Chapter 4.2

Do vitamin D level and dietary calcium intake modify the association between loop diuretics and bone health?

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ABSTRACT

Loop diuretics (LD) may affect bone health by inhibiting renal calcium reuptake. However, whether vitamin D status and dietary calcium intake modify the association between LD and bone outcome is unclear. Therefore, this study aimed to evaluate whether vitamin D level or calcium intake modify the association between LD and various indices of bone health including bone mineral density (BMD), and Trabecular Bone Score (TBS). From The Rotterdam Study, a prospective population-based cohort study, we used data from 6,990 participants aged >45yr with a DXA scan (2002-2008); 6,908 participants with femoral neck (FN)-BMD, 6,677 participants with lumbar spine (LS)-BMD and 6,476 participants with LS-TBS measurements. Use of LD was available from pharmacy dispensing records. Vitamin D (25(OH)D) level was measured in serum, and dietary calcium intake was measured with a validated food frequency questionnaire. Almost eight percent of the participants used LD. The association between LD (past-users compared to never-users) and LS-TBS was significantly different by 25(OH)D concentrations (P for interaction=0.04). A significantly lower LS-TBS among LD past-users was observed for 25(OH)D ≥50 nmol/l compared to ≤20 and 20-50 nmol/l (ß=-0.036, 95% CI -0.060; -0.013 versus ß=-0.012, 95% CI -0.036; 0.013 and ß=-0.031, 95% CI -0.096; 0.034 respectively). However, no other significant effect-modification by 25(OH)D and dietary calcium intake was found in the associations between LD-use and bone health outcomes (P-interaction > 0.13). This study suggests that the association between LD-use and indices of bone health is not consistently modified by vitamin D or dietary calcium intake.

Keywords

Loop Diuretics; Bone Mineral Density; bone geometry; Trabecular Bone Score.

INTRODUCTION

In the past decade, recognition of the importance of food and drug interactions has been growing in clinical practice (1). Especially in older people, however, more knowledge is needed because of the frequent use of medications and polypharmacy and higher risk of poor nutritional status (2). Food-drug interactions may be relevant in older people using loop diuretics (LD). Diuretics are frequently prescribed in the treatment of heart failure and hypertension (3, 4), and they have been shown to influence calcium homeostasis and bone metabolism.

Thiazide diuretics have shown to have a protective effect in preserving bone mass and in decreasing the risk of fractures (5). Yet, these effects have been shown to vary in relation to dosage, duration of treatment and do not last long after discontinuation of treatment (6-8). In contrast, few studies have been carried out assessing the effect of loop diuretics (LD) on skeletal health. Some studies suggest that LD can have a negative impact on bone turnover by increasing urinary calcium excretion (9, 11-13) whereas other show no association between LD-use and bone health on long-term use (6-13).

These conflicting findings may be the result of differences in calcium intake and vitamin D levels across the studied population, considering that bone mineral deposition/formation and bone resorption can be relatively normal as long as serum calcium and phosphate and calciotropic hormone levels like vitamin D and parathyroid hormone (PTH) are normal (14). Vitamin D has a major role in calcium homeostasis through three mechanisms: I) increased intestinal absorption of calcium, II) reduced renal excretion of calcium by stimulating resorption of the distal tubules and III) deposition and mobilization of calcium from bones (15). An inverse association between LD-use and serum 25(OH)D level has been reported before (16). LD-users have a lower concentration of 25(OH)D than non-users (17). As a result vitamin D deficiency may amplify any potential adverse effects of LD on bone health due to increased urinary calcium losses. Also, the use of LD may be harmful for bone health through increased plasma PTH and $1.25(OH)_2D$ levels as a result of increased renal calcium losses (13). Higher calcium loss may increase bone turnover resulting in a negative calcium balance. Furthermore, in case of very low external calcium supply from diet or supplementation, high levels of $1.25(OH)_2D_3$ mobilise the bone calcium reservoir for serum calcium homeostasis at the (temporary) expense of bone mass and strength (18). Accordingly, in people with a low calcium intake, the effect of LD on bone loss and increased fracture risk may be enhanced (10).

Therefore, in view of the frequent use of LD and a high prevalence of osteopenia and osteoporosis in the ageing population, in combination with a higher risk of mal-

nutrition and vitamin D deficiency in older individuals, it is important to evaluate whether vitamin D level or calcium intake may modify the association between loop diuretics and various indices of bone health including bone mineral density (BMD) and Trabecular Bone Score (TBS) in elderly population.

MATERIALS AND METHODS

Study Population

This study was embedded in the first three cohorts of The Rotterdam Study (RS-I, RS-II and RS-III), an ongoing, population-based cohort study in Ommoord, a suburb of Rotterdam, the Netherlands (19). Since January 1990 participants of 55 years and over were recruited for RS-I (N=7,983). In 2000, the study was extended to 3,011 participants (RS-II). Later in 2006, the study was extended with a third cohort of participants of 45 y and older (RS-III). Overall response for all three cycles at baseline was 72% (14,926 of 20,744) (19). Participants were interviewed at home by a trained research assistant, after which they were invited for a physical examination and dietary assessment at the research center. The study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of the Netherlands Ministry of Health, Welfare and Sports, and approval has been renewed every 5 years. From all subjects written informed consent was obtained. More details on the main objectives, design and diagram of examination cycles of the Rotterdam Study (RS) have been published elsewhere (19). Because of a possible persisting effect on bone, users of bisphosphonates were excluded from the study. For the current analysis, 6,908 participants with data available for femoral neck (FN)-BMD, 6,677 participants with lumbar spine (LS)-BMD and 6,476 participants with LS-TBS were included from the fourth examination of the first cohort (RS-I-4, 2002-2004), the second examination of the second cohort (RS-II-2, 2004-2005) and the first examination of the third cohort (RS-III-1, 2006-2008) (**Figure 1**).

