High Plasma Sphingosine 1-phosphate Levels Predict Osteoporotic Fractures in Postmenopausal Women: The Center of Excellence for Osteoporosis Research Study

Mohammed-Salleh M. Ardawi[1,2], Abdulrahim A. Rouzi[1,3], Nawal S. Al-Senani[1,3], Mohammed H. Qari[1,4], Ayman Z. Elsamanoudy[2], Shaker A. Mousa[1,5]

[1]Center of Excellence for Osteoporosis Research, Faculty of Medicine, King Abdulaziz University Hospital, King Abdulaziz University, Jeddah;
[2]Department of Clinical Biochemistry, Faculty of Medicine, King Abdulaziz University Hospital, King Abdulaziz University, Jeddah;
[3]Department of Obstetrics and Gynecology, Faculty of Medicine, King Abdulaziz University Hospital, King Abdulaziz University, Jeddah;
[4]Department of Hematology, Faculty of Medicine, King Abdulaziz University Hospital, King Abdulaziz University, Jeddah, Saudi Arabia;
[5]The Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, Rensselaer, NY, USA

Corresponding author

Ayman Z. Elsamanoudy
Department of Clinical Biochemistry, Faculty of Medicine, King Abdulaziz University Hospital, P.O. Box 20724, Jeddah 21465, Saudi Arabia
Tel: +966-59-506-2375
Fax: +966-6952063
E-mail: ayman.elsamanoudy@gmail.com

KSBMR
Korean Society for Bone and Mineral Research

Background: Higher sphingosine 1-phosphate (S1P) plasma levels are associated with decreased bone mineral density (BMD), and increased risk of prevalent vertebral fracture. So, we hypothesized that postmenopausal women with increased baseline plasma S1P levels have a greater risk for future incident fracture (osteoporosis-related fractures [ORFs]). **Methods:** This study was conducted in a prospective longitudinal cohort of 707 women recruited in 2004 and followed up annually for a mean period of 5.2 ± 1.3 years. They were postmenopausal (aged ≥ 50 years). The primary outcome measure was the time to the first confirmed ORF event using radiographs and/or a surgical report. **Results:** The plasma S1P levels (µmol/L) were significantly higher in the women with incident fracture (7.23 ± 0.79) than in those without ORFs (5.02 ± 0.51; $P < 0.001$). High S1P levels were strongly associated with increased fracture risk. After adjustment for age and other confounders, the hazard ratio (HR) was 6.12 (95% confidence interval [CI], 4.92-7.66) for each 1-standard deviation increase in plasma S1P levels. The women in the highest quartile of S1P levels had a significant increase in fracture risk (HR, 9.89; 95% CI, 2.83-34.44). Results were similar when we compared plasma S1P levels at the 1-year visit. **Conclusions:** The associations between plasma S1P levels and fracture risk were independent of BMD and other confounders. These findings demonstrate that high plasma S1P level at baseline and at years 1 to 5 is a strong and independent risk factor for future [ORFs] among postmenopausal women and could be a useful biomarker for fracture risk assessment in this population.

Key Words: Osteoporosis, Osteoporotic fractures, Postmenopausal, Sphingosine 1 phosphate

INTRODUCTION

Incident fracture (osteoporosis-related fractures [ORFs]) have major economic and social impacts, with serious morbidities and increased mortality following hip and/or vertebral fractures.[1,2] Many postmenopausal women at risk of fracture

will be missed based on bone mineral density (BMD) assessment alone,[1,2] a finding that is related to the multiple determinants of bone fragility, including age, fracture history, and deterioration of bone quality.[2] Identifying new biomarker(s) independent of BMD to improve fracture prediction is needed. Bone turnover markers (BTMs), particularly those of bone resorption, can predict fracture risk among postmenopausal women.[3] Such prediction may improve if BTMs are assessed along with BMD.[4,5] One possible new biomarker is plasma sphingosine 1-phosphate (S1P).

S1P is a bioactive lysophospholipid, autocrine-paracrine signaling molecule that regulates multiple cellular processes, including migration, proliferation, differentiation, and apoptosis, in many cell types.[6-8] S1P plays an important role in bone metabolism as a coupling factor between bone resorption and formation. It is also produced by osteoclasts to stimulate proliferation, migration, and survival of osteoblasts, possibly leading to bone formation stimulation.[6-8] Conversely, S1P augments osteoclastogenesis via induction of receptor activator of nuclear factor-κB ligand (RANKL) in osteoclasts and T-cells.[9] A concentration gradient of S1P is formed between circulation and bone tissue whereby osteoclast precursor cell movement is facilitated by chemo-repulsion mediated by S1P receptor 2 (S1PR2) signaling.[10] Such observations suggest that S1P affects osteoclasts, leading to increased bone resorption.[8] Furthermore, S1PR2-deficient mice exhibit moderate osteoporosis due to a decrease in osteoclastic bone resorption.[11] Recently in a case-control study, Kim et al.[12] demonstrated markedly higher S1P levels in 69 postmenopausal women with vertebral fractures than those in 69 non-fractured age and body mass index (BMI) matched controls, with S1P levels inversely correlated with BMD at various sites and positively correlated with bone resorption markers (BRMs). These data suggest that circulating S1P level could serve as a new biomarker of fracture risk. However, Heilmann et al.[13] using systemic FTY720 (an S1P analog) treatment did not improve fracture healing in experimental mice although several *in vitro* studies demonstrated osteoanabolic effects [14,15] and improved bone defect healing.[16]

We hypothesized that postmenopausal women with higher circulating S1P levels have a greater ORF risk. We examined the association between plasma S1P levels and ORF risk in a prospective cohort of postmenopausal women aged ≥50 years in the Center of Excellence for Osteoporosis Research (CEOR) study.

