

Oral Supplementation With 25(OH)D₃ Versus Vitamin D₃: Effects on 25(OH)D Levels, Lower Extremity Function, Blood Pressure, and Markers of Innate Immunity

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ABSTRACT

To test the effect of 25(OH)D₃ (HyD) compared to vitamin D₃ on serum 25-hydroxyvitamin D levels (25(OH)D), lower extremity function, blood pressure, and markers of innate immunity. Twenty healthy postmenopausal women with an average 25(OH)D level of 13.2 ± 3.9 ng/mL (mean ± SD) and a mean age of 61.5 ± 7.2 years were randomized to either 20 μg of HyD or 20 μg (800 IU) of vitamin D₃ per day in a double-blind manner. We measured on 14 visits over 4 months, 25(OH)D serum levels, blood pressure, and seven markers of innate immunity (eotaxin, interleukin [IL]-8, IL-12, interferon gamma-induced protein 10 kDa [IP-10], monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein beta [MIP-1β], and "Regulated upon Activation, Normal T-cell Expressed, and Secreted" [RANTES]). At baseline and at 4 months, a test battery for lower extremity function (knee extensor and flexor strength, timed up and go, repeated sit-to-stand) was assessed. All analyses were adjusted for baseline measurement, age, and body mass index. Mean 25(OH)D levels increased to 69.5 ng/mL in the HyD group. This rise was immediate and sustained. Mean 25(OH)D levels increased to 31.0 ng/mL with a slow increase in the vitamin D₃ group. Women on HyD compared with vitamin D₃ had a 2.8-fold increased odds of maintained or improved lower extremity function (odds ratio [OR] = 2.79; 95% confidence interval [CI], 1.18–6.58), and a 5.7-mmHg decrease in systolic blood pressure (*p* = 0.0002). Both types of vitamin D contributed to a decrease in five out of seven markers of innate immunity, significantly more pronounced with HyD for eotaxin, IL-12, MCP-1, and MIP-1 β. There were no cases of hypercalcemia at any time point. Twenty micrograms (20 μg) of HyD per day resulted in a safe, immediate, and sustained increase in 25(OH)D serum levels in all participants, which may explain its significant benefit on lower extremity function, systolic blood pressure, and innate immune response compared with vitamin D₃. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: 25-HYDROXYVITAMIN D; VITAMIN D₃; BLOOD PRESSURE; FUNCTIONAL DECLINE; MARKERS OF IMMUNITY; POSTMENOPAUSAL WOMEN

Introduction

Desirable thresholds for 25(OH)D have been discussed recently, both for bone health and non-skeletal endpoints. Although the 2010 Institute of Medicine (IOM) recommendations

support a 25(OH)D threshold of 20 ng/mL for bone health,⁽¹⁾ the International Osteoporosis Foundation, in their 2010 position paper on vitamin D, recommends a threshold of 30 ng/mL for optimal fall and fracture reduction,⁽²⁾ consistent with the most recent recommendations by the U.S. Endocrine Society

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published in June 2011.⁽³⁾ The recommendations by the International Osteoporosis Foundation (IOF) and the Endocrine Society are based on two meta-analyses of double-blind randomized controlled trials that suggested fall reduction required a threshold of at least 24 ng/mL⁽⁴⁾ and fracture reduction a threshold of at least 30 ng/mL.⁽⁵⁾

Regarding general health endpoints, the Endocrine Society states that while evidence from randomized controlled trials is lacking, numerous epidemiological studies have suggested that a 25(OH)D blood level of 30 ng/mL and above may have additional health benefits in reducing the risk of common cancers, autoimmune diseases, type 2 diabetes, cardiovascular disease, and infectious diseases.^(6–10) In contrast, the IOM concludes that there is no evidence that a 25(OH)D threshold greater than 20 ng/mL has any benefit to health.⁽¹¹⁾

Although no consensus has been achieved as to whether 20 ng/mL or 30 ng/mL is the optimal threshold of vitamin D, as reflected in these most recent recommendations, most agree that many people are vitamin D-deficient and need vitamin D supplements to meet their vitamin D requirement.⁽¹²⁾ The most common form of dietary supplementation used today is cholecalciferol or vitamin D₃ and most healthy adults reach a target range of 20 ng/mL with 600 to 800 IU vitamin D per day. However, the target range of at least 30 ng/mL may require 1800 IU to 4000 IU vitamin D₃ per day.⁽¹³⁾

As an alternative strategy to enhance 25(OH)D levels, supplementation with the 25(OH)D₃ metabolite itself (HyD) has been suggested. Compared to vitamin D₃, HyD has hydrophilic properties, has a much shorter half-life of 8 to 11 hours after oral administration, and causes a rapid increase in serum 25(OH)D levels.^(14,15) In earlier studies, oral intake of HyD resulted in a potent increase in serum 25(OH)D levels and parathyroid hormone suppression.^(16–21) Further, some smaller trials reported a benefit on bone density among groups of cardiac and kidney transplant patients, and elderly hip fracture patients^(19,22,23); although this was not confirmed in a larger trial among 438 seniors.⁽¹⁸⁾ However, the treatment dose of HyD in the larger trial was low with 15 µg per day.⁽¹⁸⁾ Finally, in laboratory fracture-healing studies, HyD improved mechanical strength of the fractured bone in old rats.⁽²³⁾

The goal of this study was to assess the efficacy of the 25(OH)D₃ metabolite (HyD) compared to vitamin D₃ in increasing 25(OH)D serum levels. Further, we aimed to investigate the effects of HyD compared to vitamin D₃ on blood pressure, lower extremity function, and markers of innate immunity.