Dietary intake and serum 25(OHD) level

Dietary data were collected at baseline (between 1989 and 1993 in RS-I-1, between 2000 and 2001 in RS-II-1 and between 2006 and 2008 in RS-III-1) using a validated semi-quantitative Food Frequency Questionnaire (FFQ) managed by a trained dietician, at the study center (20, 21). For RS-I-1 and RS-II-1, a two-stage 170-items FFQ was used (during first stage, participants mentioned on 170 food item, which foods they consumed at least twice a month in the preceding year, and in the second stage,

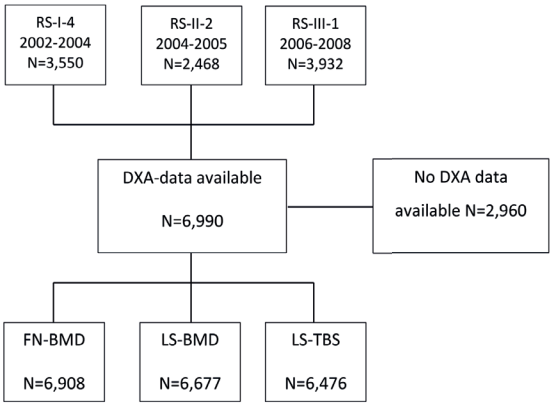

Figure 1. Flow-chart of sub-cohorts included in the study

dietician identified how often and in which amounts the foods were consumed). For RS-III-1, a one-stage 389-items FFQ was used. Dietary intake of nutrients (incl. total energy and dietary calcium intake) was determined using the Dutch Food Composition Tables (NEVO) from 1993, 2001 and 2006, using standardized portion sizes (20, 22). Intake of calcium was adjusted for energy corresponding to the residual method (23). Serum 25(OH)D was measured in the blood collected at the same time as dietary data, between 1990 and 2008 using electrochemiluminescence immunoassay (COBAS, Roche Diagnostics GmbH). The sensitivity of the test was 10 nmol/L, the range of serum 25(OH)D concentrations was from 7.5 nmol/L to 175 nmol/L, the within-run accuracy was less than 7.8%, and the intermediate precision accuracy was less than 13.1% (24, 25). Vitamin D deficiency was defined as a serum 25(OH)D level ≤ 50 nmol/L according to the current recommendations for older adults aged >70 years by the Institute of Medicine and the Dutch Health Council (26). In addition, we used also a vitamin D deficiency threshold <20 nmol/L in our analysis and showed the stratification analysis in categories of <20 nmol/L, between 20-50 nmol/L and >50 nmol/L.

Loop diuretics

As of 1st January 1991, pharmacy records of prescriptions were collected via all pharmacies in the Ommoord region with details on product name, ATC-code, number of tablets/capsules in each prescription, and prescribed daily number (27). LD-use (ATC-code C03C) was determined from baseline to the date of the DXA scan [19] and defined in three different categories: current-users, past-users and never-users. Current-users were defined when the participant had a drug exposure period between the date of the DXA scan and 120 days prior to the performed DXA scan. Past-users were defined when the participant had a drug exposure period more than 120 days

prior to the performed DXA scan. In addition, if the participants had no drug exposure from baseline till the DXA scan, the participants were considered as never-users. The duration of LD-use among current-users was categorized into 1-120 days, 120-365 days, and more than 365 days. Never-use of LD was used as the reference category.

Assessment of co-variables

Co-variables related to lifestyle, body composition and socioeconomic status (SES) were included. Weight (kg) and height (cm) were measured at study entry. BMI was calculated as weight divided by height squared (kg/m^2). During home interviews physical activity (PA) was assessed by means of the Zutphen Physical Activity Questionnaire (28). Metabolic equivalent of tasks were calculated (MET hours/week) according to time spent in categories of light, moderate and vigorous activity (28). Socioeconomic status variables (i.e., educational level and income level), smoking (yes/no), pack years, use of alcohol, prevalence of Coronary Heart Diseases (CHD), stroke and Diabetes Mellitus (DM) were assessed by home interview. Also the use of bisphosphonates was determined using pharmacy dispensing records in the same way as the use of LD. Serum calcium, magnesium and sodium were measured in blood samples by the Department of Clinical Chemistry of the Erasmus Medical Center using the Roche/Hitachi cobas c501 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Outcome assessment

Femoral neck and lumbar spine BMD was measured at RS-I-4, RS-II-2 and RS-III-1 (between 2002 and 2008) by dual energy X-ray absorptiometry (DXA) using a ProdigyTM fan-beam densitometer (GE Lunar Corp, Madison, WI, USA for all participants (19). The DXA-derived Trabecular Bone Score (TBS), which is measured in the Lumbar Spine (LS-TBS) is a grey-level texture measurement that correlates with 3D parameters of bone micro-architecture, connectivity density, trabecular separation and trabecular number (29). LS-TBS predicts future fractures (all type) independent of areal BMD [30]. Moreover, recent studies have shown that LS-TBS may be an applicable measure of trabecular bone integrity to study in regard to lifestyle factors that are adaptable, such as a dietary intake (31, 32). LS-TBS was derived from the same lumbar DXA scans that BMD was obtained from and it was analyzed using TBS iNsight software (version 1.9; Medimaps, Geneva, Switzerland) at the Bone Disease Unit of the University of Lausanne (Lausanne, Switzerland). LS-TBS was calculated for a subgroup of RS-I-4, RS-II-2 and RS-III-1 and represents the mean value of the individual vertebral measurements from L1 to L4. Subjects with a BMI higher than 37 kg/m2 were excluded from the study since LS-TBS measurements in morbidly obese persons are not ac-

curate. Furthermore, the LS-TBS was standardized according to sex using the residual method, due to technical differences between LS-TBS across sexes. The method of LS-TBS calculation has been described in detail elsewhere (32).

