Thresholds for serum 25(OH)D concentrations with respect to different outcomes

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Context: Vitamin D is essential for bone health. In addition, vitamin D has recently been proposed to play a role in the pathophysiology of many chronic diseases. Despite the large amount of studies published on vitamin D, the threshold for a sufficient serum 25(OH)D concentration is still debated and may differ according to outcomes and subgroups.

Objective: To estimate thresholds for serum 25(OH)D concentration with respect to different outcomes and for different subgroups.

Design, setting and participants: Observational data from the Longitudinal Aging Study Amsterdam were used, an ongoing population-based Dutch cohort study (N=1164, mean (SD) age 75.2 (6.5) years).

Main outcome measures: falling, fractures, hypertension, cardiovascular disease, blood pressure, parathyroid hormone (PTH), grip strength, physical performance, functional limitations, body mass index (BMI) and mortality. To determine thresholds, spline curves were used. Visual inspection and the statistical best fit of the spline regression models were used together to estimate the best estimate of the thresholds.

Results: Thresholds for serum 25(OH)D concentrations in the whole sample ranged from 46 nmol/l (PTH) to 68 nmol/l (hypertension). On average, women, the oldest old (≥75 years) and individuals with high BMI (>25kg/m^2), had lower thresholds compared to men, the youngest old (65–75 years) and individuals with low-to-normal BMI (<25kg/m^2).

Conclusion: The results indicate that thresholds for serum 25(OH)D may vary according to different outcomes and subgroups. This study does not support the high thresholds (>75 nmol/l) as advised by some experts, and the higher requirements in women, older persons and those with high BMI.

Vitamin D is essential for bone health (1). The main and best known function of vitamin D is to increase the calcium absorption from the gut to facilitate bone mineralization (1). In addition, vitamin D has recently been proposed to play a role in the function of many other systems, such as the immune and cardiovascular system (2, 3), although causality has not yet been proven for most non-classical outcomes (4).

Despite the large amount of studies published on vitamin D, there is still discussion on the threshold of desired 25-hydroxyvitamin D (25(OH)D) concentration. The Endocrine Society advocates levels of at least 75 nmol/l (5), whereas the Institute of Medicine advises minimal levels between 30 to 50 nmol/l (6). Different national guidelines also advise various levels. Usually, the thresholds are based on optimal bone health and the level at which parathyroid hormone (PTH) concentrations stop to decline (7). Several years ago, a review on the evidence on thresholds of serum 25(OH)D was published (8). The authors stated that levels above 100 nmol/L were necessary to reach max-

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Abbreviations:

imal bone mineral density (BMD) or the lowest risk for fractures, whereas for muscle strength a serum level above 40 nmol/l was desirable (8). In contrast, a study in Korean individuals revealed that levels of around 50 nmol/l was sufficient to maintain bone health (9).

In previous studies, low vitamin D status was associated with higher blood pressure (BP) (10), higher mortality (11), higher fracture and falls rates (12), higher BMI (13) and lower physical performance (14, 15), but thresholds were not determined systematically for every outcome. Furthermore, it is known that mean serum 25(OH)D concentrations differ between men and women, low and high age and for individuals with different body mass index (BMI) (1, 12, 16). However, it is not clear whether the same thresholds are applicable to these subgroups.

Therefore, this study aimed to explore the thresholds of serum 25(OH)D with respect to different outcomes. In the current study, a threshold is not the point at which an outcome does not occur anymore, but it is defined as the serum 25(OH)D concentration from which the risk for the specific outcome or outcome value starts to level off. Although, causality has not yet been proven for most non-skeletal outcomes, the results of large clinical trials on these outcomes will available in the next years. Many observational studies are pointing towards a possible causal relationship. Therefore, we decided to include these outcomes in the current study. In addition, we examined whether these thresholds differ between several subgroups, ie, men and women, different age and BMI groups. We hypothesized that women and older individuals would have higher thresholds, because women and older subjects have a higher fracture risk (17). This higher fracture risk may be caused by lower calcium intake and thereby requiring higher serum 25(OH)D to stimulate sufficient absorption of calcium from the gut. Furthermore, vitamin D is fat-soluble and therefore 25(OH)D may be more stored in fat in persons with high BMI, also possibly leading to higher thresholds.