Patients and Methods

Participant population

For the study, we recruited 20 white postmenopausal women 50 to 70 years of age, in general good health. Other inclusion criteria were serum 25(OH)D level between 8 to 24 ng/mL, body mass index between 18 and 29 kg/m², and ability of giving informed consent. Exclusion criteria were composed of medical contraindications to vitamin D supplements, medical conditions, or medication use that would alter pharmacokinetics of study products or otherwise interfere with the study. Specific exclusion

criteria are listed in Appendix 1. All subjects were recruited at the Centre on Aging and Mobility, University Hospital Zurich, Zurich, Switzerland. This study was approved by the Zurich Cantonal Ethical Committee and Swiss Medic. Written informed consent was obtained from all study participants. The trial has been registered at the international trial registry (NCT00718276).

Clinical trial protocol

Age-eligible women, who were willing to participate, were invited for a screening visit at which demographic characteristics were collected, health status and habits were evaluated against inclusion/exclusion criteria, and fasting blood was drawn to assess serum levels of 25(OH)D, calcium, creatinine, and albumin, and glucose levels. Women who passed eligibility criteria signed informed consent and were randomly assigned to one of four groups: 20 µg HyD daily, 20 µg (800 IU) vitamin D₃ daily, 140 µg HyD weekly, and 140 µg (5600 IU) vitamin D₃ weekly. All supplements were taken orally. We randomized five participants to each treatment group. The dose of vitamin D₃ was chosen to compare to the current standard for vitamin D₃ (20 µg = 800 IU/d). The same dose was chosen for HyD, which, based on our review of the literature, offered an optimal balance of efficacy and safety.^(16,21) We randomized 15 additional women to one-time bolus groups, which were only included in a pharmacokinetic analysis, while clinical endpoint evaluations for this manuscript are restricted to the daily and weekly treatment groups (*n* = 20). As the results between daily and weekly HyD and vitamin D₃ supplementation did not differ significantly, the daily and weekly groups for each supplementation were combined for the analysis.

DSM Nutritional Products, Ltd., Switzerland, manufactured crystalline 25(OH)D₃ (HyD[®]) and vitamin D₃ and formulated both as identical spray-dried powders stabilized with DL-alpha tocopherol. Study capsules (hard gelatin capsules) were provided by DSM Nutritional Products, Ltd., Switzerland, and contained HyD or cholecalciferol (vitamin D₃), respectively. Subjects, investigators, study physician, and nurses were aware of the treatment regimen (daily or weekly), but blinded with regard to the type of treatment (HyD or vitamin D₃). The physiotherapist who assessed all functional endpoints was blinded to both regimen and type of treatment.

Clinical visits

Participants attended one screening visit and 14 clinical visits during a 4-month trial period. During the first week of the study, participants attended five daily visits. The baseline visit (visit 2) included 24-hour pharmacokinetic profiling for HyD and vitamin D₃, with repeated measurements of serum levels of calcium, creatinine, and 25(OH)D. Additionally, a baseline level of intact parathyroid hormone (PTH), albumin, insulin, fasting glucose, 1,25(OH)₂D, and immunity markers (eotaxin, interleukin [IL]-8, IL-12, interferon gamma-induced protein 10 kDa [IP-10], monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein beta [MIP-1β], "Regulated upon Activation, Normal T-cell Expressed, and Secreted" [RANTES]) were assessed. Further, all subjects underwent functional testing of lower extremities, including validated and standardized procedures for

knee flexor/extensor strength, timed up-and-go test, and repeated sit-to-stand test.⁽²⁴⁾ The remaining four daily visits of the first week (visits 3–6) included measurements of circulating 25(OH)D, immunity markers, albumin, serum calcium, and creatinine.

The following eight biweekly visits (visits 7–14) included the same measurements as visits 3 to 6; however, some visits included additional measurements. PTH was measured at visits 8, 10, and 12 (weeks 3, 7, and 11, respectively); visit 10 (week 7) included measurements of fasting glucose and insulin. In addition to the first clinical visit, 1,25(OH)₂D was measured two more times during the follow-up period: on visits 9 and 14 (weeks 8 and 17, respectively). During week 16, participants underwent two final clinical visits. Visit 14 included 24-hour pharmacokinetic profiling and extended set of measurements identical to that for visit 2 (baseline visit).

Biomarker assays

We assessed serum 25(OH)D concentration by a sensitive and selective assay based on liquid chromatography coupled to tandem mass spectrometry detection (HPLC-MS/MS), which selectively measures 25(OH)D₃ and was validated in an international NIST comparison (www.nist.gov; see Supporting Information for detailed methods or contact the authors). 1,25-hydroxyvitamin D was assessed by a radioimmunoassay. Intact PTH was measured in EDTA blood by means of an electrochemiluminescence immune assay.⁽²⁵⁾ Insulin levels were measured by a chemiluminescence assay.⁽²⁶⁾ Multiparametric kits for cytokine and chemokine determinations were purchased from BIO-RAD Laboratories (Hercules, CA, USA) and used in the LiquiChip Workstation IS 200 (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Measurements included: serum concentration for IP-10, eotaxin, IL-8, IL-12, MCP-1, MIP-1 β , and RANTES.