Statistical analyses

First, for all variables normal distribution was examined by visual check of histograms. When necessary, data were log transformed. Linear regression analysis (cross-sectional) was used to assess the association between LD-use (past-, current-use and the duration of 1-120 days, 120-365 days and >365 days) and FN-BMD, LS-BMD and LS-TBS. All the analyses were adjusted for age and sex and cohort (model 1). Thereafter, the co-variables were added additionally to model 2, based on literature relevance as well as principles of causal inference combined with the change-in-effect-criterion of ≥10% (33, 34). Potential confounders were BMI, smoking (pack-years), alcohol intake (g/day), SES (education and job), total physical activity (METhours/wk) and comorbidities (prevalence of CHD, stroke and DM). For the analysis of the duration (continuous), the model was additionally adjusted for the past-users. An earlier study showed that in subjects with osteopenia/normal BMD levels, TBS is significantly associated with vertebral fractures (35). Even though BMD and TBS are correlated, they present different aspects of bone health (35). For that reason, as sensitivity analyses, we wanted to assess how the association between LD and BMD may depend on measures of bone architecture (i.e. TBS) and how a potential association between LD and TBS is dependent on BMD. So, the association between LD and FN-BMD and LS-BMD were additionally adjusted for LS-TBS and vice versa (model 3). In addition, in all analyses, sensitivity analyses was performed with additional adjustment for serum vitamin D, season of blood collection of vitamin D: (winter (September till end of February) and summer (March till end of August)) serum calcium, magnesium and sodium concentrations.

To assess whether effect-modification by serum 25(OHD) level or dietary calcium intake was present in the association of LD (ever-use) on FN-BMD, LS-BMD and LS-TBS, we evaluated the effect-modification by serum 25(OH)D level and energy adjusted dietary calcium intake on FN-BMD, LS-BMD and LS-TBS in model 2 (P for interaction<0.10), since the beta did not changed after additional adjustment in model 3. Thereafter, we stratified the analyses of LD-use (LD never-use, current-use and past-use with never use as reference category) and bone outcomes according to serum 25(OH)D level, in subsequent categories of ≤20, between 20-50 and ≥50 nmol/L) and energy adjusted dietary intake of calcium in following categories: ≤950 mg/day, 950-1200 mg/day and ≥1200 mg/day. The association between LD and LS-TBS, LS-BMD and FN-BMD was also evaluated by sex through effect-modification.

To account for missing data in co-variables (varied from 1.6% to 66.8%), we used a multiple imputation approach (n=10 imputations, 10 iterations). Briefly, as described by Sterne et al, multiple imputation is created on the correlation between each variable and missing values with other participant characteristics [36]. Linear regression analyses were then separately accomplished in each of the 10 datasets [36]. Beta's were pooled by taking the average of the effect sizes of the 10 imputed datasets. The pooled standard errors and respective 95% Confidence Intervals (CI) were then calculated by using Rubin's rules (37). For details of the multiple imputation, see supplement **supplemental tables S1 and S2**. The statistical software package of SPSS 24.0 was used for the statistical analyses (SPSS Inc., Chicago, Illinois, USA). For all the analyses except the effect-modification analysis, p-values of < 0.05 were considered statistically significant.

RESULTS

Population characteristics

Baseline characteristics are presented for the total population, LD-users and non-users (**Table 1**) and baseline characteristics before and after multiple imputation are shown in **supplemental table S2**. The median age, on the DXA scan date, was 65.0 years [57.0-99.0 IQR] for the total population (n=6,990). Of the total population, 7.8% (n=543) ever used LD from baseline till the DXA scan with a mean duration of 51.2 days (325.1 SD). The median age of the 543 LD-users was 77.0 years [52.0-99.0 IQR] and 64.0 years [51.0-97.0 IQR] for the LD non-users (**Table 1**).

Table 1. Characteristics total population, users and non-users of LD

	Total population (n=6,990)	LD ever users (n=543)	LD never users (n=6,447)	p-value LD ever- vs. never users
Age (years)[b]	65.0 [57.0-99.0]	77.0 [52.0-99.0]	64.0 [51.0-97.0]	**<0.001***
Sex (%women)	3,985 (57.0)	309 (56.9)	3,675 (57.0)	0.92
BMI (kg/cm2)[a]	27.6 (4.1)	28.9 (4.7)	27.5 (4.7)	**0.002***
Cohort (%)				**<0.001***
RS-I	2,799 (40.0)	437 (80.5)	2,360 (36.6)	
RS-II	980 (14.0)	62 (11.4)	918 (14.2)	
RS-III	3211 (46.0)	44 (8.1)	3,167 (49.1)	

Table 1. Characteristics total population, users and non-users of LD (*continued*)

