abcam, CA, USA). The analysis was performed according to the manufacturer's instructions.

RNA expression and qPCR

Total RNA was extracted from BMAs using TRIZOL (Invitrogen, CA, USA). Reverse transcription of 1 µg total RNA was performed by using the Easy Script First-Strand cDNA Synthesis Super Mix kit (TransGen Biotech, Beijing, China). qPCR was performed on a StepOne™ Real-Time PCR Systems (Applied Biosystems) using SYBR Green PCR Master Mix ThermoFisher (MA, USA). GAPDH was used as the reference gene. The primers used for the analysis were listed in Table S1.

L-serine treatment for animals

The animals were randomized and intragastrically treated with fluoxetine (10 mg/kg body weight), L-serine (10 mg/kg body weight) or both. Other groups were given an equal amount of 0.9% saline solution. The total sample size estimate was 6 per group based on the assumed effect size of 0.6, with the expected α and power values set as 0.05 and 0.8, respectively. We actually used 8 animals per group. The treatment lasted 5 months for rabbits and 6 weeks for rats. The BMD of the right femur, the metabolomic analysis of BMAT, total ceramides, sphingomyelins, and S1P in BMAT were analyzed at the end of the experiment.

L-serine treatment for postmenopausal patients with long-term fluoxetine use

The study was approved by the Institutional Review Board of Jiangsu Vocational College of Medicine (2019-007). It was also registered on the Chinese Clinical Trial Registry (ChiCTR1900023505) and conformed to the World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects. The inclusion criteria were female subjects between 45 and 55 years old and were in perimenopause or menopause (change in the regularity of menses in the past 12 months, or no period in the past 12 months). Patients had a confirmed diagnosis of major depression disorder (MDD) and were prescribed to take Prozac tablets (fluoxetine, 20 mg/d) before the study. Patients were asked not to change their diet and therapy throughout the whole study. Exclusion criteria comprised the co-existence of other major diseases, including severe diabetes, coronary vascular diseases, malignant tumors, and renal failure. We required at least 13 subjects per

Table 1 Baseline characteristics for the participants in the clinical trial.

Characteristic	Fluoxetine + Placebo	Fluoxetine + Serine	P value
N	20	20	NA
Sex	F	F	NA
Age, years	47.2 ± 5.3	48.4 ± 4.5	0.45
BMI, kg/m ²	27.2 ± 3.8	27.8 ± 4.9	0.67
Weight change during the study (kg)	−0.54 ± 3.89	0.62 ± 4.12	0.37
Menopausal status, n (%)			
Postmenopausal	12 (60%)	13 (65%)	1.00
Late perimenopausal	8 (40%)	7 (35%)	1.00
Current calcium supplement user, n (%)	5 (25%)	4 (20%)	1.00
Current vitamin D supplement user, n (%)	5 (25%)	5 (25%)	1.00
Total lumbar spine BMD, g/cm ²	0.94 ± 0.23	0.91 ± 0.17	0.64
Total hip BMD, g/cm ²	0.94 ± 0.17	0.99 ± 0.22	0.43
Diagnosis			
MDD	20	20	N/A
Montgomery-Åsberg depression rating scale	28.6 ± 9.1	30.2 ± 7.8	0.55
Use of other medications that may modify fracture risk			
Proton-pump inhibitor, n (%)	3 (15%)	2 (10%)	1.00
Thiazolidinediones, n (%)	1 (5%)	1 (5%)	1.00

Data were mean ± sem or the values with percentages. Student's *t*-test, Mann-Whitney *U* test or Two-proportion *z*-test was used.

group to detect a large effect size with 80% power. A total of 40 patients were enrolled in this study, and the written informed consent was obtained from all the subjects. The participants' baseline characteristics were described in Table 1.

Patients were randomly divided into two groups: (1) Fluoxetine (20 mg/d) + L-serine (250 mg/kg/d): L-serine was given for 12 months at the dose of 250 mg/kg/d (divided into 2 dietary supplements of L-serine powder, mixed with food and/or drinks). This dose was selected based on previous observations³⁰. (2) Fluoxetine (20 mg/d) + Placebo (250 mg/kg/d): the placebo (dextrose powder) had the identical appearance, taste, and smell to L-serine powder. The trial was conducted between May 2017 and August 2018, and the treatment lasted for 12 months.

The subjects underwent DEXA scans of their lumbar spine (L1–L4) and hip using a Horizon DXA system (Hologic, MA, USA) at the beginning and the end of the trial. Magnetic resonance imaging (MRI) of the lumbar spine was performed on a Siemens 3.0 T MRI scanner (MAGNETOM Skyra, Erlangen, Germany). Blood was taken in the morning after overnight fasting for about 12 hours and the serum was prepared. Serum carboxy-terminal telopeptide of type I collagen (CTX-I) concentrations were measured using a serum CrossLaps® ELISA kit (IDS Ltd., London, UK).