Compliance and safety

We assessed adherence to daily and weekly treatment regimen by capsule count at each study visit. Those on the weekly treatment regimen took one-half of their capsules at biweekly visits under a nurse's supervision. In addition, adherence was assessed by measuring serum concentrations of 25(OH)D at the end of the study.

Adverse events were monitored by interview during each study visit. Serum calcium levels and urinary calcium excretion (calcium/creatinine ratio in spot urine) were assessed at each visit and on the two pharmacokinetic days at each time point.

Statistical analysis

The statistical analysis of clinical outcomes is focused on women who were on daily or weekly HyD or vitamin D₃ treatment ($n = 20$) and was based on intent to treat. Results of exploratory analyses showed that the effects of daily and weekly treatment regimens with either HyD or vitamin D₃ were not significantly different. Based on that, the original four groups were consolidated into two groups: those treated with HyD and those treated with vitamin D₃. Each group included 10 participants. Comparability of treatment groups with respect

to important demographic characteristics, lower extremity function indicators, and plasma levels of immunity markers at baseline were assessed by one-way ANOVA.

We used generalized estimation equations (GEE) models to compare the odds of maintaining or improving lower extremity function during the 4-month follow-up between the HyD and vitamin D₃ treatment groups. For these models, a dichotomous variable was created indicating those whose results worsened versus those whose results improved or stayed the same for the duration of the follow-up period. Amount of change in lower extremity function was assessed by linear models comparing the results of functional tests (knee extensor and flexor strength, the timed up-and-go test, and the repeated sit-to-stand test) between the groups at the end of follow-up. The models controlled for the results of the same tests at baseline, age, and body mass index (BMI). Parameters that were assessed at multiple clinical visits (systolic and diastolic blood pressure, plasma levels of 25(OH)D and immunity markers) were analyzed by linear mixed models.

Results

Table 1 shows baseline characteristics of the study population. Groups randomized for treatment with vitamin D₃ and HyD were comparable to each other with respect to all investigated characteristics. Participants randomized to the vitamin D₃ group tended to be older than those in HyD group (63.5 versus 59.5 years of age), had higher BMI (25.5 versus 23.2 kg/m²), and higher average blood pressure (126/75 versus 119/70 mmHg); average blood levels of insulin and PTH were somewhat lower than in the HyD group. None of these differences, however, were statistically significant (Table 1).

Serum 25(OH)D levels and serum 1,25-dihydroxyvitamin D levels

Changes in circulating 25(OH)D levels in both treatment groups throughout the 4 months follow-up are depicted in Figure 1. From as early as the second day of follow-up women on oral HyD treatment experienced substantially higher serum levels of 25(OH)D levels than those treated with vitamin D₃. In the HyD group, the average circulating level of 25(OH)D continued to increase more rapidly than in the vitamin D₃ group for the entire follow-up period, reaching by the last clinical visit 69.5 ng/mL versus 31.0 ng/mL in the vitamin D₃ group (mean difference 39.0 ng/mL, $p < 0.0001$; Table 2). 25(OH)D levels shifted to above 30 ng/mL in all participants of the HyD group at 35 days of follow-up, while in the vitamin D₃ group about 50% of participants remained below this threshold throughout the 4 months of follow-up (Fig. 1). Both treatments resulted in an increase in 1,25(OH)₂D levels, but this increase was more pronounced with HyD. Average circulating 1,25(OH)₂D rose by 19.5 pg/mL in the HyD group and by 2.5 pg/mL in the vitamin D₃ group ($p = 0.004$; Table 2).

Blood pressure and lower extremity function

Treatment with HyD compared with vitamin D₃ lowered systolic blood pressure on average by 5.7 mmHg ($p = 0.002$) over

Table 1. Baseline Characteristics of the Study Population

Characteristic	Vitamin D ₃ (n = 10)		HyD (n = 10)		p
	Mean	SD	Mean	SD	
Anthropometrics					
Age (years)	63.45	7.78	59.48	6.27	0.22
BMI (kg/m ²)	25.49	3.38	23.24	3.22	0.15
Functional tests					
Systolic blood pressure (mmHg)	126.30	14.27	118.70	10.90	0.20
Diastolic blood pressure (mmHg)	75.30	8.19	70.00	5.56	0.11
Knee flexion strength (N)	180.64	34.99	202.57	29.89	0.15
Knee extension strength (N)	292.40	41.85	286.92	76.37	0.84
Timed up-and-go test (second)	7.44	1.34	7.48	0.54	0.93
Repeated sit-to-stand test (second)	8.34	2.41	7.24	1.57	0.24
Blood plasma/serum levels					
25(OH)D (ng/mL)	14.18	3.61	12.28	4.08	0.28
1,25(OH) ₂ D (pg/mL)	38.61	12.10	33.02	13.63	0.36
Serum calcium (mmol/L)	2.27	0.08	2.26	0.07	0.71
Parathyroid hormone (pg/mL)	54.87	10.71	63.22	16.37	0.19
Insulin (mIU/L)	2.01	2.09	5.59	5.92	0.33
Serum glucose (mmol/L)	5.33	0.43	5.28	0.61	0.83
Eotaxin plasma level (pg/mL)	27.48	8.97	34.01	13.78	0.23
IL-8 (pg/mL)	2.90	0.84	3.04	0.68	0.68
IL-12 (pg/mL)	4.80	2.58	6.74	3.93	0.21
IP-10 (pg/mL)	309.30	187.44	278.80	190.82	0.72
MCP-1 (pg/mL)	13.69	5.24	16.29	8.37	1.00
MIP-1β (pg/mL)	29.57	8.91	36.86	26.48	0.43
RANTES (pg/mL)	32.52	24.63	43.24	48.64	0.54

Values are mean ± SD; values of *p* are based on *t* test for normally distributed variables and on Wilcoxon rank-sum test for those that were not normally distributed.