METHODS

1. Study design

The CEOR study is an ongoing prospective, population-based cohort of 912 women aged ≥50 years residing in the Jeddah area of Western Saudi Arabia who were recruited in 2004. The study investigated the determinants of bone loss and fractures among healthy postmenopausal women. The rationale and design of the study as well as sampling and data collection have been described previously.[5,17] The baseline evaluation included 3,015 women. At enrollment, women were eligible for the study if they were aged ≥50 years, had been postmenopausal for >1 year (with serum FSH levels >15 mIU/L), were independently mobile and on an unrestricted diet. Exclusion criteria included a cancer history (n=11), regardless of type and stage) and current diagnosis of or prior therapy for osteoporosis (n=15). Women were also ineligible at enrollment if they had diseases or conditions that could interfere with bone metabolism or if they used anti-osteoporotic treatments (n=36), bisphosphonates, estrogen, calcitonin, fluoride salts, calcitriol, strontium or selective estrogen-receptor modulators). Women were also excluded if they had fever (oral temperature ≥38.0°C) or abnormal findings on complete blood cell counts including platelets and/or leukocytes (n=305). Women with abnormal hepatic, renal or thyroid function tests were also excluded (n=1,736). All women provided written informed consent for study participation. The study was approved by the Human Research Ethics Committee of CEOR, King Abdulaziz University. During the follow-up, of the 912 women initially recruited (fulfilling inclusion criteria), 148 were lost to follow-up, 20 died and 37 withdrew. The present study investigated 707 healthy postmenopausal women (mean age, 61 years; range, 53-93 years) from the CEOR cohort at the sixth annual follow-up for whom blood and urine samples were obtained at the baseline and follow-up visits. Women were followed with the intention to treat from the baseline visit to fracture, completion of the follow-up period, withdrawal from the study, or loss to follow-up or death, if no fracture had occurred. The participants received vitamin D and calcium supplementation to maintain the serum 25-hydroxy-vita-
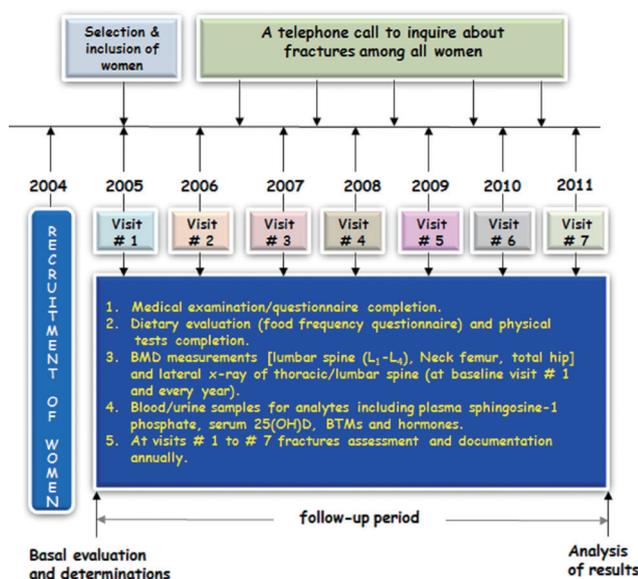
Fig. 1. Study design and measurements.

min D (25[OH]D) concentration >50 nmol/L and eura para-thyroidism. Among the women who completed the study, plasma S1P and BTM levels, along with other analytes, were evaluated during the baseline visit, the 1-year follow-up visit, and annually thereafter (Fig. 1). Additional baseline assessments (using a questionnaire) included information on medical history, menopausal age, years since menopause, socioeconomics, education level, family history of fragility/fracture(s), and occurrence of a fall(s) during the past year. Information on smoking, physical activity (PA), vitamins and medication use were also recorded. Height and weight, as well as hip and waist circumferences, were measured while the women were wearing lightweight clothing with no shoes. BMI and the waist-to-hip ratio were also calculated. Cumulative PA scores from 0 to 27 were constructed based on the 7-day PA record described previously.[1] Women were classified into either sedentary (PA-score ≤14) or moderate/high activity (PA-score >14) groups. Hand-grip strength was measured using a JAMAR-Plus hand dynamometer (Sammons Preston, Bolingbrook, IL, USA) on both hands. Dietary intake of calories, protein, calcium and vitamin D were assessed using a food-frequency questionnaire.[17]

2. Assessment of fractures

Research physicians at CEOR monitored the enrolled women for incident fractures, which were recorded using CEOR's information system network. Two research physicians classified fracture events independently according to the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10).[18] A radiologist and/or an orthopedic surgeon reviewed all coded events for final fracture classification. Only low-trauma fractures (i.e., falls from standing height or less) were considered and classified as incident fragility fractures. Incident fractures were reported during annual follow-ups, and all peripheral fractures were confirmed by radiographs or a surgical report, as described previously.[17]