	Total population (n=6,990)	LD ever users (n=543)	LD never users (n=6,447)	p-value LD ever- vs. never users
Loop diuretics use (%ever use)	543 (7.8)	543 (100)	0	NA
Duration (days)[a]	NA	194 [40-893]	0	
25(OH)D (nmol/l)[b]	54.4 [36.5-76.0]	42.3 [28.8-62.3]	55.4 [37.2-76.7]	**<0.001***
Cutoff (%):				
<50 nmol/L	2,580 (44.2)	247 (45.5)	2,333 (36.2)	**<0.001***
>50 nmol/L	3,257 (55.8)	156 (28.7)	3,101 (48.1)	
Season measurement (%):				0.41
Spring	1,928 (27.6)	147 (27.0)	1,854 (28.8)	
Summer	1,143 (16.4)	82 (15.1)	1,107 (17.2)	
Autumn	2,219 (31.7)	177 (32.5)	2,126 (33.0)	
Winter	1,432 (20.5)	138 (25.4)	1,360 (21.0)	
Dietary calcium intake (mg/day)[a]	1126.5 (388.9)	1189.2 (394.1)	1120.8 (394.1)	0.81
Tertiles (%):				
<950.0 mg/day	1,691 (24.2)	103 (19.0)	1,588 (24.6)	
950-1200 mg/day	1,504 (21.5)	120 (22.1)	1,384 (21.5)	
>1200 mg/day	1,795 (25.7)	186 (34.3)	2,609 (25.0)	
Lumbar Spine TBS[a]	1.24 (0.13)	1.17 (0.12)	1.25 (0.12)	0.32
Femoral Neck Bone Mass Density (g/cm2)[a]	0.91 (0.15)	0.86 (0.15)	0.91 (0.15)	0.98
Lumbar Spine Bone Mass Density (g/cm2)[a]	1.14 (0.21)	1.15 (0.22)	1.14 (0.20)	0.40
Smoking (%)				**0.003***
Never smoker	2,080 (29.8)	157 (28.9)	1,921 (29.8)	
Current smoker	1,358 (19.4)	58 (10.6)	1,302 (20.2)	
Former, non-smoker	3,506 (50.2)	319 (58.8)	3,185 (49.4)	
Pack years[a]	9.4 (18.6)	20.3 (28.0)	8.5 (28.0)	**<0.001***
Education category (%)				**<0.001***
Low education	3,575 (51.6)	319 (58.7)	3,256 (50.9)	
Higher education	3.353 (48.4)	219 (40.3)	3,134 (49.1)	
Alcohol Intake (g/day)[b]	7.3 [0.8-20.0]	3.1 [0.1-15.7]	7.9 [0.9-20.0]	**0.11**
PA (MET hours/week)[b]	70.5 [39.4-103.9]	70.8 [47.6-100.1]	70.4 [38.5-104.4]	**0.84**
Energy intake (Kcal/day)[a]	2176.6 (710.2)	1956.9 (533.8)	2195.2 (533.8)	**<0.001***
Comorbidities (%yes)	1,112 (15.9)	150 (27.6)	963 (14.9)	**<0.001***
CHD	315 (4.5)	69 (12.7)	246 (3.8)	
Stroke	129 (1.8)	13 (2.4)	116 (1.8)	
DM	790 (11.3)	98 (18.0)	691 (10.7)	

[a]mean (SD); [b]median (IQR); %=percentage; * p-value<0.05.

Table 2. Linear regression of the use of LD and FN-BMD and LS-TBS in RS-I-4, RS-II-2 & RS-III-1

	Model 1 β (95%CI)	Model 2 β (95%CI)	Model 3 β (95%CI)
LS-TBS[a] (6,476)			
LD never-use (5,972)	*Reference*	*Reference*	*Reference*
LD past-use (n=310)	**-0.031 [-0.044; -0.017]***	**-0.019 [-0.033; -0.006]***	**-0.025 [-0.038; -0.012]***
LD current-use (n=194)	**-0.018 [-0.034; -0.001]***	-0.011 [-0.028; 0.006]	-0.015 [-0.032; 0.002]
Duration of LD-use categories[b]			
Never users (n=5,972)	*Reference*	*Reference*	*Reference*
Current users 1-120 days (n=37)	-0.008 [-0.027; 0.011]	-0.0005 [-0.043; 0.042]	-0.003 [-0.044; 0.039]
Current users 121-365 days (n=27)	-0.026 [-0.048; 0.003]	-0.026 [-0.070; 0.018]	-0.041 [-0.084; 0.002]
Current users >365 days (n=310)	-0.019 [-0.039; 0.001]	-0.010 [-0.031; 0.010]	-0.013 [-0.032; 0.007]
LS-BMD (n=6,677)			
LD never-use (6,146)	*Reference*	*Reference*	*Reference*
LD past-use (n=321)	**0.048 [0.026; 0.071]***	**0.029 [0.006; 0.052]***	**0.038 [0.016; 0.060]***
LD current-use (n=210)	**0.037 [0.010; 0.065]***	0.021 [-0.008; 0.050]	0.025 [-0.003; 0.052]
Duration of LD-use categories[b]			
Never users (n=6,146)	*Reference*	*Reference*	*Reference*
Current users 1-120 days (n=41)	0.020 [-0.040; 0.081]	0.012 [-0.059; 0.083]	0.014 [-0.054; 0.082]
Current users 121-365 days (n=32)	**0.072 [0.004; 0.140]***	0.068 [-0.003; 0.139]	**0.075 [0.007; 0.143]***
Current users >365 days (n=137)	**0.034 [0.0004; 0.068]***	0.012 [-0.022; 0.047]	0.016 [-0.017; 0.049]
FN-BMD (n=6,908)			
LD never-use (6,376)	*Reference*	*Reference*	*Reference*
LD past-use (n=319)	**0.016 [0.001; 0.031]***	0.006 [-0.009; 0.021]	0.010 [-0.005; 0.025]
LD current-use (n=213)	0.006 [-0.012; 0.024]	-0.006 [-0.025; 0.013]	-0.006 [-0.024; 0.013]

Table 2. Linear regression of the use of LD and FN-BMD and LS-TBS in RS-I-4, RS-II-2 & RS-III-1 (*continued*)

	Model 1 β (95%CI)	Model 2 β (95%CI)	Model 3 β (95%CI)
Duration of LD-use categories[b]			
Never users (n=6,376)	*Reference*	*Reference*	*Reference*
Current users 1-120 days (n=44)	0.020 [-0.019; 0.058]	0.024 [-0.021; 0.069]	0.021 [-0.025; 0.067]
Current users 121-365 days (n=32)	0.035 [-0.011; 0.080]	0.019 [-0.028; 0.066]	0.023 [-0.023; 0.069]
Current users >365 days (n=137)	-0.005 [-0.027; 0.018]	-0.019 [-0.042; 0.003]	-0.018 [-0.040; 0.004]

Model 1: sex, age, cohort. Model 2: additional adjusted for BMI, Alcohol, smoking, SES, PA and comorbidities (for duration of LD-use additional adjusted for past users). Model 3: + LS-TBS (for the analysis of FN-BMD)/FN-BMD and LS-BMD (for the analysis of LS-TBS); *p<0.05; [a]standardized according to sex by residual method; [b]Past users depicted in the rows above.