Materials and Methods

Study participants

Data for this study were collected within the Longitudinal Aging Study Amsterdam (LASA). LASA is an ongoing cohort study in a representative sample of the Dutch older individuals. The sampling and data collection procedures are described elsewhere in detail (18). Briefly, a random age- and sex stratified sample was drawn from population registries of 11 municipalities in three regions in the Netherlands. The study started in 1992 and 3107 individuals of 55–85y were included. In 2002 and in 2012, two additional cohorts were started. For this study, data from the second cycle of the first cohort, which took place in 1995/1996, were used. Participants who were 65 years and older on January first 1996 were invited for a medical interview in 1995/1996 (N = 1509). In 1320 participants, serum 25(OH)D was measured. We were not able to use the data of 156 participants due to missing values for the outcome variables. Therefore, the study sample consisted of 1164 participants.

LASA was approved by the Medical Ethics committee of the VU University Medical Center and all participants gave informed consent.

Outcome measures

The following variables were considered as outcome measures: falls, fractures, PTH, BP, cardiovascular disease, BMI, grip strength, physical performance, functional limitations, and mortality. These variables were chosen because many, mostly cross-sectional studies, showed an association between vitamin D status and these outcomes(3;10–15;19;20).

Falls were prospectively recorded by using a fall and fracture calendar for three years. Every three months participants had to send back their calendar to the study center. A fall was determined as "an unintentional change in position resulting in coming to rest at a lower level or at the ground" (21). Recurrent faller was defined as an individual who fell at least two times within six months (22). Times to first and second fall were calculated; for participants who were lost to follow-up or deceased, time until drop-out was calculated.

Fractures were recorded by using two different methods. For the first three years of follow-up, fractures were reported with a fall and fracture calendar (1995/96–1998/99), which had to be sent back to the study center every three months. Second, questions on the occurrence of fractures in the last three years were asked during the interviews of the next measurement cycles (1998/9–2005/6). So in total, a ten-year fracture follow-up period is available. The date and type of fracture were verified by general practitioners or in hospitals. When a participant was lost to follow-up or deceased, information on the occurrence of fractures was asked from the general practitioners. Fractures of the hand, head, finger, foot and toe were excluded, because these are not likely to be osteoporotic. Time to first fracture was calculated or, when no fracture occurred, the time to end of follow-up, death or to loss of follow up. Participants were thus only in the analyses for the time they were at risk.

PTH was measured by using an immunoradiometric assay (IRMA) (Incstar Corp., Stillwater, MN, USA). The interassay coefficient of variation was 12%.

Blood pressure (mmHg) was measured using an automatic device (Omron model HEM-706; Omron Corporation, Tokyo, Japan) after at least five minutes of rest at the upper left arm in sitting position. *Mean arterial pressure (MAP)* was calculated using the following formula: diastolic pressure + 1/3 * (systolic pressure–diastolic pressure).

Hypertension was defined as systolic pressure > 160mmHg, and/or diastolic pressure > 90mmHg, and/or the use of antihypertensive medication according to guidelines for older individuals of the Dutch general practitioners (23).

Cardiovascular disease was based on self-report. During the medical interview participants were asked questions on whether they had a chronic cardiovascular disease.

BMI (kg/m^2) was calculated from measured weight and measured height in square meters. Body weight (kg) was measured without upper cloths and shoes using a calibrated balance beam scale. Body height (m) was measured using a stadiometer.

Grip strength (kilograms) was measured using a calibrated strain-gauged dynamometer (Takei TKK 5001, Takei Scientific Instruments Co. Ltd, Tokyo, Japan). Participants had to perform two maximum efforts with each hand, when standing with their hand alongside their body. Mean grip strength was calculated as the mean of the maximum scores of the right and left hand.

Physical performance was determined using three different tests; the walking test in which participants had to walk 3 m, turn around and walk back as quickly as possible, the chair stand test, in which participants had to stand up from and sit down on a chair without using their hands for five times as quickly as possible and the tandem stand test, in which they had to stand in tandem position (one foot directly in front of the toes of the other foot) for a maximum of ten seconds. The scores of the individual test were summed and the total scores ranged from 0–12, with 12 points indicating the best performance.

Functional limitations were assessed by asking questions on the ability to perform activities of daily life: using their own or public transportation, cutting their own toenails, dressing and undressing themselves, walking outside for five minutes without stopping, sitting down and standing up from chair, walking up and down a staircase of 15 steps without resting. A dichotomization was made between having limitations in any of these activities vs no limitations in any these activities.

The exact *date of death* was collected from the municipality registries where participants where living. Time until death from 1995/1995 to 2005/2006 was calculated.

The analyses on fractures, falling, recurrent falling, and mortality were based on longitudinal data, whereas the other outcomes were cross-sectional.