We excluded all non-ORFs (i.e., accident-related fractures such as a traffic road accident or a fall from a height higher than a chair or the first rung of a ladder or severe trauma; and all finger, toe, skull, and facial fractures). The specific fractures and fracture follow-up times were recorded at different sites during a follow-up period when a woman sustained an incident first fracture. The follow-up period was calculated as the time from the baseline visit to the first fracture, death, or the end of the study, whichever occurred first.[17] For women lost during the follow-up, the period was computed as the time from enrollment to the date of last contact. For incident vertebral fractures, lateral X-ray films of the thoracic and lumbar spine were obtained at baseline and annual visits for all women, and at the end of the study. All prevalent and incident vertebral fractures were identified by a radiologist as described by Genant et al.[19] A prevalent fracture was defined as a vertebral body with a semi-quantitative grade ≥1. Any new vertebral fracture was identified by an increase of at least one grade in a vertebral body that was defined as normal at baseline. We excluded vertebral fractures due to major trauma, and/or vertebral deformities with non-osteoporosis causes (e.g., osteoarthritis and/or bone diseases). All vertebral and non-vertebral fractures were included in the final analyses.

3. Assessment of BMD

The BMD (lumbar spine [L1-4] and the mean of the proximal right and left femur [total and sub-regions]) was measured by dual energy X-ray absorptiometry using the Lunar Prodigy Model (Lunar Corp., Madison, WI, USA). Quality-control procedures were performed according to the manufacturer's recommendations.[17]

4. Measurement of plasma S1P levels and analytes

During the baseline and follow-up visits, venous blood samples were collected between 9:00 and 11:00 a.m. after an overnight fast under standardized conditions. Second-void morning urine samples were collected on the same day of blood sampling. The blood was centrifuged, and plasma (sodium citrate- or EDTA-treated), serum, and urine samples were stored in liquid nitrogen at -190℃ at the Biobank Unit of CEOR until they were analyzed. Plasma (treated with sodium citrate) S1P was measured using a competitive ELISA kit (Echelon Biosciences, Salt Lake City, UT, USA) according to the manufacturer's instructions.[20] All plasma samples showing hemolysis or clotting were discarded. However, no sample was collected immediately following any ORF. For women with ORFs, samples (n=9 women) were collected at least 3 months following the incident, and adjustments were made for the collection time. [17] The lower detection limit was 0.06 µmol/L with intra-assay and inter-assay coefficients of variations equaling 6.3% and 5.9%, respectively. Duplicate samples were assayed, and all results were reported as means. Plasma samples were diluted by a factor of 1/10. Further validation studies were performed for this assay. Linearity was assessed by serially diluting samples with sample diluents (dilutions 1:15, 1:20, and 1:30) and comparing observed values with expected values (observed recoveries range, 101%-109%) (data not shown). Additionally, recovery of spiked standards was tested by adding different S1P concentrations (three different concentrations) to eight different human plasma samples presented with various levels of endogenous S1P. Spiked recovery ranged from 90% to 106% (data not shown). No sample tested had S1P values below the lower limit of detection or above the upper limit of quantitation corresponding to 2.0 µmol/L. Other biochemical tests were performed at the Diagnostic laboratory of CEOR according to standard methods as described previously [21,22].

5. Fracture risk assessment tools evaluation

BMD (g/cm^2) was determined as described before.

Part of the withdrawn fasting blood sample was used for serum preparation to measure the biochemical BTMs. Moreover, urine samples were obtained in morning. Serum bone alkaline phosphatase (s-bone ALP), serum procollagen type 1 N-terminal propeptide (s-P1NP) , urinary cross-linked C-terminal telopeptide of type 1 collagen (CTX) and serum N-terminal telopeptide of collagen type I (NTx) were measured using competitive inhibition enzyme linked immunoassay by the commercially available kits.

Serum follicle stimulating hormone (FSH), luteinizing hormone, E2 and intact-parathyroid hormone were also, measured by commercially available immunoassays using Elecsys autoanalyzer (Roche Diagnostics GmbH, D-68298. Mannheim, Germany). The intra- and inter-assay coefficient of variations (CVs) were less than 4.0%. Serum 25(OH)D was measured by direct competitive chemiluminescence immunoassay using LIASON autoanalyzer (DiaSorin Inc., Stillwater, MN,. USA). The intra- and inter-assay CVs were 7.8% and 3.8% respectively.

6. Statistical analysis

Results are presented as means±standard deviation (SD), and categorical variables are expressed as frequencies. Data were analyzed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance or the Mann-Whitney test was used, as appropriate, to examine differences among the groups for different variables and χ^2 test for categorical variables to test for significant differences among groups. Correlation analysis was performed using Pearson's test. Partial correlation analysis was performed to adjust for various possible confounders. The relationship between incident fracture risk and various plasma S1P levels was evaluated using SD score changes and quartile-based analysis. The SD score was calculated using the formula $(S1P_i–S1P_m)\div SD$, where $S1P_i$ is the individual S1P level, $S1P_m$ is the mean cohort S1P level, and SD is the SD of cohort S1P levels. This calculation was used to determine the change in incident fracture risk for each incremental increase of 1-SD in S1P levels. Similar calculations were made for BTMs. Plasma S1P and BTMs were categorized into Quartiles, and the lowest Quartiles were compared to the fourth (highest) or other Quartiles. Quartiles were calculated for the following variables: age, years since menopause, dietary calcium intake, serum 25(OH)D, PA score, hand-grip strength, and BMD measurement at all sites. Cox proportional hazards regression analysis was used to estimate fracture risk. Data were used to estimate the hazard ratios (HRs) by Quartile and per SD increase in plasma S1P and other BTMs, and results were reported as