Statistical analysis

Data analysis except specified was conducted using GraphPad Prism 8.0 (Prism, CA, USA). Shapiro–Wilk test was used to check the normal distribution of the data. One-way ANOVA (parametric) or Kruskal–Wallis (non-parametric) followed by Post hoc Tukey's or Duncan's test was used for multiple comparisons. The student's *t*-test (parametric, two-sided) or Mann–Whitney *U* test (non-parametric) was used to calculate the significance levels between two groups of the continuous variables. Two-proportion *z*-test was used for the analysis of two proportions. $P < 0.05$ was considered significantly different. Data were presented as means \pm sem from at least 3 replicates.

Results

Long-term treatment with fluoxetine accelerates bone loss in both OVX rabbits and OVX rats subjected to CUMS

To test the effect of long-term fluoxetine treatment on bone remodeling, we treated female OVX rabbits with fluoxetine for 5 months. We found that fluoxetine administration (10 mg/kg/d) significantly accelerated the reduction of BMD in OVX rabbits (Fig. 1a).

To further confirm the long-term adverse effect of fluoxetine on bone mass, we created a rat model of postmenopausal depression (Supplementary Fig. S1A). We showed that rats with OVX and CUMS demonstrated depressive-like behaviors, which were corrected by fluoxetine treatment for 6 weeks (Supplementary Fig. S1B and S1C). However, rats treated with fluoxetine for 6 weeks showed dramatically lower BMD and bone volume/tissue volume (BV/TV) compared with the controls treated with saline (Fig. 1b, c).

Inhibition of acid sphingomyelinase (ASM) by fluoxetine in BMAT is associated with bone loss

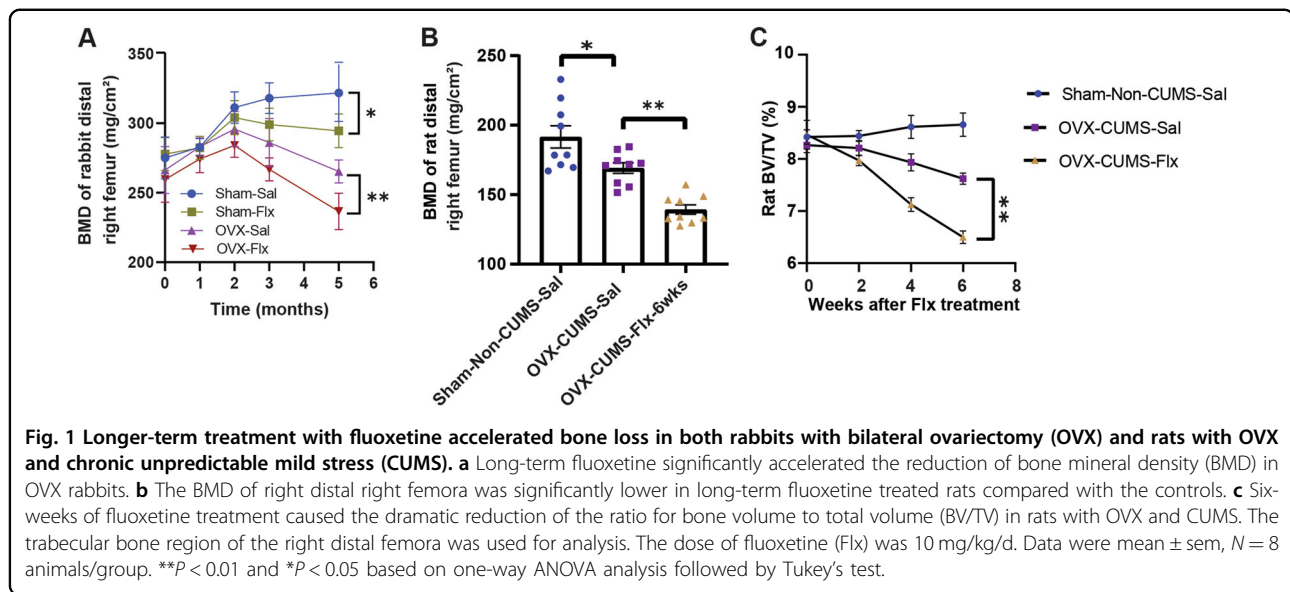
BMAT is increasingly recognized as the regulator of bone metabolism¹⁸. We next explored the post-OVX metabolomic changes in BMAT of rabbits at 0, 1-month and 3-month. The detailed results were described in the Supplementary materials (Supplementary results and Fig. S2). The key finding of this experiment was that sphingolipids metabolism is the main metabolic pathway disturbed in BMAT during the progression of bone loss in rabbits with OVX (Fig. 2a). We then analyzed the changes of total sphingomyelins (SMs), ceramides, and sphingosine-1-phosphate (S1P) after long-term of fluoxetine treatment in both rabbits and rats. As shown in Fig. 2b, c, the fluoxetine administration led to the accumulation of total SMs and a significant decrease of total ceramides in BMAT in both sham and OVX rabbits. In addition, we noticed a dramatic reduction of S1P in response to fluoxetine (Fig. 2d). Spearman correlation analysis showed that the reduction of BMD was

significantly correlated with the increase of total SMs concentration and the decrease of the S1P level in BMAT (Fig. 2e). Similarly, we found that total SMs in BMAT was significantly higher in OVX-CUMS rats treated with fluoxetine for 6 weeks compared with the saline group (Fig. 2f). In contrast, total ceramides and S1P in BMAT were dramatically reduced after long-term treatment with fluoxetine in OVX-CUMS rats (Fig. 2g, h). S1P and ceramide species were the top metabolites positively correlated with BMD, while, SMs species were negatively correlated with BMD changes in BMAT of rats (Fig. 2i). The above results suggested that fluoxetine causes bone loss through the disruption of sphingolipids metabolism in BMAT.