Serum 25(OH)D measurements

Morning blood samples were taken from the participants, who were allowed to take tea or toast, but no dairy products. Samples were centrifuged and stored at –20°C until determination in 1997/1998. A competitive binding protein assay was used (Nichols Diagnostics, San Juan Capistrano, CA, USA). The interassay coefficient of variation was 10%.

Subgroups

Age and sex were derived from population registries. BMI was calculated as described above. To define two subgroups of BMI, the common cut-off point of 25 kg/m^2, which is near the median BMI, was used. Age was dichotomized around the median age, namely 75 years.

Statistical analysis

Serum 25(OH)D was used as a continuous independent variable in all analyses. Thresholds were estimated in four steps:

First, spline plots were made to determine the relationship between serum 25(OH)D and the outcomes. For continuous variables, the splines curves plotted the outcome variable against serum 25(OH)D, whereas the outcome was Log Odds or Log Hazards for dichotomous and longitudinal outcomes, respectively. Subsequently, it was determined whether there was a clinically relevant difference in outcome between high and low serum 25(OH)D concentrations in our sample. The magnitude of the clinically relevant difference was based on the literature if possible. For BP (diastolic, systolic and MAP), a difference of 6 mmHg was considered relevant (24) and for grip strength this was 6 kg (25). A 5% weight loss is often considered as clinically relevant in the literature (26), therefore a change in BMI of approximately 1 point was used in this study. For physical performance, a difference of 1 point in total score was considered as clinically relevant (27). For differences in odds and hazard ratios, a minimal difference of 0.5 was considered as relevant. When a clinically relevant difference in outcomes (risks or values) was not observed between high and low serum 25(OH)D, no further steps were performed.

Second, on the basis of the spline curves, a nonlinear relationship had to be confirmed, because no threshold will exist in a linear association. This was done by using the nonlinearity test within spline regression analysis. A p-value < 0.2 was considered as nonlinear.

Third, the threshold of serum 25(OH)D was estimated visually and was defined as the point at which the outcome value started to increase or decrease less rapidly (28) or the lowest value on the spline curve was chosen in case of a U-shaped relationship.

Fourth, these visually determined thresholds were used in further analyses to determine the definite thresholds by the following procedure. Spline regression analyses were performed using three knots. The first and third knots remained the fixed standard knots based on the distribution of values (the 10th and 90th percentile). A range of possible values around the visually determined threshold value (+/- 10 nmol/l) was explored for the second knot. The value used as second knot which resulted in the model with the best fit, was defined as the definite threshold value (28). The best model fit was assessed by the highest C-index for dichotomous outcome variables and for survival analyses, and by the R^2 for continuous outcome variables. To evaluate the performance of the models we choose for measures that reflect discrimination as these are considered more important for clinicians. Furthermore, the C-index for discrimination is closely related to the R2. With these measures we were able to use comparable performance measures independent of the type of model.

Table 1 provides a summary of this procedure. An adjustment for confounders was not made in order to represent the true

Table 1. Summary of the procedure to determine the thresholds

Step		Goal
1.	Spline curves	Confirmation of a clinically relevant difference
2.	Spline regression -- test of non-linearity	Confirmation of a non-linear association ($P < 0.2$)
3.	Spline curves -- visual inspection	Visually determination of the threshold
4.	Spline regression -- best model fit	Determination of the definite threshold

values of the general population. Dichotomous outcomes, ie, the risk of falling, being a recurrent faller, functional limitations, hypertension and cardiovascular disease, were analyzed using logistic regression analyses within the spline analyses. Continuous outcomes, ie, grip strength, physical performance, PTH, systolic and diastolic pressure, MAP, and BMI, were analyzed using spline regression analyses based on linear regression. These spline regression analyses are only based on linear regression, and are not assuming a linear relationship as linear regression itself does. The longitudinal outcomes, ie, time to first fracture, to first and second fall and to death, were analyzed using Cox-regression analyses.

Results

The characteristics of the study sample are shown in Table 2. In total, 1164 individuals were analyzed and their mean (SD) age was 75.2(6.5) years. Men and women were similarly distributed. Mean (SD) serum 25(OH)D was 54.5(24.1) nmol/l. Serum 25(OH)D concentrations <25 nmol/l, 25–50 nmol/l, 50–75 nmol/l, and ≥ 75 nmol/l were present in 10.1%, 36.2%, 34.9%, and 18.9% of the individuals, respectively.