HRs with estimation of 95% confidence intervals (CIs). In the Cox model, the follow-up time describes the time from the baseline visit to (a) the first fracture; or (b) completion of the follow-up period; or (c) death, if no fracture had occurred. The first fracture was considered the analysis endpoint. HRs were computed by comparing women with and without a fracture. Cox regression analysis was performed for different times: 2.0, 3.0, 5.0, and 6.0 years after collecting samples of the baseline visit. All fracture risk estimates were adjusted for age, BMI, and other confounders as appropriate. The population attributable risk (PAR) proportion was calculated according to the formula $\{Px(RR-1)\div[1+Px(RR-1)]\}\times100$, where P is the percentage of the population exposed, and RR is the relative risk.[23]

RESULTS

1. Baseline characteristics

Baseline characteristics of the participants are presented in Table 1. During the mean follow-up period of 5.2 ± 1.3 years, 138 women sustained ORFs: 44 vertebral fractures and 104 non-vertebral fractures (52 wrist, 12 hip, 21 rib, 8 humerus, 4 ankle, 5 metatarsal, and 2 pelvic) were documented. Women who had no fracture (n=569) were used as age and BMI matched control group in all analyses. As expected, women with ORFs were older, had higher number of years since menopause, lower values for BMD and lower hand-grip strength and PA-score values. Plasma S1P levels were significantly higher in women with ORFs than in women without fractures (7.23 ± 0.79 vs. 5.02 ± 0.51

Table 1. Baseline characteristics of postmenopausal women studied according to fracture

	No fracture (n=569)	Fracture present (n=138)	P-value
Age (year)	59.4 ± 5.7	67.6 ± 7.1	<0.001
Years since menopause (year)	9.4 ± 5.5	14.7 ± 6.9	<0.001
Parity	4.6 ± 2.9	4.7 ± 3.1	0.533
BMI (kg/m^2)	32.8 ± 6.3	32.6 ± 7.5	0.692
WHR	0.88 ± 0.08	0.90 ± 0.08	0.114
Fragility fracture in father (%)	3.0	5.1	0.220
Fragility fracture in mother (%)	9.3	8.7	0.370
Current smokers (%)	4.7	3.6	0.226
Dietary calcium intake (mg/day)	542 ± 199	495 ± 177	0.009
Hand-grip strength (kg)	15.7 ± 2.9	13.8 ± 3.2	<0.001
Physical activity score	14.4 ± 2.2	12.9 ± 1.9	<0.001
Serum creatinine (mmol/L)	63.9 ± 15.4	65.5 ± 18.2	0.199
Serum 25(OH)D (nmol/L)	35.2 ± 23.3	30.9 ± 20.6	0.044
Plasma S1P (μmol/L)	5.02 ± 0.51	7.23 ± 0.79	<0.001
Serum bone-ALP (U/L)	16.22 ± 3.81	16.10 ± 4.46	0.635
Serum P1NP	47.20 ± 16.24	49.28 ± 15.82	0.665
Plasma CTx (pg/mL)	361.5 ± 40.22	368.3 ± 47.30	0.002
Urinary NTx (nmol/mmol creatinine)	61.36 ± 11.08	63.75 ± 19.06	0.141
BMD (g/cm^2)			
Lumbar spine (L1-L4)	1.009 ± 0.163	0.987 ± 0.164	0.156
Femur neck	0.851 ± 0.134	0.812 ± 0.126	0.002
Total hip	0.888 ± 0.141	0.852 ± 0.137	0.005

The data is presented as mean±standard deviation.
To convert 25(OH)D values to ng/mL divide by 2.496; to convert creatinine values to mg/dL, divide by 88.4.
BMI, body mass index; WHR, waist-to-hip ratio; 25(OH)D, 25-hydroxy-vitamin D; S1P, sphingosine 1-phosphate; ALP, alkaline phosphatase; P1NP, procollagen type 1 N-terminal propeptide; CTX, C-terminal telopeptide of type 1 collagen; NTx, N-terminal telopeptide of collagen type I; BMD, bone mineral density.

Table 2. Plasma sphingosine 1-phosphate levels in the study participants at baseline and 1, 2, 3, 4, and 5 years

| | Baseline | Plasma S1P (µmol/L) | | | | | 5-year change (%) |
		1	2	3	4	5	
Women with fractures (n=138)	7.23±0.79	7.32±0.82	7.51±0.91	7.68±0.88	7.75±1.02	7.79±0.96	+7.75±0.88[a]
Women without fractures (n=569)	5.02±0.51	5.12±0.56	5.24±0.62	5.49±0.57	5.72±0.81	5.63±0.74	+12.2±0.64[a]
All women (n=707)	5.49±1.06	5.58±0.82	5.71±1.09	5.87±0.96	6.04±1.11	6.02±1.04	+9.7±0.78[a]

The data is presented as mean±standard deviation. The change in plasma S1P is percent change of baseline.
[a]All changes in plasma S1P levels (repeated-measures analysis of variance) are significant at $P<0.001$.
S1P, sphingosine 1-phosphate.