We then analyzed the effects of fluoxetine on the expression of key enzymes in sphingolipids metabolism in BMAT of OVX rabbits, including sphingosine kinase 1 (SPHK1), alkaline ceramidase 1 (ACER1) and acid sphingomyelinase (ASM). We found that fluoxetine significantly reduced the expression of SPHK1, ACER1, and ASM in the BMAT of both sham and OVX rabbits (Fig. 2j). Since fluoxetine was reported to be a potent inhibitor of ASM³¹, we then tested the role of ASM in bone turnover and mineralization using ASM knockout (*Smpd1*^{-/-}) mice. μ CT analysis of the trabecular bone region of right distal femora revealed that at 20 weeks, ASM KO mice had significantly lower bone volume/tissue volume (BV/TV) and the trabecular number (Tb. N) compared with the wild type (Fig. 2k–m). In contrast, the trabecular spacing (Tb. Sp) was dramatically larger in ASM KO mice (Fig. 2n). These results suggested that inhibition of ASM by fluoxetine leads to the disruption of sphingolipids metabolism in BMAT and accelerates bone loss.

Fluoxetine stimulates RANKL secretion by BMAs through COX-2/PGE2 pathway

To further elucidate how the inhibition of ASM by fluoxetine accelerates the bone loss, we first treated the rabbit BMAs primary culture with different doses of fluoxetine (0, 0.005, 0.01, 0.05, 0.1, 0.5 and 1 μ M) in vitro. We found that fluoxetine treatment reduced the intracellular level of S1P in BMAs in a dose-dependent manner (Fig. 3a). BMAT is considered an essential regulator of bone turnover and reabsorption through the secretion of adipokines, inflammatory factors, and other molecules¹⁸. We next tested the effect of fluoxetine addition on the secretion of adipokines and cytokines by BMAs. As shown in Fig. 3b, fluoxetine treatment caused a significant increase of receptor activator of nuclear factor-kappa-B ligand (RANKL). Moreover, RANKL secretion by BMAs increased gradually with the increase of fluoxetine addition in a dose-dependent manner (Fig. 3c). In contrast, the secretion of leptin, IL-6, and TNF-alpha was not changed after fluoxetine treatment (Supplementary Fig. S3).



CDDP is a compound that could upregulate the expression of ASM. We showed that the increase of RANKL secretion by fluoxetine was completely normalized by the addition of CDDP in the culture of BMAs (Fig. 3b). This confirmed the link between the inhibition of ASM by fluoxetine and the increased RANKL secretion by BMAs.

Since fluoxetine treatment also reduced the S1P level in BMAs (Fig. 3a), we then hypothesized that the upregulation of RANKL secretion by fluoxetine in BMAs is negatively regulated by the intracellular S1P. To test the hypothesis, we first added S1P to the culture of BMAs along with fluoxetine. After 24 h treatment, we found the level of RANKL in the media in Flx + S1P group was closer to the untreated control and significantly lower than that in fluoxetine alone group (Fig. 3d). L-serine is the precursor of sphingolipids metabolism. Similar to the direct addition of S1P, the increase of RANKL secretion by fluoxetine was normalized with the co-addition of 1 μ M of L-serine (Fig. 3d). We next analyzed the effect of fluoxetine on RANKL gene expression in BMAs. We found that fluoxetine treatment induced the significant overexpression of RANKL in BMAs, which was normalized by S1P or L-serine treatment (Fig. 3e). COX-2/PGE2 and TNF- α are known to upregulate the expression of RANKL³². Since TNF- α expression was not altered by fluoxetine treatment (Supplementary Fig. S3C), we then tested whether S1P negatively regulates COX-2 expression and PGE2 production. Fluoxetine alone significantly increased the expression of COX-2 in BMAs (Fig. 3f). The co-addition of S1P to the culture resulted in a prominent decrease in COX-2 protein expression in a dose-dependent manner (Fig. 3f). Similarly, PGE2 production in the fluoxetine group was significantly higher than the untreated controls (Fig. 3g). PGE2 concentration decreased dramatically with