The median serum 25(OH)D level of the LD-users was 42.3 nmol/L [28.8-62.3 IQR] and for LD non-users, 55.4 nmol/l [37.2-76.7 IQR]. LD-users showed a significantly lower serum 25(OH)D level than non-users (p<0.001). The mean dietary calcium intake of the LD-users was 1189 mg/day (394 SD) and 1121 mg/day (394 SD) for LD non-users. Results of the linear regression analysis of LD-use and indices of bone health are shown in **table 2** and discussed below.

Loop diuretics and LS-TBS

Compared to LD never-use, current-use of LD was only associated with LS-TBS in the crude model (ß=-0.018, 95% CI: -0.034; -0.001) and past-use of LD was associated with lower LS-TBS, in model 1 (ß=-0.031, 95% CI: -0.044; -0.017). Adjustment for co-variates in model 2 attenuated the association somewhat whereby the effect size was 30% lower. The analysis of categories of the duration of LD-use among users and LS-TBS, showed no significant associations (**Table 2**).

Loop diuretics and LS-BMD

Current-use of LD in 210 participants was associated with significantly higher LS-BMD compared to never-use of LD in the crude model (ß=0.037, 95% CI: 0.010; 0.065). Past-use of LD was associated with higher LS-BMD compared to never-use of LD in the crude model, model 2 and fully adjusted model (model3) (ß=0.048, 95% CI: 0.026; 0.071, ß=0.029, 95% CI: 0.006; 0.052 and ß=0.038, 95% CI: 0.016; 0.060). In the analyses of the duration of LD-use, current-use of LD between 121-365 days showed a significantly higher LS-BMD in fully adjusted model (ß=0.075, 95% CI: 0.007; 0.143 **table 2**).

Loop diuretics and FN-BMD

LD current-users showed no significant association with FN-BMD compared to never-use of LD. However, past-use of LD showed a significantly higher FN-BMD only in the crude model (ß=0.016, 95% CI: 0.001; 0.031, **table 2**).

Additional adjustment for serum vitamin D, season of blood collection of vitamin D, serum calcium, magnesium and sodium did not change the results (data not shown).

Furthermore, we evaluated the association between LD and LS-TBS, LS-BMD and FN-BMD by sex and found no evidence that the association between LD and LS-TBS was significantly different according to sex (P-interaction=0.83). However, the association

Table 3. Linear regression of LD-use (yes/no) on FN-BMD and LS-TBS for 25(OH)D level in categories (≤20, 20-50 and ≥50 nmol/l) in model 2

	25(OH)D ≤20 nmol/l (n=302)	25(OH)D 20-50 nmol/l (n=2,240)	25(OH)D ≥50 nmol/l (n=3,225)	p-value interaction term
	Β (95%CI)	Β (95%CI)	Β (95%CI)	
LS-TBS[b] (6,476)				
LD never-use	*Reference*	*Reference*	*Reference*	**0.04***
LD past-use (n=310)	-0.012 [-0.036; 0.013]	-0.031 [-0.096; 0.034]	**-0.036 [-0.060; -0.013]***	
LD current-use (n=194)	0.001 [-0.036; 0.039]	-0.029 [-0.099; 0.041]	-0.014 [-0.046; 0.017]	
	25(OH)D ≤20 nmol/l (n=287)	**25(OH)D 20-50 nmol/l (n=2,146)**	**25(OH)D ≥50 nmol/l (n=3,108)**	**p-value interaction term**
	Β (95%CI)	**Β (95%CI)**	**Β (95%CI)**	
LS-BMD (n=6,677)				
LD never-use	*Reference*	*Reference*	*Reference*	0.30
LD past-use (n=321)	0.079 [-0.054; 0.148]	0.003 [-0.034; 0.041]	0.038 [-0.003; 0.080]	
LD current-use (n=210)	-0.052 [-0.157; 0.053]	**0.044 [0.002; 0.087]***	0.039 [-0.016; 0.095]	
	25(OH)D ≤20 nmol/l (n=267)	**25(OH)D 20-50 nmol/l (n=2,054)**	**25(OH)D ≥50 nmol/l (n=3,056)**	**p-value interaction term**
	Β (95%CI)	**Β (95%CI)**	**Β (95%CI)**	
FN-BMD (n=6,908)				
LD never-use	*Reference*	*Reference*	*Reference*	0.13
LD past-use (n=319)	0.027 [-0.037; 0.092]	-0.006 [-0.031; 0.019]	0.008 [-0.020; 0.036]	
LD current-use (n=213)	-0.022 [-0.089; 0.045]	-0.004 [-0.031; 0.023]	0.015 [-0.021; 0.051]	

*p<0.05; **p<0.10; [b]standardized according to sex by residual method

Table 4. Linear regression of LD-use (yes/no) on FN-BMD and LS-TBS for dietary calcium intake categories (≤950, 950-1200 and ≥1200 mg/day) in model 2