µmol/L; $P<0.001$) with no significant difference in BTMs except for plasma CTX (p-CTX) ($P=0.002$). The mean plasma S1P values at baseline and at different time-point measurements are shown in Table 2. The plasma S1P levels remained stable during the follow-up period with only minor but significant increases (1.94%/year; $P<0.001$) for all women studied. Approximately 76% to 93% of women remained in the same plasma S1P Quartile at baseline and at the 5-year interval (data not shown). Of those at the highest Quartile at baseline, 89% remained at the highest Quartile also 5 years later (data not shown). Women who had fractures during the follow-up period had constantly high plasma S1P at baseline and at different time-point measurements (Table 2).

Stratifying the participants into three groups according to the number of incident fracture (i.e., no fracture, single or multiple fractures) demonstrated that plasma S1P levels (µmol/L) were significantly higher in women with single (7.03±0.71) or multiple fractures (7.92±0.64) than in those without fractures (5.02±0.51), (P-value for trending $P<0.001$). After adjustment for BMD value of the lumbar spine (L1-4) or total hip, statistical significance persisted. Plasma S1P levels in vertebral fractures (7.12±0.87) was not significantly different from women with non-vertebral fractures (7.27±0.75).

2. Plasma S1P levels in relation to fracture risk and BMD

High plasma S1P levels were associated with increased incident fracture risk (Table 3). After adjusting for age and other potential confounders, the ORF risk for each increment increase of 1-SD of the S1P level was greater than 6-fold among postmenopausal women. Women in the highest Quartile (risk group) had an increased incident fracture risk, so the risk was more than 10-fold as high as the risk in the lower three Quartiles combined (reference

Table 3. Hazard ratios for osteoporosis-related fractures by sphingosine 1-phosphate cut-off values

Analysis	Continuous (per SD analysis)[a]	Quartile-specific analysis[b]
Plasma S1P (µmol/L)		
Unadjusted	6.22 (5.02–7.82)	10.41 (2.92–33.37)
Adjusted for age	6.07 (4.87–7.58)	9.92 (2.84–34.58)
Multivariate 1[c]	6.12 (4.92–7.66)	9.89 (2.83–34.44)

The data is presented as HR (95% confidence interval) and all values were statistically significant.
[a]S1P levels were analyzed as a continuous measure. The HR is for each increment of 1-SD above the mean value. [b]S1P levels were analyzed as a dichotomous variable. The HR for women in the highest quartile of S1P levels, as compared with women in the three lower quartiles. [c]The analysis included adjustments for age, body mass index, physical activity score, dietary calcium intake, serum 25(OH)D, hand-grip strength, and bone mineral density of total hip.
SD, standard deviation; S1P, sphingosine 1-phosphate; HR, hazard ratio; 25(OH)D, 25-hydroxy-vitamin D.

group) (Table 3). Significant negative correlations were observed between the plasma S1P levels and BMD at different sites studied, and the adjustments for controlling factors strengthened the statistical significance (Table 4). When we included BMD in multivariate regression analyses, risk estimates were not significantly affected (Table 3). Figure 2 shows the cumulative incidence of fractures in the cohort according to the specific Quartile of the plasma S1P level.

3. Possible confounding variables

Circulating S1P levels were positively correlated with age (r=0.339; $P<0.001$), age at menopause (r=0.103; $P=0.006$), BMI (r=0.212; $P<0.001$), and years since menopause (r=0.189 $P<0.001$). Furthermore, in the women of the present cohort, plasma S1P showed a significant positive correlation with BRMs: p-CTX (r=0.166; $P<0.001$) and urinary NTx (u-NTx) (r=0.253; $P<0.001$) (data not shown). Conversely, bone formation markers (BFMs) s-P1NP (r=-0.014; $P=0.708$) and s-bone ALP (r=0.028; $P=0.457$) were not associated

Table 4. Correlation between plasma sphingosine 1-phosphate levels and bone mineral density at various sites, bone turnover markers and other potential confounding factors among postmenopausal women studied

Variable	γ	P-value[a]	γ	P-value[b]
Bone mineral density (g/cm^2)				
Lumbar spine (L1-4)	-0.073[c]	0.043[c]	-0.096[c]	0.031[c]
Neck femur	-0.085[c]	0.023[c]	-0.101[c]	0.001[c]
Trochanter	-0.071[c]	0.058[c]	-0.085[c]	0.036[c]
Total hip	-0.078[c]	0.038[c]	-0.094[c]	0.002[c]
Bone turnover markers				
s-bone ALP (U/L)	0.028	0.457	0.031	0.446
s-P1NP (μg/L)	-0.014	0.708	0.012	0.760
p-CTX (pg/mL)	0.166	<0.001	0.170	<0.001
u-NTx (nmol/mmol creatinine)	0.253	<0.0001	0.260	<0.0001
Others				
s-25(OH)D (nmol/L)	-0.027	0.468	-0.018	0.560
s-PTH (pmol/L)	0.028	0.456	0.030	0.462
s-FSH (IU/L)	-0.033	0.385	-0.023	0.411
s-LH (IU/L)	-0.061	0.103	-0.072	0.094
s-E$_2$ (pg/mL)	-0.091[c]	0.016[c]	-0.098[c]	0.011[c]