Table 3 presents the results for the analyses on the estimation of the thresholds for serum 25(OH)D. In the analyses in the whole sample, the optimal serum 25(OH)D concentrations ranged from 46 (PTH) to 68 nmol/l (hypertension). Thresholds could not be determined for risk of falling, cardiovascular disease, systolic and diastolic BP, mean arterial pressure, BMI and fractures, because of a lack of clinical relevance or nonlinearity of the associations (see Table 3 for reason per outcome). For the BP variables, there appeared to be a threshold, but the difference in BP between the optimal and worst point was only around 2–4 mmHg and thus considered as clinically nonrelevant. For the other variables without a threshold, the p-value for nonlinearity was > 0.2, and therefore a nonlinear relationship was not assumed. Figures 1 and 2 provide some examples of the determination of the thresholds.

When analyzing the different subgroups, differences in thresholds of serum 25(OH)D were observed (Table 3). On average, but not for all outcomes, the threshold for women was lower than for men. Thresholds for men ranged from 48 to 68 nmol/L, whereas for women the range was 40 to 58 nmol/L. The same trend was observed for individuals <75 years of age (51–72 nmol/l) compared to ≥ 75 years (38–61 nmol/l) and for individuals with low-to-normal BMI (46–76 nmol/l) compared to high BMI (42–64 nmol/l).

Discussion

This study showed that thresholds for serum 25(OH)D vary between different subgroups and outcomes. It was found that thresholds were lower for women, the oldest old ($\geq$75years), and higher BMI ($\geq$25 kg/m^2), compared to men, younger old and low-to-normal BMI, respectively. Thresholds for serum 25(OH)D concentrations in the whole sample ranged from 46 (PTH) to 68 (hypertension) nmol/l. In addition, no clear thresholds could be estimated for BP indices, cardiovascular disease, fractures and BMI,

Table 2. Sample characteristics

		Sex		Age		Body Mass Index	
	LASA study sample 1995/1996	Men	Women	<75 yr	≥ 75 yr	<25 kg/m^2	≥ 25 kg/m^2
N	1164	583	581	596	568	400	764
Age (years)	75.2 (6.5)	75.3 (6.5)	75.2 (6.5)	69.8 (2.8)	81.0 (3.7)	75.5 (6.7)	75.1 (6.4)
< 75 yr	596 (51.2)	291 (49.9)	305 (52.5)	-	-	202 (50.5)	394 (51.6)
≥ 75 yr	568 (48.8)	292 (50.1)	276 (47.5)	-	-	198 (49.5)	370 (48.4)
Gender							
Men (n, %)	583 (50.1)	-	-	291 (48.8)	292 (51.4)	211 (52.8)	372 (48.7)
Women (n, %)	581 (49.9)	-	-	305 (51.2)	276 (48.6)	189 (47.3)	392 (51.3)
Serum 25(OH)D (nmol/liter)	54.5 (24.1)	59.0 (24.5)	49.9 (22.9)	61.7 (23.5)	46.9 (22.8)	57.1 (24.9)	53.1 (23.6)
PTH (pmol/liter)	3.2 [2.5–4.3]	3.1 [2.4–4.3]	3.2 [2.5–4.2]	2.9 [2.3–3.8]	3.5 [2.6–4.5]	3.0 [2.3–4.0]	3.2 [2.5–4.3]
Any fracture (n, %)	134 (11.5)	45 (7.7)	89 (15.3)	59 (9.9)	75 (13.2)	41 (10.3)	93 (12.2)
Time to any fracture (days)	1341 [564–2383]	1134 [605–1784]	1541 [528–2669]	1694 [615–3009]	1095 [547–1705]	1968 [997–3017]	1095 [469–1790]
Falling (n, %)	635 (54.62)	286 (49.1)	349 (60.1)	308 (51.7)	327 (57.6)	223 (55.8)	412 (53.9)
Time to first fall (weeks)	40 [16–83]	45.5 [16–83]	38 [16–84]	39 [16–81]	41 [16–83]	41 [20–79]	39 [14–83]
Recurrent faller (n, %)	297 (25.5)	146 (25.0)	151 (26.0)	124 (20.8)	173 (30.5)	117 (29.3)	180 (23.6)
Time to second fall (weeks)	61 [30–99]	62 [30–98]	59 [30–102]	61 [20–89]	63 [33–108]	61 [35–92]	61 [27–102]
Hypertension (n, %)	743 (63.8)	370 (63.5)	373 (64.2)	348 (58.4)	395 (69.5)	205 (51.2)	538 (70.4)
Blood pressure (systolic) (mmHg)	153.4 (25.7)	153.3 (25.1)	153.5 (26.3)	150.7 (24.3)	156.2 (26.8)	149.2 (25.7)	155.6 (25.4)
Blood pressure (diastolic) (mmHg)	83.5 (13.5)	83.8 (14.1)	83.1 (12.9)	84.7 (13.1)	82.1 (13.8)	81.3 (12.7)	84.6 (13.8)
Mean arterial pressure (mmHg)	106.7 (15.9)	106.9 (15.8)	106.6 (16.0)	106.7 (15.1)	106.8 (16.6)	103.9 (15.6)	108.2 (15.8)
Cardiovascular disease (n, %)	278 (23.9)	165 (28.3)	113 (19.4)	86 (14.4)	192 (33.8)	80 (20.0)	198 (25.9)
Body Mass Index (kg/m^2)	26.8 (4.0)	26.2 (3.3)	27.4 (4.6)	26.9 (3.8)	26.8 (4.3)	22.7 (1.9)	28.9 (3.1)
< 25 kg/m^2	400 (34.4)	211 (36.2)	189 (32.5)	202 (33.9)	198 (34.9)	-	-
≥ 25 kg/m^2	764 (65.6)	372 (63.8)	392 (67.5)	394 (66.1)	370 (65.1)	-	-
Deceased (n, %)	469 (42.6)	300 (51.5)	196 (33.7)	143 (24.0)	353 (62.1)	177 (44.3)	319 (41.8)
Time to death (weeks)	279 [161–404]	269 [143–404]	302 [178–403]	280 [146–409]	278 [166–398]	268 [136–385]	289 [172–406]
Grip strength (kg)	28.6 (9.9)	36.0 (7.9)	21.2 (4.8)	31.1 (10.1)	26.1 (9.0)	28.0 (9.2)	29.0 (10.2)
Physical performance (0–12)	7.5 (3.1)	8.1 (2.8)	7.0 (3.2)	8.7 (2.5)	6.3 (3.2)	7.8 (3.1)	7.4 (3.1)
Functional limitations (n, %)	630 (54.1)	277 (47.5)	353 (60.8)	241 (40.4)	389 (68.5)	175 (43.8)	455 (59.6)