	Dietary calcium intake ≤950 mg/day (n=1,822)	Dietary calcium intake 950-1200 mg/day (n=1,600)	Dietary calcium intake ≥1200 mg/day (n=1,875)	p-value interaction term
	ß (95%CI)	ß (95%CI)	ß (95%CI)	
LS-TBS[b] (6,476)				
LD never-use	*Reference*	*Reference*	*Reference*	0.58
LD past-use (n=310) (yes/no)	-0.018 [-0.047; 0.010]	-0.022 [-0.050; 0.005]	-0.015 [-0.037; 0.007]	
LD current-use (n=194) (yes/no)	-0.009 [-0.044; 0.025]	0.007 [-0.028; 0.043]	-0.015 [-0.043; 0.013]	
	Dietary calcium intake ≤950 mg/day (n=1,732)	**Dietary calcium intake 950-1200 mg/day (n=1,548)**	**Dietary calcium intake ≥1200 mg/day (n=1,838)**	**p-value interaction term**
	ß (95%CI)	**ß (95%CI)**	**ß (95%CI)**	
LS-BMD (n=6,677)				
LD never-use	*Reference*	*Reference*	*Reference*	0.87
LD past-use (n=321) (yes/no)	0.017 [-0.034; 0.068]	0.039 [-0.008; 0.086]	0.026 [-0.012; 0.063]	
LD current-use (n=210) (yes/no)	0.038 [-0.023; 0.100]	0.045 [-0.016; 0.106]	0.016 [-0.031; 0.064]	
	Dietary calcium intake ≤950 mg/day (n=1,695)	**Dietary calcium intake 950-1200 mg/day (n=1,509)**	**Dietary calcium intake ≥1200 mg/day (n=1,771)**	**p-value interaction term**
	ß (95%CI)	**ß (95%CI)**	**ß (95%CI)**	
FN-BMD (n=6,908)				
LD never-use	*Reference*	*Reference*	*Reference*	0.99
LD past-use (n=319) (yes/no)	0.001 [-0.032; 0.034]	0.087 [-0.023; 0.039]	0.008 [-0.017; 0.034]	
LD current-use (n=213) (yes/no)	-0.003 [-0.043; 0.037]	0.024 [-0.015; 0.064]	-0.025 [-0.057; 0.007]	

*p<0.05; **p<0.10; [b]standardized according to sex by residual method

between LD and LS-BMD and FN-BMD was significantly different according to sex (P-interaction for both=0.04) **(Supplemental table S3)**.

Serum 25(OH)D level and dietary calcium intake in the association of the use of loop diuretics and FN-BMD, LS-BMD and LS-TBS

Effect-modification analysis of serum 25(OH)D level and dietary calcium intake in the association between LD-use on FN-BMD and LS-TBS are shown in **tables 3 and 4**. P-value for interaction terms varied from 0.04 to 0.99. Serum 25(OH)D level was an effect modifier in the association between LD use and LS-TBS (P for interaction=0.04). After stratification in model 2, the group of serum 25(OH)D level ≥50 nmol/L showed a significantly lower LS-TBS compared to serum 25(OH)D ≤20 nmol/L and between 20-50 nmol/L for LD past-use (ß=-0.036, 95% CI: -0.060; -0.013 vs. ß=-0.012, 95% CI: -0.036; 0.013 and ß=-0.031, 95% CI: -0.096; 0.034, respectively, **table 3**).

There was no effect modification by serum 25(OH)D level on the association between LD-use and FN-BMD and LS-BMD (**Table 3**). After stratification, for the analysis of the association between LD and LS-BMD, participants with serum 25(OH)D level between 20-50 nmol/L had a significantly higher LS-BMD compared to participants with serum 25(OH)D level ≤20 and ≥50 nmol/l for LD current use, however there was no significant interaction (P for interaction=0.30) (**Table 3**).

No significant effect modification by dietary calcium intake was observed on the association between use of LD and FN-BMD, LS-BMD and LS-TBS (**Table 4**). After stratification of dietary calcium intake in categories of intake ≤950, 950-1200 and ≥1200 mg/day, no significant associations were found with FN-BMD, LS-BMD and LS-TBS (**Table 4**).

DISCUSSION

In this study, we observed a modest increase in BMD of the lumbar spine and a modest decrease in TBS in LD past-users, therefore our study conclude that if any, LD use does not have a strong association with bone health, in a population with a high calcium intake. Furthermore, this study found effect-modification by serum 25(OH)D level in the association between LD and LS-TBS. However, after stratification on serum 25(OH)D, no consistent findings were found in the association between LD-use and bone outcomes, suggesting no strong modifying effect of serum 25(OH)D on these associations. Additionally, no effect-modification was found by dietary calcium intake.

In our study, current LD-use showed a negative association with LS-TBS, however not significant.

In contrast to the findings of the association between LD and LS-BMD, we found that a history of LD was associated with a decreased LS-TBS if LD was used in the past. To our knowledge there have been no studies on LD-use and LS-TBS. TBS is a measurement related to bone microarchitecture provides skeletal information that is not captured from the standard BMD measurements (31). Also, TBS might be an appropriate measure to study in regard to lifestyle factors that are adaptable, such as dietary intake (31, 32). Other studies have reported lower TBS among individuals with primary hyperparathyroidism (38). And as mentioned, LD's increase the plasma PTH and 1.25(OH)2D levels as a result of increased renal calcium losses (11). Thus a possible explanation for our finding is that LS-TBS may be decreased by secondary hyperparathyroidism caused by long-time LD-use (13, 39). However, we could not confirm this possible pathway in our current study, since we do not have the availability of serum PTH. Other explanation for the opposite direction between BMD and TBS due to potential residual confounding (i.e. body composition and health status) in the association between LD and TBS. Further studies are needed to explore the association between LD and overall bone health, especially TBS.