To convert 25-hydroxyvitamin-D values to ng/mL divide by 2.496.
[a]P-values were determined by Pearson's correlation analysis with respect to plasma S1P levels. [b]P-values were determined by partial correlation analysis with respect to plasma S1P levels adjusted for age, body mass index, physical activity-score, current smoking, hand-grip strength and dietary calcium intake. [c]The statistically significant.
s-bone ALP, serum bone alkaline phosphatase; s-P1NP, serum procollagen type 1 N-terminal propeptide; p-CTX, plasma C-terminal telopeptide of type 1 collagen; u-NTx, urinary N-terminal telopeptide of collagen type I; s-25(OH)D, serum 25-hydroxy-vitamin D; s-PTH, serum parathyroid hormone; s-FSH, serum follicle stimulating hormone; s-LH, serum luteinizing hormone; s-E2, serum estradiol; S1P, sphingosine 1-phosphate.

with plasma S1P, respectively, before or after adjustment for covariates (Table 4).

4. How long can plasma S1P predict fractures?

Circulating S1P levels could predict ORFs 1 year from the baseline visit with HR (95% CI, 6.36 [5.08-7.94]) for a 1-SD increase and 10.23 (2.87-34.46) for levels lower than the highest Quartile (data not shown). HRs for incident fracture prediction was of similar magnitude or slightly lower but remained significant when the follow-up was extended to 2, 3, and 5 years after enrollment (Table 5). There was no apparent increase in the risk of incident fractures in Quartile 2 as compared with quartile 1, but it became significant for Quartiles 3 and 4 (Fig. 3). There were positive significant correlations between plasma S1P levels measured at baseline visit and at 1 year (r=0.812; P<0.001), 2
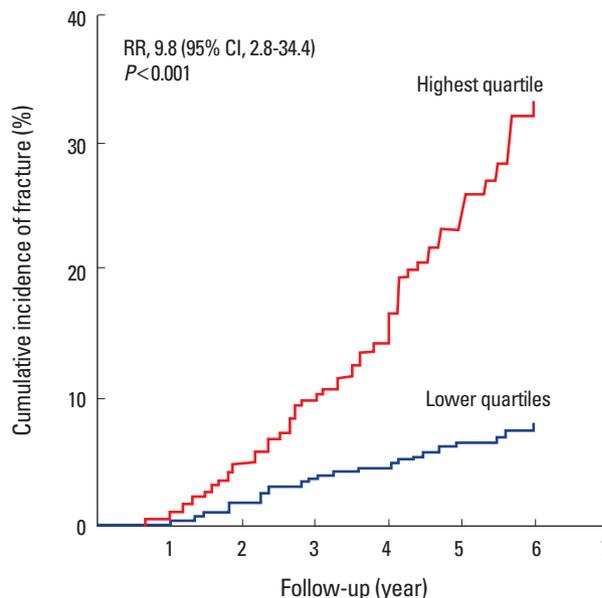

Fig. 2. Cumulative incidence of fractures among the studied women with sphingosine 1-phosphate levels in the highest quartile compared with that in all other quartiles. CI, confidence interval.

Table 5. Hazard ratio with 95% confidence interval per standard deviation or highest quartile of plasma sphingosine1-phosphate levels (μmol/L) for osteoporosis-related fractures from baseline (time point zero) to 2, 3, and 5 years after baseline

Fractures	HR (95% CI)	
	Per SD	Highest quartile
0-2 years (n=51)	5.96 (4.82-7.42)	9.94 (2.89-32.76)
0-3 years (n=87)	6.14 (4.96-7.98)	9.88 (2.76-32.44)
0-5 years (n=110)	6.32 (5.10-7.94)	10.61 (2.95-31.66)
0-mean follow-up years (n=148)	6.22 (5.02-7.82)	10.41 (2.92-33.37)

All values were statistically significant. Women with fractures are compared with women without fractures. Follow-up starts from baseline visit, and the annual follow-up times are 2, 3, and 5 years and the complete period was 5.2±1.3 years. HR is given per SD increase or per highest quartile of plasma sphingosine 1-phosphate (μmol/L). The lowest quartiles are used as the reference group (HR, 1.0).
HR, hazard ratio; CI, confidence interval; SD, standard deviation.

years (r=0.851; P<0.001), 3 years (r=0.802; P<0.001), and 5 years (r=0.846; P<0.001), after the basal visit.