HyD = 25(OH)D₃; BMI = body mass index; IL = interleukin; IP-10 = interferon gamma-induced protein 10 kDa; MCP-1 = monocyte chemotactic protein-1; MIP-1β = macrophage inflammatory protein beta; RANTES = Regulated upon Activation, Normal T-cell Expressed, and Secreted.

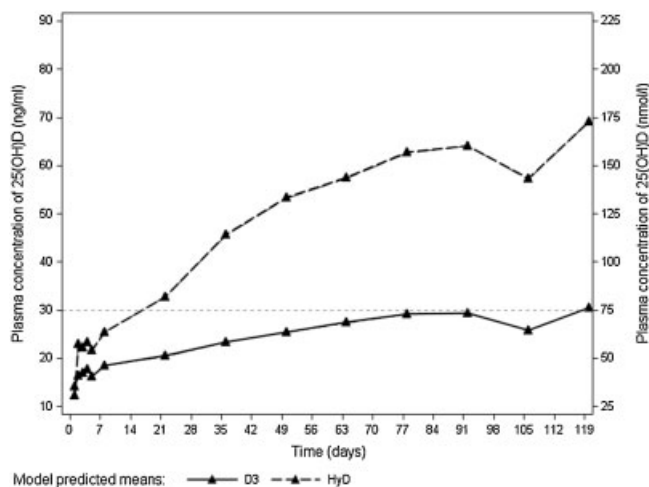


Fig. 1. Change in 25(OH)D over time with HyD versus vitamin D₃. Dashed line indicates the 30 ng/mL (75 nmol/L) threshold of 25(OH)D serum concentration. Graph shows individual and model predicted means of serum 25(OH)D levels across 14 clinical visits by treatment. Predicted means are adjusted for age, BMI, and baseline 25(OH)D levels. 25(OH)D levels shifted to above 30 ng/mL in all participants of the HyD group at 35 days of follow-up, while in the vitamin D₃ group about 50% of participants remained below this threshold throughout the 4-month follow-up.

4 months, independent of age, BMI, and baseline systolic blood pressure (Table 3). Systolic blood pressure in the HyD group progressively decreased for the first 3 weeks of follow-up and then stabilized at a lower level for the remainder of follow-up; in the vitamin D₃ group, on the other hand, systolic blood pressure remained virtually constant for the entire 4-month follow-up (Fig. 2). Diastolic blood pressure did not differ significantly between the treatment groups and remained constant for the duration of the study (Table 3).

Among the lower extremity function endpoints, only knee extension strength differed significantly between the treatment groups at 4-month follow-up with a 17% better knee extension strength compared with those treated with vitamin D₃ (*p* = 0.03; Table 4), independent of age, BMI, and baseline knee extension strength. Results for the other functional endpoints, including knee flexion strength, timed up-and-go test, and repeated sit-to-stand test, the HyD treatment group yielded somewhat better results (Table 4), but differences between the groups were not statistically significant. Across all tests of lower extremity function, participants in the HyD treatment group had a significantly higher probability to maintain or improve their lower extremity strength/function characteristics (odds ratio [OR] = 2.79; 95% confidence interval [CI], 1.18–6.58) during the 4-month follow-up period than those treated with vitamin D₃.