Values are mean (SD), N (%) or median [interquartile range]

Table 3. Threshold values in nmol/liter with respect to different outcomes and subgroups

		Sex		Age		Body mass index	
	Total group	men	women	<75 yr	≥ 75 yr	<25 kg/m^2	≥ 25 kg/m^2
Dichotomous							
Risk of falling	**	**	*	**	**	*	*
Risk of recurrent falling	48	55	40	53	38	76	46
Hypertension	68	68	49	*	61	58	*
Cardiovascular disease	**	**	**	**	48	54	42
Functional limitations	57	**	51	72	**	**	53
Continuous							
PTH	46	68	45	52	55	47	62
Systolic blood pressure	*	*	**	*	*	76	*
Diastolic blood pressure	*	*	*	*	*	*	*
Mean arterial pressure	*	*	*	*	*	*	*
Grip strength	65	48	49	**	58	66	64
Physical performance	60	50	58	51	55	46	63
Body Mass Index	**	45	**	**	**	-	-
Survival							
Mortality	56	49	51	71	47	59	62
Fractures	**	**	41	*	*	**	**
Falling	59	54	*	**	*	**	61
Recurrent falling	49	56	42	51	40	76	45

* no clinically relevant difference, ** no non-linear association

values are in nmol/liter.

because of lack of clinical relevance or a linear relationship.

To the best of our knowledge, this is the first study to show remarkable differences in thresholds for serum 25(OH)D between different subgroups. The underlying mechanisms why women, the older old and individuals with high BMI have lower thresholds have to be studied in further research. A priori, we hypothesized that these groups would have higher thresholds. On the other side, it can also be hypothesized that in individuals with high BMI, the serum 25(OH)D concentration less well reflects the total body 25(OH)D because it is stored in fat cells (29). Therefore, the threshold for serum 25(OH)D can be lower in these individuals, since there will be 25(OH)D available from the adipocytes. In addition, older individuals may have lower thresholds compared to younger individuals, because it can be imagined that other variables, such as chronic diseases, have a greater influence on the studied outcomes than serum 25(OH)D. In addition, it cannot be excluded that unmeasured confounding factors, such as vitamin D binding protein levels or calcium intake, play a role. Surprisingly, previous studies on associations between vitamin D status and several outcomes in LASA revealed that when an interaction was found, the association was most significant within the subgroup with the highest thresholds found in the current study. For example, serum 25(OH)D was only longitudinally associated with expiratory peak flow in men (30), with the one year risk of recurrent falling (31) and six-year fracture risk (12) in individuals 65–75 years, and with quantitative ultrasound parameters and BMD only in individuals with low-to-normal BMI (32). The highest mean serum 25(OH)D levels were also found in these subgroups; this may partly explain why the thresholds were also higher. However, if a lower threshold was only found due to lower mean values, then one would expect that the line of the graph was still increasing or decreasing until the highest serum 25(OH)D concentrations observed in our study without reaching a plateau or without showing an U-shaped curve. Therefore a really lower threshold for these subgroups is suggested compared to their counterparts. However, further research is needed to clarify underlying mechanisms and to establish whether the same thresholds exists in populations with higher concentrations of serum 25(OH)D.