5. BTMs level in relation to fracture risk and the prediction of fractures

Baseline BRMs were associated with fracture risk: the HRs per SD for p-CTX, and u-NTx were 1.29, and 1.41, respectively (data not shown). These values persisted after adjustment for age and other potential covariates. When fracture risk was analyzed for Quartiles, women in the highest Quar-

Table 6. Factors independently associated with all osteoporosis-related fractures in relation to population-attributable risk among 707 Saudi postmenopausal women

Risk factor	Cut-off	Population-attributable risk (%)	P-value
Physical activity score	≤first quartile (≤12.61)	49.5	<0.001
Age	≥60 years	54.4	<0.001
Hand-grip strength	≤first quartile (≤13.88 kg)	55.3	0.011
BMD total hip	≤first quartile (≤0.784 g/cm^2)	38.0	0.002
Plasma sphingosine 1-phosphate	≥fourth quartile (≥5.82 μmol/L)	31.5	<0.001
Dietary calcium intake	≤first quartile (≤391 mg/day)	26.4	0.020
Serum 25(OH)D level	≤first quartile (≤17.90 nmol/L)	19.9	0.027
Past year history of falls	Women with history of falls	16.2	0.028

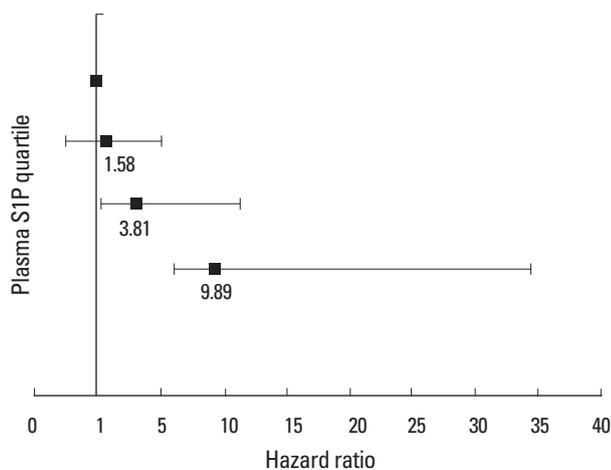BMD, bone mineral density; 25(OH)D, 25-hydroxy-vitamin D.

Fig. 3. Multivariable-adjusted hazard ratios for the risk of osteoporosis-related fractures, according to the quartile of plasma sphingosine 1-phosphate (S1P) concentration. Plasma S1P (μmol/L) quartiles were: Q$_1$<4.85; Q$_2$=4.86-5.24; Q$_3$=5.25-5.81; and Q$_4$≥5.82. The y axis is on a log scale. The reference group is quartile 1. The I bars denote 95% confidence intervals.

tiles of BRMs exhibited higher fracture risk. These values persisted following adjustment for age and other potential covariates (data not shown). However, BFMs s-P1NP and s-bone ALP were not associated with fracture risk either when analyzed per SD increase or in quartiles of the BFMs and none was able to predict incident fractures (data not shown). Plasma CTX, and u-NTx levels were able to predict ORFs 1 year from baseline visit with HRs (95% CIs, 1.28 [1.26-1.54]; 1.36 [1.08-1.50]) for 1-SD increase and 1.46 (1.10-1.97), and 1.38 (1.10-1.80) for levels lower than the highest quartiles, respectively (data not shown). The HRs for incident fractures prediction was of similar magnitude or slightly higher but remained significant when the follow-up period was extended to 2, 3, and 5 years (data not shown).

6. PAR

The incident fracture risk attributable to plasma S1P levels (in the highest Quartile) was estimated at 31.5%. The PAR for incident fracture independent risk factors among women studied is detailed in Table 6.

DISCUSSION

Our study is novel because it is the first longitudinal cohort being followed for 5.2±1.3 years with annual sampling and measurement of S1P as well as assessment of incident fractures at each visit. Thus, the fracture risk was not based on one measurement but on several determinations of the predictor plasma S1P. This study also includes a large number of patients (n=707), and both vertebral and non-vertebral fractures were assessed. This study is therefore more informative than that reported by Kim et al.[12], which used a cross-sectional sampling approach of 69 women with vertebral fractures only as a case-control study compared to 69 postmenopausal women without vertebral fractures and based on only one measurement of S1P.

In this study, we report a significant association between increased plasma S1P and incident fracture risk in a population of postmenopausal women followed prospectively for 5.2±1.3 years. We demonstrated that S1P levels were significantly higher with increasing number of incident fractures even after adjustment for possible confounders. A novel aspect of this study is an examination of the relationship between the risk of fracture and plasma S1P levels in postmenopausal women. The association was independent of the known risk factors for fragility fractures, including BMD, prior fractures, recent falls, smoking, low PA-score, and nutritional deficiencies, suggesting that plasma S1P

could be a new potential biomarker of fracture risk independent of BMD in postmenopausal women.

Generally, plasma S1P levels in our cohort were similar to those reported for Korean postmenopausal women using the same assay method.[24] Our study is also consistent with the most recent report describing higher plasma S1P levels associated with a greater risk of vertebral fractures in Korean postmenopausal women (a case-control study of 69 women with vertebral fractures versus 69 women without fractures).[12] BRMs (p-CTX and u-NTx) were significantly associated with ORFs for the same follow-up period, with much lower HR values than those obtained for plasma S1P. No association was obtained for BFMs and fracture risk. Our observations of BTMs in relation to the prediction of ORFs are consistent with those of our recent study [5] and those of Ivaska and colleagues [25] and others [4].