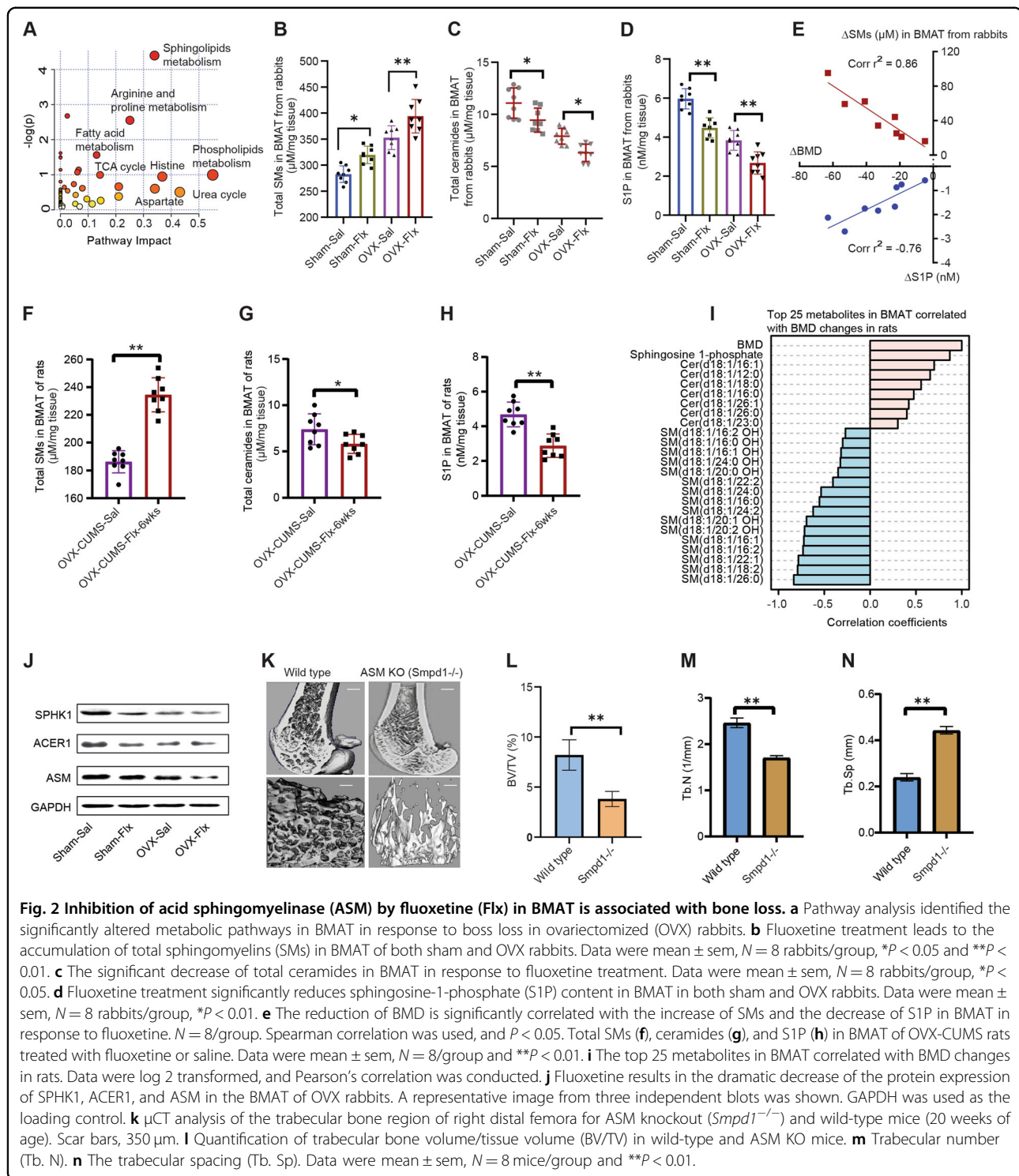
the increased addition of S1P to BMAs culture (Fig. 3g). These data suggested the expression of COX-2/PGE2 is negatively regulated by S1P level in BMAs.

To further confirm the direct linkage between fluoxetine-induced changes in BMAs and the bone loss, we treated RAW 264.7 cells with BMAT supernatant to check the formation of osteoclasts. We found that the osteoclastogenesis of RAW264.7 cells was stimulated by the supernatant of BMAT from OVX-CUMS rats received 6 weeks of fluoxetine treatment (OVX-CUMS-Flx) (Fig. 3h). In contrast, the formation of osteoclasts was inhibited by BMAT supernatant from OVX-CUMS-Flx-Ser (OVX-CUMS rats with fluoxetine and L-serine) (Fig. 3i). The number of TRAP-positive cells (Osteoclasts) was significantly higher in the OVX-CUMS-Flx group than that in the OVX-CUMS-Flx-Ser group (Fig. 3j).

Based on the previously known facts and our data here, a proposed schematic diagram of fluoxetine-induced bone loss was shown in Fig. 3k. Briefly, fluoxetine treatment inhibits ASM and thus disrupts sphingolipids metabolism in BMAs and leads to the decrease of ceramides and its subsequent metabolite S1P. The significant reduction of S1P causes the overexpression of RANKL through the upregulation of the COX-2/PGE2 pathway. The increased RANKL secretion from BMAs promotes osteoclast formation and eventually leads to bone loss.