Previous studies on the estimation of thresholds are scarce and mainly based on bone outcomes and PTH concentrations. On the one hand the use of PTH as an outcome measure can be justified because PTH stimulates bone loss (33). On the other hand, PTH concentrations fluctuate with dietary habits, time of the day, renal function and physical activity (33–37). Therefore, a wide range of thresholds have been found (20–110 nmol/l) (33). In our study, thresholds for serum 25(OH)D concentrations based on PTH levels are more or less in the same range as these were for other outcomes, although, in the whole sample the thresholds were lower based on PTH than based on other outcomes. In addition, several previous studies on thresholds are based on the postintervention serum 25(OH)D levels of clinical trials supplementing vitamin D (8) and therefore it is difficult to compare these results with our population-based observational study. In the future, dose-response clinical trials supplementing vitamin D, will be necessary to establish definite thresholds, but the results of the current study can be considered as a first step in exploring thresholds for different outcomes and subgroups. This information can be used when designing these dose-response clinical trials.

When considering the results, it is remarkable that thresholds could be estimated for hypertension, but not for any of the other BP variables. These variables mainly

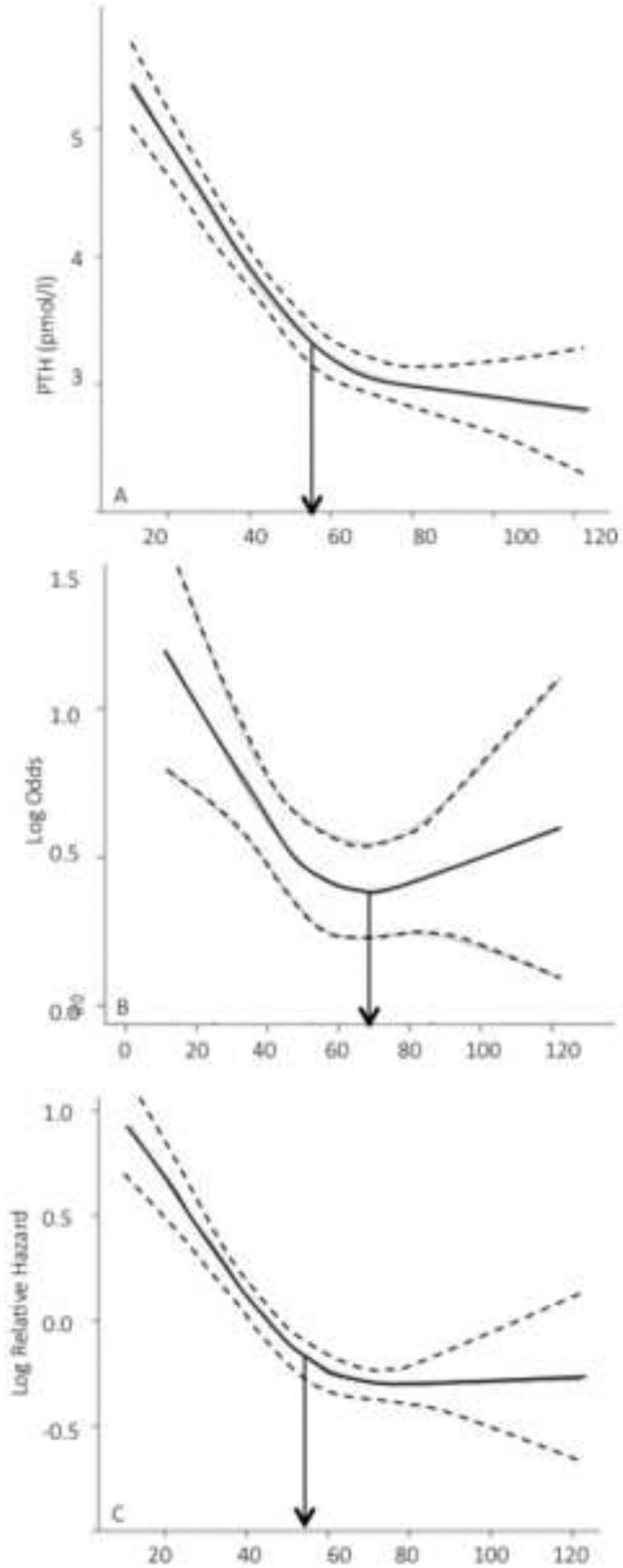

Figure 1. The determination of the threshold values for three different outcomes, one for each type of statistical analysis. Small dotted lines are 95% confidence intervals. The arrows indicate the visually determined thresholds. X-axis represents serum 25(OH)D in nmol/l; y-axis respresents the outcome variables A. linear regression: parathyroid hormone (in pmol/l), visually 56 nmol/l, after statistical analyses 46 nmol/l B. logistic regression: hypertension (log odds), visually 67 nmol/l, after statistical analyses 68 nmol/l C. cox regression: mortality (log relative hazard), visually 55 nmol/l, after statistical analyses 56 nmol/l

failed to pass the criterion with regard to clinical relevance. This can be explained by the fact that individuals using antihypertensive drugs were also classified as having hypertension, whereas in the other variables only the measured BP values were taken into account. In case of well-controlled hypertension, the BP will be comparable to individuals without hypertension and therefore no clear association can be found. Also in previous research within LASA, no significant association between vitamin D status and BP was observed (38).