Table 2. Treatment Effects on Biomarkers

	Repeated measurement analysis using all time points (means (SE) across follow-up)			End of follow-up comparison (means (SE) at the end of follow up)		
	Vitamin D ₃	HyD	Difference	Vitamin D ₃	HyD	Difference
25(OH)D level (ng/mL)	22.48 (0.81)	40.85 (0.82)	<i>p</i> < 0.0001	30.99 (1.59)	69.47 (1.58)	<i>p</i> < 0.0001
1,25(OH) ₂ D level (pg/mL)	42.44 (1.56)	45.98 (1.47)	<i>p</i> = 0.15	40.50 (2.91)	53.06 (2.76)	<i>p</i> = 0.004
Serum calcium (mmol/L)	2.28 (0.02)	2.28 (0.02)	<i>p</i> = 0.97	2.27 (0.03)	2.27 (0.03)	<i>p</i> = 0.97
Urinary calcium/creatinine ratio	0.41 (0.03)	0.36 (0.03)	<i>p</i> = 0.37	0.33 (0.06)	0.33 (0.06)	<i>p</i> = 0.98
PTH (pg/mL)	53.42 (2.48)	49.94 (2.48)	<i>p</i> = 0.36	51.68 (3.43)	43.00 (3.43)	<i>p</i> = 0.09
Insulin (mIU/L)	6.02 (0.48)	6.33 (0.43)	<i>p</i> = 0.69	6.61 (0.69)	6.21 (0.59)	<i>p</i> = 0.69
Serum glucose (mmol/L)	5.04 (0.07)	5.19 (0.07)	<i>p</i> = 0.16	5.03 (0.10)	5.14 (0.10)	<i>p</i> = 0.40
Plasma eotaxin level (pg/mL)	23.23 (1.23)	20.81 (2.29)	<i>p</i> = 0.43	30.91 (1.61)	21.07 (1.90)	<i>p</i> = 0.0002
Plasma IL-8 level (pg/mL)	1.07 (0.05)	1.14 (0.05)	<i>p</i> = 0.35	1.55 (0.12)	1.35 (0.11)	<i>p</i> = 0.22
Plasma IL-12 level (pg/mL)	1.23 (0.08)	1.14 (0.08)	<i>p</i> = 0.49	2.63 (0.32)	0.98 (0.18)	<i>p</i> < 0.0001
Plasma IP-10 level (pg/mL)	294.40 (37.71)	393.75 (37.90)	<i>p</i> = 0.10	230.71 (59.76)	279.31 (59.75)	<i>p</i> = 0.57
Plasma MCP-1 level (pg/mL)	7.11 (0.74)	6.17 (0.75)	<i>p</i> = 0.40	12.11 (1.19)	7.68 (1.21)	<i>p</i> = 0.01
Plasma MIP-1β level (pg/mL)	19.85 (0.78)	19.98 (0.79)	<i>p</i> = 0.91	25.57 (1.79)	19.85 (1.43)	<i>p</i> = 0.01
Plasma RANTES level (pg/mL)	31.90 (5.32)	38.37 (5.33)	<i>p</i> = 0.44	26.68 (8.81)	24.47 (8.82)	<i>p</i> = 0.86

All analyses controlled for the results of the same tests at baseline, age, and BMI. All parameters were analyzed by repeated measurement analysis in addition to the end of study comparison. Plasma levels of 25(OH)D and immunity markers were assessed in all 14 visits. Insulin and fasting glucose levels were assessed only at visits 2 (day 2) and 10 (day 63). 1,25-dihydroxyvitamin D levels were assessed at visits 2 (day 2), 9 (day 50), and 14 (day 120).

SE = standard error; HyD = 25(OH)D₃; PTH = parathyroid hormone; IL = interleukin; IP-10 = interferon gamma-induced protein 10 kDa; MCP-1 = monocyte chemoattractant protein-1; MIP-1β = macrophage inflammatory protein beta; RANTES = Regulated upon Activation, Normal T-cell Expressed, and Secreted; BMI = body mass index.

PTH, glucose, insulin, serum calcium, and urinary calcium excretion

Effects of study treatments on circulating concentrations of PTH, glucose, and insulin were not significantly different. However, while glucose and insulin levels stayed stable in both groups for the entire follow-up period, PTH levels decreased in both groups. Figure 3 illustrates that HyD may cause a more rapid decrease in PTH concentration than does vitamin D₃. During the 4-month period average PTH levels decreased by 5.7 pg/mL in the vitamin D₃ group and by 18.1 pg/mL in the HyD group; a statistically significant difference was likely missed due to the small sample size.

As a safety measure, serum calcium was measured at each study visit. Serum calcium level remained stable throughout the entire 4 months of follow-up and no participant had at any time point a serum calcium level above 2.6 nmol/L (reference range: 2.19–2.60 nmol/L). Similarly, there was no significant difference

in urinary calcium excretion between groups assessed as the calcium/creatinine ratio in spot urine.

Markers of immunity

Treatment effects were significantly different between the treatment groups for four of the seven investigated markers of immunity over the follow up period (Table 2) with a significantly more pronounced suppression of eotaxin, IL-12, MCP-1, and MIP-1β with HyD compared to vitamin D₃ at the last visit at 4 months. Notably, however, treatment with either form of vitamin D led to decrease in circulating levels of the markers indicating lowering the level of innate immune activity by both HyD and vitamin D₃. In the combined group of participants treated with either form of vitamin D, the overall declining trend had two distinct phases: a relatively precipitous decrease during the first 5 days of follow-up, which was not different between the daily and weekly application, and somewhat slower decline during the 98-day

Table 3. Treatment Effect on Blood Pressure

	Repeated measurement analysis using all time points (means (SE) across follow-up)			End of follow-up comparison (means (SE) at the end of follow up)		
	Vitamin D ₃	HyD	Difference	Vitamin D ₃	HyD	Difference
Systolic blood pressure (mmHg)	117.0 (1.0)	111.4 (1.0)	<i>p</i> = 0.002	119.6 (2.6)	112.4 (2.6)	<i>p</i> = 0.06
Diastolic blood pressure (mmHg)	69.7 (0.8)	69.5 (0.8)	<i>p</i> = 0.83	67.8 (1.8)	70.8 (1.8)	<i>p</i> = 0.24

Treatment with HyD lowered systolic blood pressure on average by 5.7 mmHg (*p* = 0.002) compared to vitamin D₃ across all time points, adjusted for age, BMI, and baseline systolic blood pressure. Diastolic blood pressure did not differ significantly between the treatment groups.

SE = standard error; HyD = 25(OH)D₃; BMI = body mass index.