L-serine treatment corrects the acceleration of bone loss induced by fluoxetine in both animal models and postmenopausal women with MDD

L-serine is the precursor for sphingolipids biosynthesis. We next investigated whether L-serine supplement could correct the acceleration of bone loss caused by long-term fluoxetine use. We first treated the OVX rabbits with



fluoxetine (10 mg/kg body weight) and L-serine (10 mg/kg body weight) for 5 months and compared with the untreated OVX rabbits and sham controls. Metabolomic analysis of BMAT followed by 3-D PLS-DA revealed the distinct separation of L-serine treated group from the saline control (OVX-Flx-Ser vs. OVX-Flx-Sal) (Fig. 4a).

More specifically, L-serine treatment significantly increased the levels of total ceramide (Supplementary Fig. S4A), ceramide species (Supplementary Fig. S4B), and S1P (Supplementary Fig. 4B) in BMAT compared with OVX-Flx-Sal group. The accelerated reduction of BMD caused by fluoxetine in rabbits with OVX was prevented by

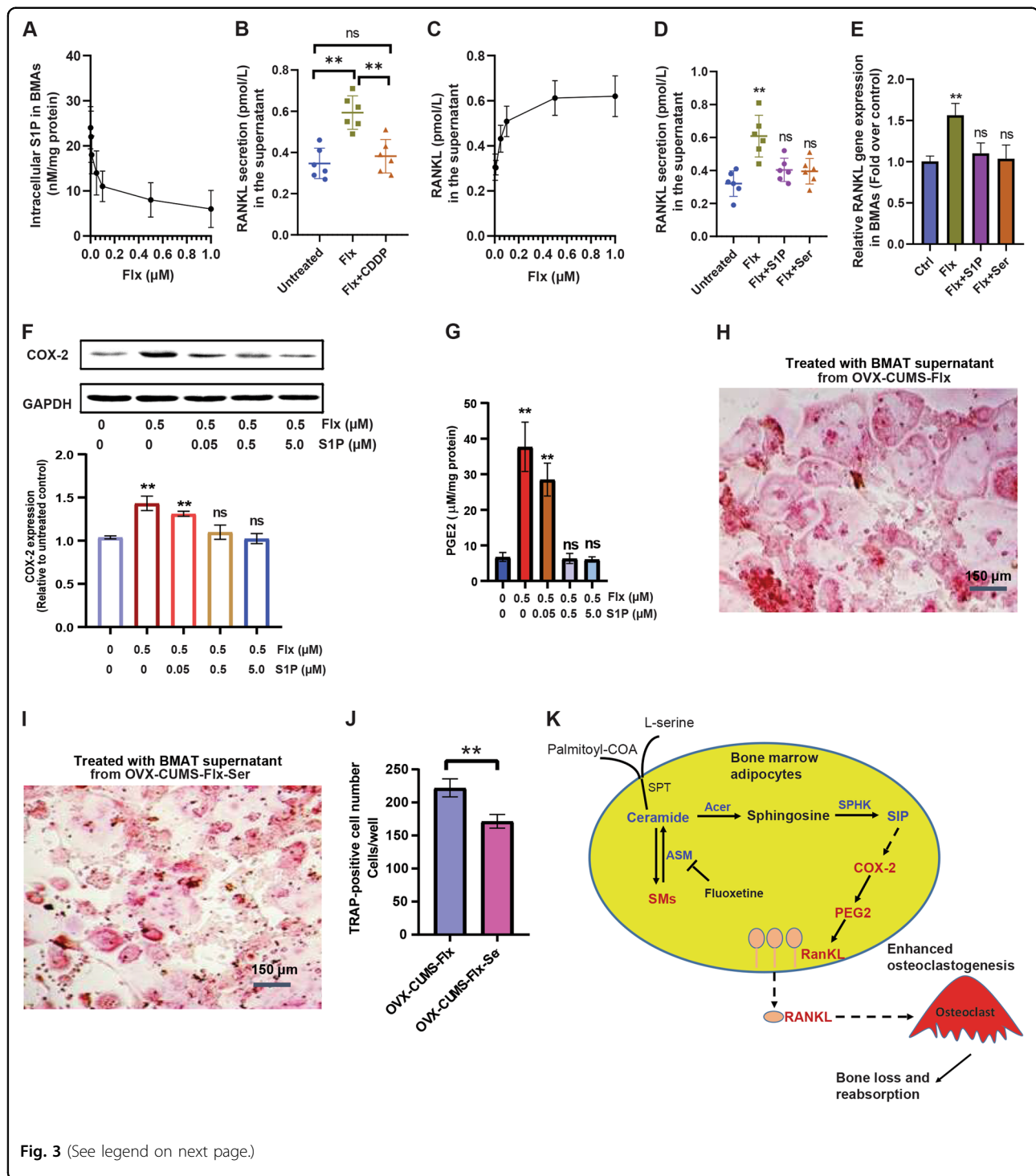


Fig. 3 (See legend on next page.)

L-serine treatment for 5 months (Fig. 4c). Similarly, L-serine normalized the rapid decline of BMD in OVX-CUMS rats caused by long-term fluoxetine treatment (Fig. 4d). Interestingly, we found that L-serine treatment did not produce dramatic antidepressant-like effects on OVX-CUMS rats (Supplementary Fig. S5). A previous study

showed that chronic L-serine treatments exert antidepressant-like effects on Wistar Kyoto rats³³. The discrepancy between our study and the previous report might be due to the differences in animal models.