Some remarks have to be made on the method of the estimation of the thresholds. A spline regression curve is a good method to show the shape of the association; it uses more information of the data, as compared to categorization of the data (39). However, it is questionable whether the thresholds found in this study rely on the distribution of serum 25(OH)D in our dataset (40). Because the serum 25(OH)D concentrations are relatively low, a high threshold could a priori not be found, due to the lack of participants with these concentrations. But on the other hand, if the threshold, in general, is in the higher concentrations of serum 25(OH)D, one would expect an ongoing increasing or decreasing line of the graph until the highest serum 25(OH)D levels in our study sample. For most of the outcomes this was not the case, because the line of the graph levels off at relatively low serum 25(OH)D concentrations. Only for cardiovascular disease and BMI there was a ongoing decreasing line with higher serum 25(OH)D levels and a high threshold is therefore still possible. As only 19% of the individuals had serum 25(OH)D concentrations $\geq$ 75 nmol/l, a reduced statistical power in the highest range of serum 25(OH)D concentrations could also have caused more imprecision and the reduced statistical power could play a role in not finding a nonlinear relationship. Therefore, to find out whether there are thresholds for these outcomes, further (longitudinal) studies in populations with higher serum 25(OH)D concentration should be performed to validate our results. Previous studies on thresholds of serum 25(OH)D usually only rely on the statistical best ratio of sensitivity and specificity, without taking the clinical relevance into account. We used a four-step approach, in which we first, systematically determined whether there was a clinically relevant difference and, second, whether it was possible to determine a threshold by using a test for nonlinearity. By using this approach, we were able to show thresholds that were of clinical relevance and that were true thresholds in a nonlinear relationship.

Legend to Figure Continued. . .