In this study, the majority of women that were classified with high plasma S1P (remaining stable over the follow-up period) during one time interval were then classified with high plasma S1P 5.2±1.3 years later. This suggests that a single measurement of plasma S1P could be sufficient to identify women at high risk for ORFs. Thus, plasma S1P metabolism, clearance, and/or production were stable among the women studied. However, we did observe minor changes in the longitudinal plasma S1P levels. Such changes may be related to aging and not analytical variability because plasma S1P measurements were performed under standardized conditions using a validated assay. Plasma levels in women are influenced by age. We observed a positive association between S1P levels and age, confirming previous studies in Korean women.[24] The S1P production increases as women age, but decreased S1P production, clearance, and/or hormonal changes may also contribute to increases in S1P levels. Further work is needed in this regard. Other biological variability, such as diet, PA, and/or other lifestyle factors, cannot be excluded.

The estimated PAR of increased S1P levels was significant, and S1P levels in the highest quartile produced 31.5% of incident fractures. The PARs were lower than those for S1P compared with the well-known fracture risk factors, including low PA-score, low hand-grip strength, and low total hip BMD, among others [5] (Table 6). High S1P levels had an effect that was equal to or greater in magnitude to established ORF risk factors for this cohort.[5,26]

The mechanisms connecting S1P levels and incident fracture risk remain unclear. S1P mediates bone destruction by inducing the formation of osteoclasts.[9,27] A gradient exists for S1P in which it is more abundant in circulation than in the bone marrow.[28,29] When the difference in S1P levels between these two compartments increases, osteoclast precursors are present in a limited amount in the bone marrow cavity, thus promoting bone resorption.[8,10,11] Accordingly, bone resorption is promoted, and BMD decreases as the plasma levels of S1P become higher in the circulation,[24] resulting in higher fracture risk.[12] Additionally, increased plasma S1P levels may lead to enhanced osteoblast-dependent osteoclastogenesis, which is usually associated with bone loss.[9] Such mechanisms are consistent with our present observations whereby higher plasma S1P levels were only associated with BRMs (Table 4), and plasma S1P levels were negatively associated with BMD at various sites studied. It is possible that one and/or both mechanisms contribute to the effects of S1P on bone. However, because S1P levels within bone marrow plasma are lower than those in circulation, the S1P systemic mechanism is suggested to be more clinically important than the autocrine mechanism. It is interesting that in the present study plasma S1P levels were associated with the risk and number of ORFs, even after additional adjustment for BMD. Such observations could be interpreted by the potential effect of S1P on bone resorption. Thus, elevated osteoclastic activity among postmenopausal women with relatively higher bone resorption results in the deterioration of bone quality and microarchitecture that will contribute to higher risk for fracture out of proportion to the decreased bone mass.[30] It is possible that compromised bone strength by higher levels of S1P may have been mainly due to deteriorated bone quality rather than low bone mass and might contribute to an increased risk of incident fractures. Taken together, these observations provide evidence that S1P controls migratory trafficking of osteoclasts precursors, dynamically regulating bone mineral homeostasis, and possibly identifying a critical control point in osteoclastogenesis.[10]

The present study has several strengths. This is the first report demonstrating that high plasma S1P levels predict incident fractures in a large prospective population-based study. The study had a large sample size for which women were randomly selected from the local population using

strict inclusion criteria. We obtained detailed lifestyle characteristics and BTM and BMD measurements along with a large number of fracture outcomes over a follow-up period of 5.2±1.3 years, plus multiple measurements of plasma S1P in relation to BTMs during annual visits. We standardized the sampling time after all of the participants fasted overnight in order to minimize pre-analytic variations due to circadian rhythm and/or food intake. Moreover, both platelets and mast cells can secrete S1P when activated by thrombin or IgE-bound antigen, respectively.[6] Such possible effects were minimized by our inclusion criteria that excluded women who may have had infectious or immune disorders during recruitment. However, neither platelets nor mast cells were shown to play a role in controlling S1P homeostasis in the blood.[31] Plasma S1P levels were measured using a commercially well-validated assay method and no significant differences were evident between basal mean plasma S1P levels and S1P levels in those who died during the follow-up period (data not shown).

The principal limitation of this study relates to the accuracy of self-reported data collection in relation to lifestyle information that may have been subject to report bias. During recruitment, women with osteoporosis were excluded from the study because we wanted to examine and treat fractures and bone loss in a cohort of healthy postmenopausal women. The effect that this exclusion may have had on the study results is unknown. Finally, the number of incident fractures is relatively low for calculating the HRs in relation to a given type of fracture.

The association between higher plasma S1P levels and ORF risk should be confirmed in other larger population studies. Proof of a causal relationship between elevated S1P levels and bone disease could be established by intervention studies that aim to lower plasma S1P levels. Indeed, it was previously demonstrated that treatment with FTY720 relieved ovariectomy-induced osteoporosis in mice by decreasing the number of mature osteoclasts that were attached to bone surfaces.[11] The mechanism of action of S1P is completely different from that of conventional therapies. Controlling the recruitment and migration of osteoclast precursors was suggested to represent a promising new therapeutic target in the treatment of bone disease,[8] including osteoporosis.

Finally, the current study could confirm the results of the previously published works of Kim et al.[32] and Bae et al.[33]. Our study could conclude that high S1P level is significantly associated with increased risk of incident fracture in postmenopausal women. Moreover, S1P in combination with BMD and biochemical markers of bone turnover could be used as a reliable predictors of the risk of incident fracture over a period extending up to 5.0 years duration.

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