We next tested the effects of L-serine oral supplementation on the bone loss of postmenopausal MDD

(see figure on previous page)

Fig. 3 Inhibition of acid sphingomyelinase by fluoxetine induces RANKL secretion in bone marrow adipocytes through the COX-2/PGE2 pathway mediated by intracellular S1P. **a** Fluoxetine treatment (0, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 μ M) resulted in a dose-dependent reduction of intracellular S1P in BMAs. Data were mean \pm sem ($N = 6$ /dose). ****** $P < 0.01$ compared with no fluoxetine addition group. **b** RANKL secretion by bone marrow adipocytes (BMAs) that received 24 h of treatment with fluoxetine (5 μ M) or the combination of fluoxetine (5 μ M) and cisplatin (CDDP, 2.5 μ M, a known compound that could upregulate ASM expression). Data were mean \pm sem ($N = 6$ /group). ****** $P < 0.01$ and ns: not statistically significant. **c** The dose-dependent RANKL secretion by BMAs in response to different levels of fluoxetine treatment (0.005, 0.01, 0.05, 0.1, 0.5 and 1 μ M). Data were mean \pm sem ($N = 6$ /dose). **d** The increase of RANKL secretion by fluoxetine in the culture of BMAs was normalized with the co-addition of S1P (0.5 μ M) and L-serine (1 μ M). Data were mean \pm sem ($N = 6$ /dose). ****** $P < 0.01$ compared with the untreated controls and ns: not statistically significant. **e** The significant upregulation of RANKL mRNA expression by fluoxetine addition in BMAs was normalized with the treatment of S1P (0.5 μ M) and L-serine (1 μ M). **f–h** The osteoclastogenesis of RAW264.7 cells was stimulated by the supernatant of BMAT from OVX-CUMS rats received 6-week of fluoxetine treatment (OVX-CUMS-Flx), while, the formation of osteoclasts was inhibited by BMAT supernatant from OVX-CUMS-Flx-Ser (OVX-CUMS rats with fluoxetine and L-serine). **f** The formation of TRAP-positive cells (Osteoclasts) stimulated by BMAT supernatant from the OVX-CUMS-Flx group. **g** TRAP-positive cells incubated with BMAT supernatant from OVX-CUMS-Flx-Ser. **h** The number of TRAP-positive cells was significantly higher in the OVX-CUMS-Flx group than that in the OVX-CUMS-Flx-Ser group. ****** $P < 0.01$ ($N = 6$ wells/group). **i** S1P negatively regulates COX-2 protein expression in BMAs with the increase of S1P concentrations from 0 to 5 μ M. Top: A representative western blot image. Bottom: the quantification of COX-2 expression from 3 replicates. Data were mean \pm sem. ****** $P < 0.01$ and $*P < 0.05$ compared with the untreated control. **j** The addition of S1P led to a significant decrease in PGE2 levels in a dose-dependent manner. Data were mean \pm sem ($N = 6$ /dose). ****** $P < 0.01$ compared with the untreated controls. **k** The proposed mechanism for fluoxetine-induced bone loss. The red color indicated an increase (or upregulation) and the blue color indicated a decrease (or downregulation) after fluoxetine treatment. The solid unbroken lines indicated the direct reactions or interactions, and the broken line indicated indirect reactions or interactions (multiple steps) between two sides.