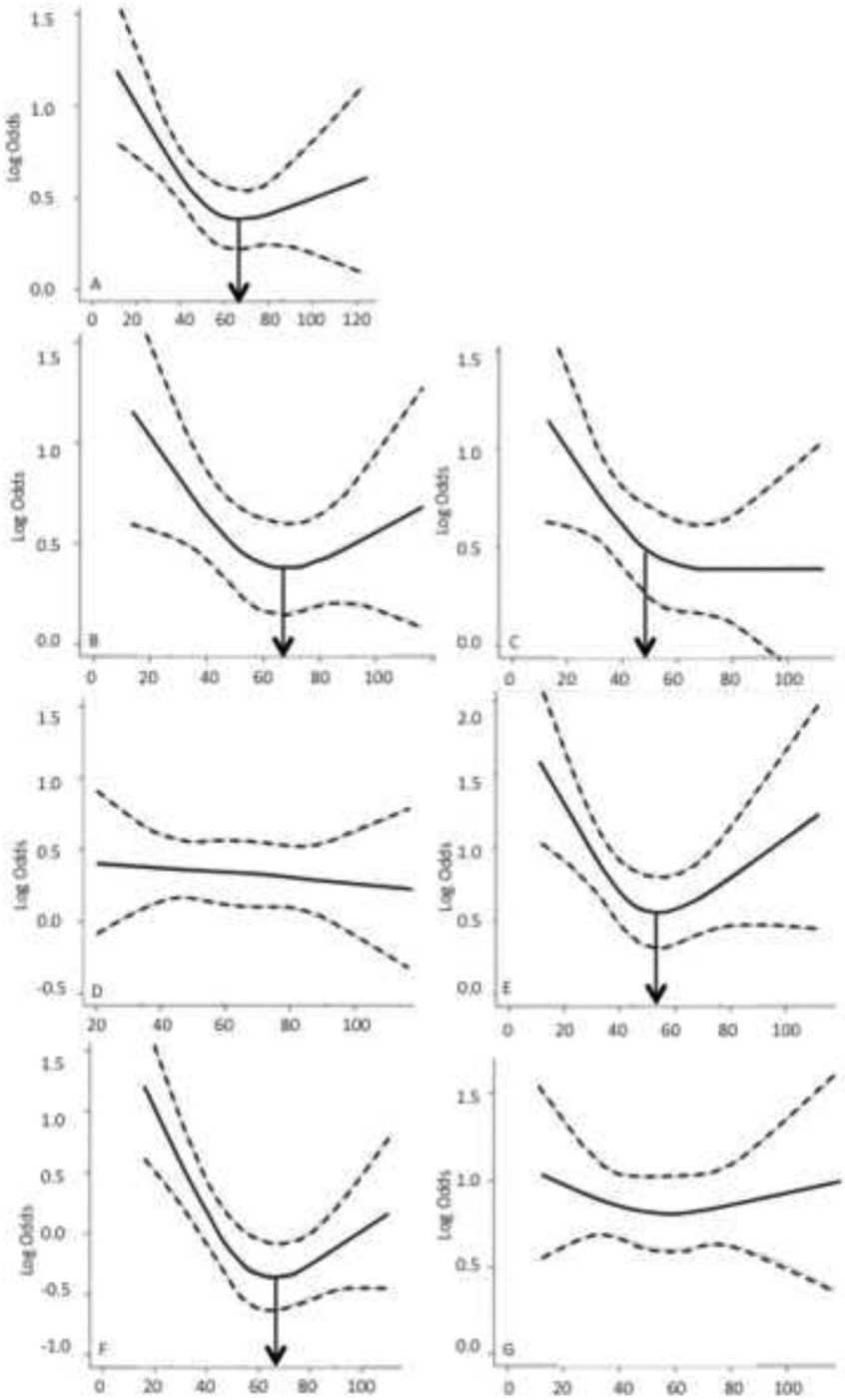

Figure 2. The determination of the threshold values for hypertension according to the different subgroups. Small dotted lines are 95% confidence intervals. The arrows indicate the visually determined thresholds. X-axis represents serum 25(OH)D in nmol/l; y-axis respresents the log odds for hypertension. A. total group: visually 67 nmol/l, after statistical analyses 68 nmol/l. B. men: visually 67 nmol/l, after statistical analyses 68 nmol/l. C. women: visually 49 nmol/l, after statistical analyses 49 nmol/l. D. age $<$ 75 years: no clinically relevant difference E. age $\geq$ 75 years: visually 54 nmol/l, after statistical analyses 61 nmol/l. F. BMI $<$ 25 kg/m^2: visually 67 nmol/l, after statistical analyses 58 nmol/l. G. BMI $\geq$ 25 kg/m^2: no clinically relevant difference.

This study has some further limitations and strengths. First, most of the analyses in this study are based on cross-sectional data. Second, some of the outcomes used, are outcomes of which the causality with regard to vitamin D is still being questioned (4). Therefore, before using the results of these analyses in updating guidelines on optimal vitamin D concentrations and on supplementation, the causal relationship with vitamin D has first to be proven. Third, data on cardiovascular disease was based on self-report. Although previous research revealed that the agreement between self-reported chronic diseases and data from general practitioners is fairly accurate (41), this could have caused some imprecision. The main strengths of this study are its large population-based study sample, the determination of many outcome variables within the same study sample, and for several outcomes a long follow-up time was available. Furthermore, the thresholds were determined systematically for all potentially relevant outcomes.

In conclusion, the main finding of this study is that thresholds for serum 25(OH)D differ according to outcome and subgroup. This indicates that future guidelines may need to consider more subgroups. In addition, this study does not support relatively high required levels of serum 25(OH)D, especially not for older persons, women, and persons with high BMI. However, the thresholds first have to be validated in other populations with other distributions of serum 25(OH)D concentrations and in dose-response clinical trials.

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