Additionally, LD-use was not associated with a lower BMD. In contrast, it has been shown that LD increases renal calcium excretion (8-11), with a potential negative effect on BMD (10, 13, 38). We expected that LD-use would result in a lower FN-BMD and LS-BMD, because treatment with LD may induce secondary hyperparathyroidism with raised bone resorption resulting in a lower BMD (39, 40). This was, however, not confirmed by our findings. Current-use of LD showed a non-significant negative association with FN-BMD and a non-significant positive association with LS-BMD. Furthermore, the study of Rejnmark et. al. in 2005 showed also a non-significantly higher LS-BMD for the group with LD (who had been treated with a LD for at least 2 years prior to inclusion in the study), compared with non-users (38). Another study of Rejnmark et. al. in 2006 showed a decrease in BMD for the LD-users compared to placebo treated for 1 year. The effect was however weakened after 6 months end of treatment. Unfortunately, we cannot confirm this result in our study. In our analysis of the association between the duration of LD and FN-BMD, we found a positive association for LD-use between 1-365 days and a negative association with FN-BMD for longer use of LD (>365 days) compared to never-users of LD, nevertheless this was not significant. Also we found an increase in LS-BMD when LD was used for 121-365 days. This suggests that LD-use might be weakly associated with higher BMD but only for a short time period.

Some studies have shown differences in vitamin D level according to LD-use. In line with our results, there is evidence that LD-users have a lower serum 25(OH)D level than non-users (34) and the inverse association between LD-use and serum 25(OH)D level has been reported before (16). To the best of our knowledge, information about the effect modification by serum 25(OH)D level on the association between LD-use and FN-BMD and LS-TBS as bone health parameters has not been reported earlier. In our study, stratified analyses did not show consistent patterns and the differences were not statistically significantly different between cut-offs of serum 25(OH)D level. However, we found evidence for potential effect modification (P-interaction<0.10) by serum 25(OH)D level for the association of LD past-use and LS-TBS. Stratified analyses showed a slightly stronger inverse association between past LD-use and LS-TBS in those with serum 25(OH)D level ≤20, however the association was not significant in strata of serum 25(OH)D level between 20-50 and above 50 nmol/L. This finding may imply that past-use of LD may have affected LS-TBS which can have a higher impact in those with extremely low 25(OH)D levels. However, because this effect-modification was not further confirmed by other categories of LD-use, further replication is needed.

Moreover, even though we adjusted our analysis for body fat mass, reduced vitamin D levels may still be confounded other measures of body composition and health status.

Our initial hypotheses was that an adequate serum 25(OH)D level and dietary calcium intake would counteract the potential adverse effects of LD on bone health. In contrast, we found no consistent significant differences between categories of dietary calcium intake, in the association between LD and FN-BMD, LS-BMD and LS-TBS. Thus, given that LD increases the renal excretion of calcium (40), the effects of LD on bone health showed unexpected results. This is in contrast with earlier studies, showing that a higher dietary intake of calcium prevented the expected decrease of FN-BMD in LD-users (38, 41, 42). Potentially, the lack of calcium modifying the association between LD and bone health in our study may result from the fact that in our study calcium intake was higher than in other studies.

Strengths and limitations

A strength in our study is the availability of data on dietary intake of calcium, serum 25(OH)D level and LD-use, and the different indices of bone health (i.e. FN-BMD as well as LS-BMD and LS-TBS). Our study also adds data to the quite unexplored field of nutrient-drug interactions in a population of community-dwelling older persons, who are at risk of musculoskeletal diseases, malnutrition and consequences of polypharmacy. However, some limitations need to be taken into account when interpreting our

results. These include the observational study design and potential residual confounding (for example, underlying disease), which prevents us from drawing conclusions regarding causality and the direction of the effect size of the association. Furthermore, as all our analyses were hypothesis-based, we did not adjust for multiple comparisons. With regard to possible type I errors, stringent interpretation of p-values should be made with caution. Likewise, potential misclassification of LD-use using pharmacy records may occur because data regarding actual compliance was lacking. Also, dietary calcium intake was assessed using self-reported dietary assessment, which is subject to bias and measurement error. Also, dietary intake was measured only once (at baseline) and as a result we were unable to account for differences in dietary habits over time (e.g. due to disease or medication use). Moreover, the complete data on calcium or vitamin D supplement use was not available, which could lead to an underestimation of dietary intake. In addition, vitamin D and calcium prescriptions was not analysed as exposure variable because of confounding by indication. Also, we did not have a comprehensive assessment of calcium homeostasis. For example, the free calcium concentration in plasma is strongly controlled through a complicated physiological system including the interaction of calcitrophic hormones such as PTH and 1,25(OH)D and only in extreme situations, will the serum calcium concentration deflect from the normal range (14). Since we did not have the availability of serum PTH, we could not investigate potential pathways between the use of LD and calcium intake and vitamin D level. Finally, our result of inconsistent effect modification by serum25(OH)D and dietary calcium intake on the associations between LD and the parameters of bone health could be explained by managing the parathyroid hormones (PTH) and 1.25 (OH)D that maintain calcium homeostasis, especially when serum calcium is reduced (43).

In conclusion, this study does not support the hypothesis that the association between loop diuretic use and indices of bone health is modified by serum 25(OH)D level and calcium intake. However, because of polypharmacy effects and a higher risk of malnutrition in elderly, further research and replication is warranted on nutrient-drug interaction on bone health (considering the subjects with osteopenia and osteoporosis and people with malnutrition), using other biomarkers as PTH and bone turnover markers as well as long term loop diuretic use.