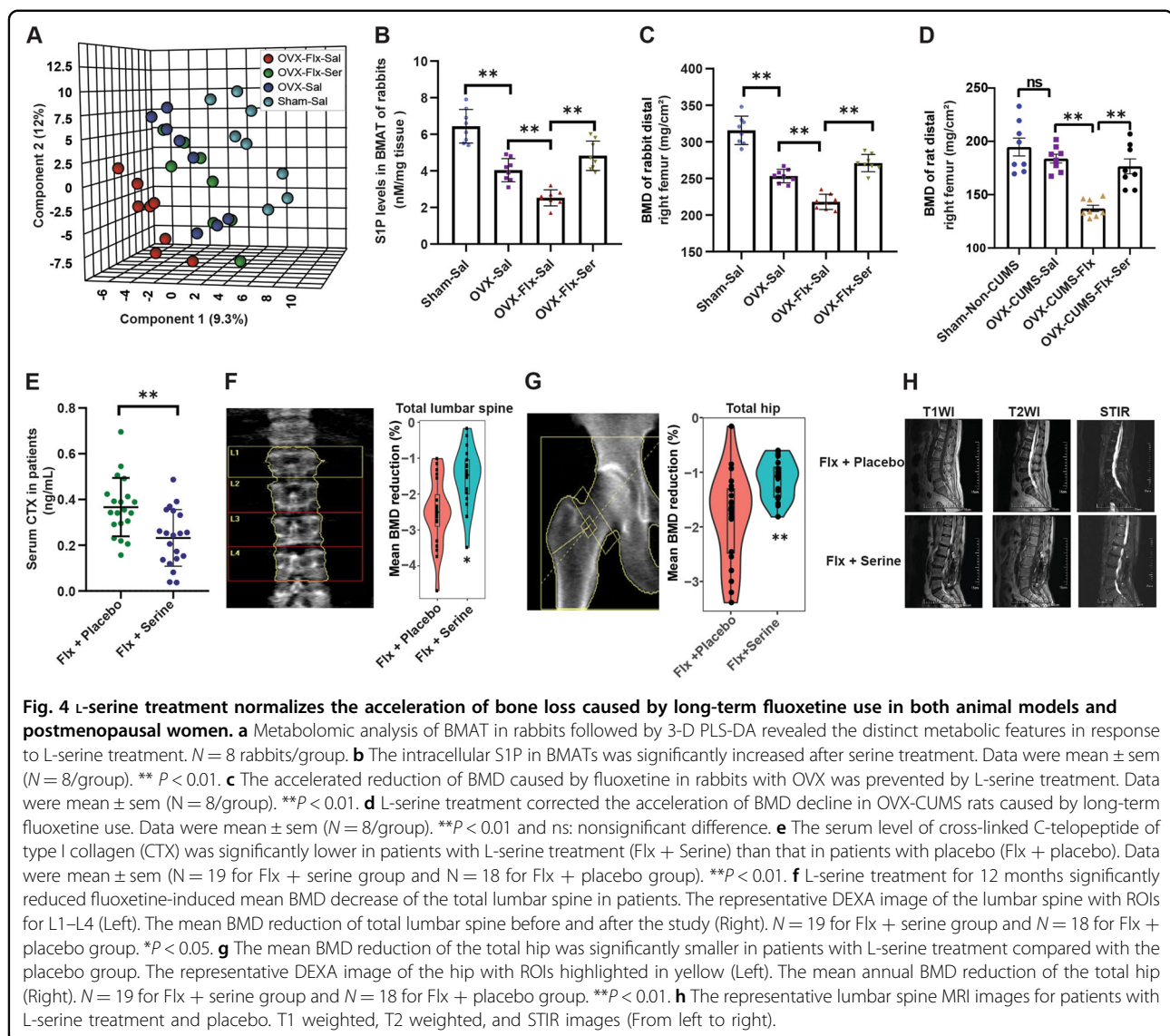
women patients who were taking fluoxetine (25 mg/d). Patients were randomly divided into 2 groups with the 12-month treatment of L-serine (250 mg/kg/d) or placebo in addition to fluoxetine. A total of 20 patients was enrolled in each group, and the baseline characteristics were well-balanced between 2 study groups (Table 1). Two participants in the placebo group and 1 subject in the L-serine treatment group lost to follow-up. No significant adverse events were reported from either group during the study. The serum CTX level is a known biomarker of bone breakdown/resorption. We found that the serum CTX level was significantly lower in the L-serine treatment group than the placebo (Fig. 4e). The primary outcome for this trial is the mean annual BMD reduction. We found that the mean BMD reduction of the total lumbar spine (Fig. 4f) and total hip (Fig. 4g) was significantly smaller in patients with L-serine treatment compared with the placebo group. In addition, we observed abnormally increased signals on T1WI and T2WI from T11-12 and L1-2 vertebral bodies in patients with placebo (Fig. 4h). The abnormally increased signals disappeared on the STIR (Fat suppression) image. These observations suggested the dramatic bone loss in these patients received the placebo. Overall, the test here suggested that oral L-serine supplementation appears to be safe in postmenopausal MDD women patients at the dose of 250 mg/kg/d and is potentially effective at slowing the bone loss caused by fluoxetine treatment.

Discussion

Both animal and human clinical studies have clearly shown that chronic use of certain antidepressants such as fluoxetine in postmenopausal women causes bone loss

and osteoporosis^{13,34}. The reduction in bone quality was independent of estrogen deficiency and primarily linked to the inhibition of the serotonin transporters by SSRIs¹⁴. However, differences in fracture risk among antidepressant classes could not be simply explained by their affinities for serotonin transport receptors⁹. In this study, we provided strong evidence that other than, or in addition to, a serotonin-mediated effect, fluoxetine accelerates bone loss through the disruption of sphingolipids metabolism in BMAT. In detail, inhibition of ASM by fluoxetine reduces cellular ceramides and S1P levels in BMAs, which then inversely regulates COX-2 expression and PGE2 biosynthesis in a dose-dependent manner. PGE2 overproduction induces the secretion of RANKL by BMAs and promotes osteoclastogenesis. The association between ASM inhibition and bone loss was further confirmed using ASM knockout mice (*Smpd1*^{-/-}), which have dramatically lower bone volume density than the wild type. In contrast, treatment with CDDP, a known chemical that upregulates ASM, reduces the secretion of RANKL by BMAs. Furthermore, we demonstrated that L-serine treatment prevents the accelerated bone loss caused by fluoxetine in both rabbits OVX model and postmenopausal women with MDD.