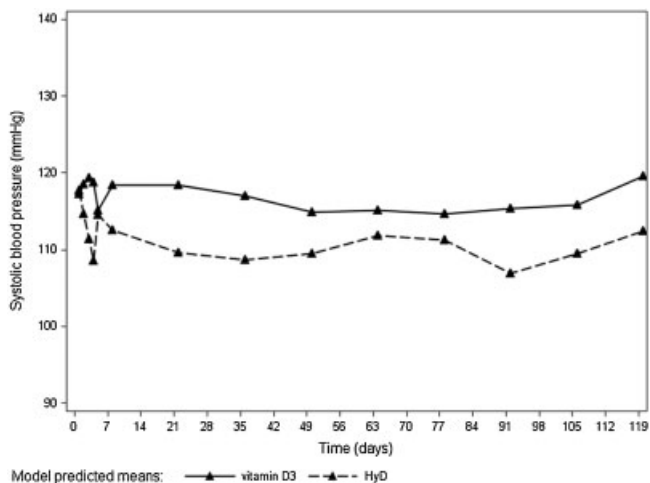


Fig. 2. Change in systolic blood pressure over time with Hyd versus vitamin D₃. Graph shows model predicted means of systolic blood pressure across 120 days of follow-up (14 clinical visits) by treatment. Predicted means are adjusted for age, BMI, and baseline systolic blood pressure. Systolic blood pressure decreased in the HyD group progressively for the first 3 weeks of follow-up (up to day 21) and then stabilized at a lower level for the remainder of follow-up; in the vitamin D₃ group, on the other hand, systolic blood pressure remained virtually constant for the entire 4-month follow-up.

period starting on day 8 (up to day 106); these two periods were separated by the brief rise of circulating levels on days 6 and 7 (Fig. 4).

Discussion

Consistent with previous studies suggesting that HyD is more potent than vitamin D₃ in raising 25(OH)D levels,^(16–21) our study shows that 20 μg HyD is significantly more efficient and more rapid in shifting healthy postmenopausal women into a desirable 25(OH)D serum level of at least 30 ng/mL compared to 800 IU (20 μg) vitamin D₃. Further, our study adds support to the

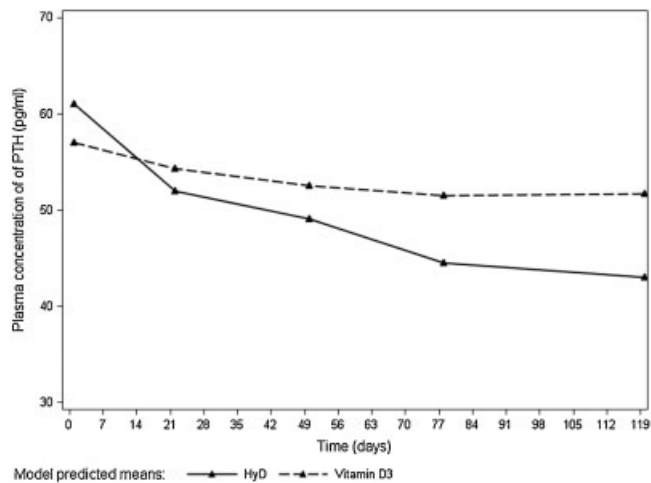


Fig. 3. Change in intact PTH levels over time with Hyd versus vitamin D₃. Graph shows model-predicted means of intact PTH levels across five clinical visits by treatment. Predicted means are adjusted for age, BMI, and baseline PTH levels. Based on this figure there is a suggestion that HyD may cause a more rapid decrease in PTH concentration than does vitamin D₃. Across the 4-month follow-up, average PTH levels decreased by 5.7 pg/mL in the vitamin D₃ group and by 18.1 pg/mL in the HyD group, but difference between PTH levels in the two treatment groups were not statistically significant throughout the follow-up period. The end of study difference approached significance with a *p* value of 0.09 (Table 2).

concept that higher 25(OH)D levels may be more desirable for nonskeletal endpoints of vitamin D, including lower extremity function, blood pressure, and immunity. Although this was a small trial, HyD treatment resulted in a rapid increase in 25(OH)D levels associated with a significant 2.8-fold higher odds of maintained or improved lower extremity function, an immediate and sustained decrease in systolic blood pressure by 5.6 mmHg, and a significantly more pronounced suppression of four out of seven markers of innate immunity. Notably, both HyD and vitamin D₃ contributed to a decrease in markers of innate immunity, indicating a benefit from both substances.

Table 4. Treatment Effect on Functional Decline

Characteristic	Vitamin D ₃ group at 4 months (<i>n</i> = 10)		HyD Group at 4 months (<i>n</i> = 10)		Difference (%)	<i>p</i>
	Mean	SE	Mean	SE		
Knee extension strength (N)	247.2	12.1	289.3	12.1	17.01	0.03
Knee flexion strength (N)	180.1	5.7	187.2	5.7	3.93	0.42
Timed up-and-go test (second)	7.9	0.4	7.3	0.4	-7.76	0.32
Repeated sit-to-stand test (second)	8.0	0.5	7.0	0.5	-12.16	0.17

At the end of the 4-month follow-up period participants in the HyD group had on average a 17% better knee extension strength compared with those treated with vitamin D₃ (*p* = 0.03), independent of age, BMI, and baseline knee extension strength. Results for the other functional characteristics were slightly better in the HyD treatment group, but differences between the groups were not statistically significant. Across all tests of lower extremity function, participants in the HyD treatment group had a significantly higher probability to maintain or improve their lower extremity strength/function characteristics (OR = 2.79; 95% CI, 1.18–6.58) during at 4-month follow-up compared with those in the vitamin D₃ group.