Supplementary Materials: Table S1: Details of the multiple imputation modelling, Table S2: Basic characteristics before and after multiple imputation.

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Author Contributions: Author's roles: S.O.A., J.C.K.d.J., A.G.U., B.H.S., and N.vd.V. discussed the hypothesis and interpreted the data. K.T., F.K., and F.R. provided the data. S.O.A., and J.K.d.J. analysed the data. A.G.U, B.H.S. and M.A.I. coordinated and directed the project. S.O.A., J.C.d.J., and N.vd.V wrote the article. S.O.A., J.C. K.d.J., K.T., F.K., F.R., M.C.Z., N.M.v.S., L.C.G.M.d.G. M.A.I., A.G.U., B.H.S. and N.vd.V. edited the article.

Informed consent: The study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of the Netherlands Ministry of Health, Welfare and Sports, and approval has been renewed every 5 years. From all subjects written informed consent was obtained.

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SUPPLEMENTARY DATA

Table S1. Details of the multiple imputation modelling

	Multiple imputation procedure
Software used	SPSS 24.0 for Windows
Imputation method	Fully conditional specification (Markov chain Monte Carlo method)
Maximum iterations	10
Imputed datasets created	10
Exposures and outcomes (not imputed, used in model as predictor)	Calcium intake, vitamin D level, LD, LS-TBS, LS-BMD and FN-BMD
Covariates (imputed)	BMI, scan age, smoking, METhours and comorbidities
Additional predictors	Sex, cohort, packyears, alcohol, use of bisphosphonate
Treatment of non-normally distributed variables	Predictive mean matching
Treatment of binary/ categorical variables	Logistic regression models

Number of missing for the variables imputed can be found in supplementary table S2.

Table S2. Basic characteristics before and after multiple imputation

	N	Missing (n)	Original data	Imputed data
Age (years)[b]	6,990	0	65.0 [57.0-99.0]	*No missing*
Sex (%women)	6,990	0	3,985 (57.0)	*No missing*
BMI (kg/cm2)[a]	6,916	74	27.6 (4.1)	27.6 (4.1)
Loop diuretics use (%yes)	6,990	0	543 (7.8)	*No missing*
Duration (days)[a]			51.2 (325.1)	
25(OH)D (nmol/l)[b]	5,837	1,153	54.4 [36.5-76.0]	*Not imputed*
Cutoff (%):				
<50 nmol/L			2,580 (44.2)	
>50 nmol/L			3,257 (55.8)	
Season measurement (%):				
Spring			1,928 (27.6)	
Summer			1,143 (16.4)	
Autumn			2,219 (31.7)	
Winter			1,432 (20.5)	
Dietary calcium intake (mg/day)[a]	4,993	1,997	1126.5 (388.9)	*Not imputed*
Tertiles (%):				
<950.6 mg/day				
951-1234.7 mg/day				
>1235 mg/day				
Lumbar Spine TBS[a]	6,476	514	1.24 (0.13)	*Not imputed*
Femoral Neck Bone Mass Density (g/cm2)[a]	6,677	313	0.91 (0.15)	*Not imputed*
Lumbar Spine Bone Mass Density (g/cm2)[a]	6,908	82	1.14 (0.21)	*Not imputed*
Smoking (%)	6,909	81		
Never smoker			2,069 (29.6)	2,080 (29.8)
Current smoker			1,352 (19.3)	1,358 (19.4)
Former, non-smoker			3,484 (49.8)	3,506 (50.2)
Pack years[b]	6,990	0	9.4 (18.6)	*No missing*
Education category (%)	6,982	8		
Low education			3,570 (51.1)	3,575 (51.6)
Higher education			3351 (47.9)	3.353 (48.4)
Alcohol Intake (g/day)[b]	6,990	0	7.3 [0.8-20.0]	*No missing*
PA (MET hours/week)[b]	5,899	1,091	70.5 [39.4-103.9]	70.5 [39.4-103.9]
Energy intake (Kcal/day)[a]	5,405	1,585	2159.7 (680.4)	2176.6 (710.2)
Comorbidities (%yes)			13.9	1,112 (15.9)
CHD	6,645	345	4.6	315 (4.5)
Stroke	6,660	330	1.2	129 (1.8)
DM	6,467	523	10.7	790 (11.3)

[a]presented as mean (SD); [b]presented as median [interquartile range]

Table S3. The association between LD and LS-BMD and FN-BMD stratified by sex

	Male (n=2,772) ß (95%CI)	Female (n=3,637) ß (95%CI)	p-value interaction term
LS-TBS[b] (6,476)			0.83
LD never-use	Reference	Reference	
LD past-use (n=310)	**-0.030 [-0.050; -0.010]***	-0.011 [-0.029; 0.007]	
LD current-use (n=194)	-0.016 [-0.042; 0.011]	-0.007 [-0.029; 0.015]	
	Male (n=2,844) ß (95%CI)	**Female (n=3,833) ß (95%CI)**	**p-value interaction term**
LS-BMD (n=6,677)			**0.04***
LD never-use	Reference	Reference	
LD past-use (n=321)	-0.003 [-0.038; 0.033]	**0.057 [0.028; 0.086]***	
LD current-use (n=210)	0.027 [-0.020; 0.074]	0.018 [-0.017; 0.053]	
	Male (n=2,985) ß (95%CI)	**Female (n=3,923) ß (95%CI)**	**p-value interaction term**
FN-BMD (n=6,908)			**0.04***
LD never-use	Reference	Reference	
LD past-use (n=319)	-0.0002 [-0.024; 0.023]	0.013 [-0.006; 0.033]	
LD current-use (n=213)	-0.008 [-0.039; 0.023]	-0.005 [-0.028; 0.019]	

***p<0.05**