Our study, for the first time, investigated the actions of fluoxetine on BMAT. The interaction between BMAT and bone is complicated. On the one hand, BMAT alters its volume in response to drug treatment, nutrition, aging, exercise, and environmental stress. For example, pharmacological FGF21 treatment induces severe bone loss by stimulating adipogenesis from bone marrow mesenchymal stem cells³⁵. Similarly, oral prednisolone intake accelerates the transformation of hematopoietic into fatty



marrow³⁶. On the other hand, BMAT secretome, including adipokines (leptin and adiponectin, etc.), inflammatory factors (IL-6 and TNF- α , etc.), and RANKL can regulate bone metabolism^{18,37,38}. The secretion profile of BMAT in response to antidepressants remains largely unknown. In this study, we found that fluoxetine causes a significant increase in RANKL secretion from primary BMAs. RANKL acts as a key regulator for the activation of osteoclastogenesis and bone loss³⁹. Lowering RANKL gene expression in BMAT inhibited osteoclastogenesis and bone resorption and thereby caused the increase of bone mass⁴⁰. Our observation in fluoxetine is similar to the previous findings in the chronic use of synthetic steroids. In these studies, the authors found that RANK expression was dramatically stimulated by dexamethasone in primary BMAs, which suggested a mechanistic link between dexamethasone and bone loss^{41,42}.

Our study highlighted the tissue-specific sphingolipids metabolism for bone remodeling. ASM is the key enzyme in the sphingolipids metabolic pathway that catalyzes the breakdown of sphingomyelins to ceramides. In this study, we show that inhibition of ASM by fluoxetine reduces the levels of all major molecular species of ceramide as well as S1P, which then leads to the increased secretion of RANKL in BMAs. The knockout of ASM (*Smpd1*^{-/-}) in mice causes significant bone loss. Similar to the mice ASM knockout model, patients with ASM deficiency (ASMD) usually have a significant decrease in BMD and bone mineral content⁴³. According to a report of the cross-sectional study, 19% of the patients with ASMD had suffered one or more bone fractures⁴⁴. ASM is one of the enzymes in the family of sphingomyelinase. Previous studies had investigated the role of neutral sphingomyelinase (nSMase2) in bone development. Inactivation of

Smpd3 (encoding nSMase2) in mice causes osteogenesis imperfecta⁴⁵. In addition, *Smpd3* expression was shown to be a critical regulator for normal bone development and mineralization⁴⁶. In chondrocytes, bone morphogenic protein (BMP) signaling upregulates nSMase2 to suppress chondrocyte maturation⁴⁷.

S1P signaling has a fundamental role in bone metabolism. A recent study reported that raising S1P levels in adult mice significantly increased bone formation, mass, and strength⁴⁸. Here, we show that reducing S1P levels in BMAT markedly increased RANKL secretion by inversely regulating COX-2 expression and PGE2 production. COX/PGE2 signaling is considered an essential mechanistic pathway by which certain drugs such as heparin induces RANKL expression leading to osteoclast resorption and bone loss⁴⁹.

L-serine is the precursor for sphingolipids biosynthesis. In a previous clinical trial, oral supplementation of L-serine (up to 400 mg/kg/d) was used to reduce the neurotoxic levels of 1-deoxysphingolipids and slow disease progression in patients with hereditary sensory neuropathy type 1 (HSAN1)^{50,51}. In this study, we treated the patients with a dose of 250 mg/kg/d and plasma L-serine level of $1000 \pm 127 \mu\text{M}$ in patients. This dose had been shown to ameliorate GRIN2B-related severe encephalopathy in a clinical trial⁵². The dose appears to be safe, and no obvious side effects were observed during the treatment. L-serine supplementation did not show any further improvement in the antidepressant effects of fluoxetine. There were no significant differences in the depression rating scores between fluoxetine with L-serine and the placebo.

In conclusion, our study provided new insights into the fluoxetine-induced bone loss. We showed that sphingolipids metabolism is the primary metabolic pathway altered in BMAT during the progression of bone loss. Inhibition of ASM, a key enzyme for the conversion of sphingomyelins to ceramides by fluoxetine or genetic loss of ASM, accelerates bone loss. Further mechanistic analysis in BMAs revealed that inhibition of ASM by fluoxetine reduces ceramides and S1P levels, leading to the marked increase of RANKL secretion through the upregulation of COX-2/PGE2 pathway. In contrast, the regulation of ASM expression by CDDP normalizes fluoxetine-induced RANKL overproduction. In addition, we reported that the acceleration of bone loss caused by chronic use of fluoxetine was prevented by L-serine treatment.

Acknowledgements

This research was supported by the Natural Science Foundation of Jiangsu Province, China (BK20151293), Qinglan Project in Jiangsu Colleges and Universities, Shanghai Medical Science and Technology Project (14411962700).

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Conflict of interest

The authors declare that they have no conflict of interest.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information accompanies this paper at (<https://doi.org/10.1038/s41398-020-0819-5>).

Received: 2 October 2019 Revised: 16 April 2020 Accepted: 22 April 2020
Published online: 12 May 2020

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