HyD = 25(OH)D₃; SE = standard error; BMI = body mass index; OR = odds ratio; CI = confidence interval.

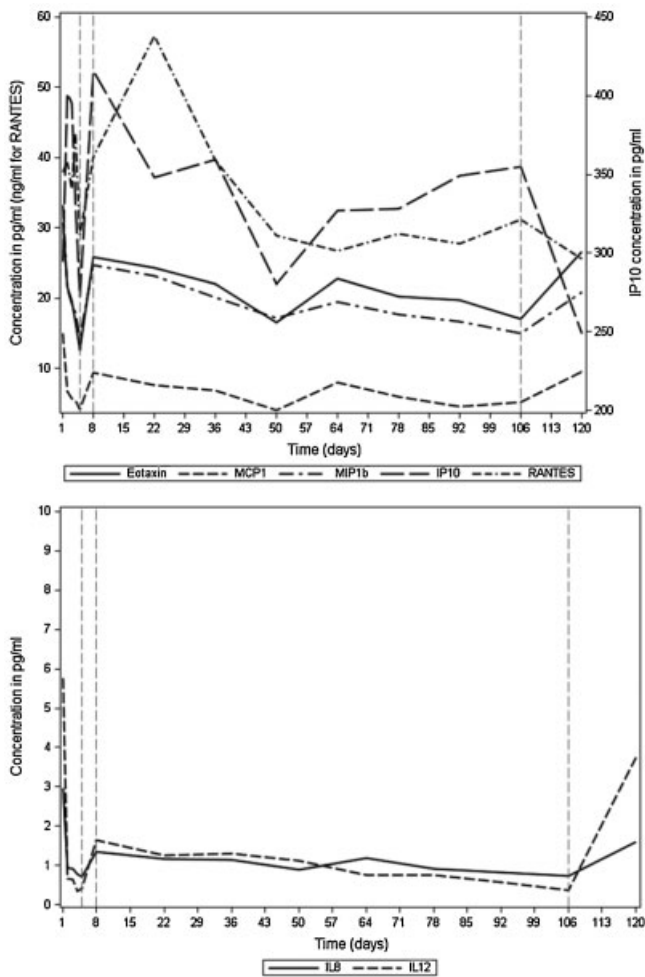


Fig. 4. Change in immunity markers over time with Hyd and vitamin D₃ combined. Graph shows model predicted means of all seven tested immunity markers across 14 clinical visits (combined $n = 20$). Predicted means are adjusted for age, BMI, and baseline levels of immunity markers. Serum concentration for IP10 (interferon gamma-induced protein 10kDa) is shown on the right y-axis in the top graph of the panel. The left y-axes show concentrations for all other markers, including eotaxin, interleukin-8 (IL-8), interleukin-12 (IL-12), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein beta (MIP-1 β), and “Regulated upon Activation, Normal T-cell Expressed, and Secreted” (RANTES). Dashed vertical lines show periods of steady decline. During the first 5 days of follow-up, plasma levels of all investigated immunity markers, except IP-10 and RANTES, decreased; and linear trends estimated by repeated measures mixed models were highly statistically significant ($p < 0.0001$) for all markers except for IP-10 and RANTES. Immunity markers augmented during a 2-day period between day 6 and 8, and after that linear decrease continued. Estimated linear trends were statistically significant ($p < 0.002$) for all markers with the exception of IP-10 for the time interval after day 8 up to day 106.

In this study, participants in the 800 IU vitamin D₃ group needed about 3 to 4 months to reach a maximum mean concentration of 31 ng/mL, leaving about 50% of the participants below the threshold level of 30 ng/mL. On the other hand, with HyD at a dose of 20 μ g, the 25(OH)D threshold of 30 ng/mL was achieved in all women already at 35 days of treatment, and after 3 to 4 months, the 25(OH)D level rose to a mean concentration maximum of 69.5 ng/mL. As demonstrated by Ilahi and

colleagues,⁽²⁷⁾ a rapid response with an early shift to the desired 25(OH)D threshold level of 30 ng/mL could also be achieved with vitamin D₃; however, only with a much higher oral bolus of 2500 μ g (100,000 IU).

Several lines of evidence support a benefit of vitamin D on blood pressure. First, in experimental studies, 1,25-dihydroxyvitamin D regulates the renin-angiotensin-aldosterone system via suppression of renin gene expression^(28,29) and has vasculoprotective effects that inhibits vascular smooth muscle cell proliferation⁽³⁰⁾ and vascular calcification.⁽³¹⁾ Second, vitamin D₃ supplementation (\sim 2000 IU/d) was found to improve endothelial function in a trial among African American individuals compared to placebo.⁽³²⁾ Third, mice lacking the vitamin D receptor (VDR) suffer from hypertension and develop heart hypertrophy.^(33,34) Fourth, clinically, in two small trials among patients with mild hypertension and older postmenopausal women, ultraviolet B (UVB) irradiation or vitamin D supplementation (800 IU/d) reduced blood pressure by 6 to 9 mmHg.^(35,36) Notably, a threshold of 25(OH)D that may confer a maximum benefit on blood pressure was suggested in two large cohorts of men and women to be at least 30 ng/mL.⁽⁹⁾ Consistent with these observations, participants treated with HyD (compared to the participants in the vitamin D₃ group) reached the 25(OH)D threshold of 30 ng/mL within 35 days of treatment and experienced a significant reduction of systolic blood pressure by 5.7 mmHg in the same time frame. In fact, the blood pressure reduction occurred in the early treatment phase and stabilized at this level for the remainder of the 4-month follow-up period. Systolic blood pressure did not change in the vitamin D₃ group, which may be explained by the much slower and smaller increase in 25(OH)D levels in the vitamin D₃ group, leaving 50% of the participants below 30 ng/mL even at 4 months of treatment.

Several lines of evidence support a role of vitamin D in muscle health. First, the VDR is expressed in human muscle tissue,^(37,38) although this was questioned recently,⁽³⁹⁾ and VDR activation may promote de novo protein synthesis in muscle.⁽⁴⁰⁾ Mice lacking the VDR show a skeletal muscle phenotype with smaller and variable muscle fibers and persistence of immature muscle gene expression during adult life.^(34,41) Second, proximal muscle weakness is a prominent feature of the clinical syndrome of vitamin D deficiency.⁽⁴²⁾ Clinical findings in vitamin D deficiency myopathy include proximal muscle weakness, diffuse muscle pain, and gait impairments such as waddling way of walking.⁽⁴³⁾ Third, vitamin D supplementation increased muscle strength and balance,^(24,44) and reduced the risk of falling in community-dwelling participants of double-blind randomized-controlled trials that gave 700 to 1000 IU vitamin D per day as summarized in a 2009 meta-analysis.⁽⁴⁵⁾ Notably, a threshold of 25(OH)D that may confer a maximum benefit on lower extremity function suggested to be at least 24 ng/mL and best 30 to 40 ng/mL based on the large National Health and Nutrition Examination Survey (NHANES) III study,⁽⁴⁶⁾ and the same thresholds were suggested for fall prevention based on the 2009 meta-analysis of double-blind randomized controlled trials (RCTs).⁽⁴⁵⁾

Consistent with the concept of optimal 25(OH)D threshold accomplishment, participants in the HyD group compared to the vitamin D₃ group improved in all of four lower extremity tests at

4-month follow-up, although statistical significance was only achieved for knee extensor strength with a 17% improvement. Improvements did not reach statistical significance for knee flexor strength (4%), for functional mobility (timed up-and-go test; 8%) and for reaction time (repeated sit-to-stand test; 12%), which may have been due to the small sample size. Overall, however, participants had a significant 2.8-fold higher odds to maintain or improve their lower extremity function with HyD compared to vitamin D₃.

Experimental and observational data support a role of vitamin D in the innate and adaptive immune response.^(34,47) 1,25-dihydroxyvitamin D treatment of cancer cells suppressed IL-8 production, which is an angiogenesis factor critical in initial tumor and metastasis development.⁽⁴⁸⁾ Further, 1,25-dihydroxyvitamin D treatment inhibited RANTES and IP-10 secretion in a concentration-dependent manner in airway smooth muscle cells.⁽⁴⁹⁾ Both chemokines play a critical role in the pathogenesis of asthma,⁽⁵⁰⁾ as do eosinophils, which are recruited to the lungs by eotaxin.⁽⁵¹⁾ Notably, low 25-hydroxyvitamin D levels were associated with elevated total immunoglobulin E (IgE) and eosinophil counts in a larger study of children with asthma.⁽⁵²⁾ Further, in patients with chronic kidney disease-associated inflammation, urinary MCP-1 protein and renal macrophage infiltration were significantly and inversely correlated with 1,25(OH)₂D levels.⁽⁵³⁾ MCP-1 is considered a key modulator of monocyte activity and was suggested to be involved in the initiation and progression of atherosclerosis.⁽⁵⁴⁾ Further, previous studies suggested that 1,25(OH)₂D₃ stimulates first-line immune defense in the innate immune system, resulting in an increase in maturation of monocyte to macrophage and an increase in MIP-1b.⁽⁵⁵⁾ Finally, IL-12, a proinflammatory cytokine that plays a central role in activation and differentiation of CD4(+) T cells into interferon- γ secreting T-helper type 1 cells, has been reported to be downregulated by 1,25-dihydroxyvitamin D treatment.^(55,56)

In our study, all tested markers of innate immunity showed some response to both types of vitamin D, but it was significantly more pronounced with HyD for four markers of innate immunity (eotaxin, IL-12, MCP-1, and MIP-1 β). The response to both types of vitamin D was very rapid in the first 5 days after treatment initiation, during which all but IP-10 and RANTES decreased, followed by an increase of all markers during a 2-day period between day 6 and 8, and a further decline thereafter. Estimated linear trends were statistically significant for all markers with the exception of IP-10 for the time interval after day 8 up to day 106. Notably, two previous trials that tested vitamin D with treatment doses of up to 40,000 IU vitamin D₃ per day among patients with multiple sclerosis (MS)⁽⁵⁷⁾ and obese patients⁽⁵⁸⁾ were not able to demonstrate any significant change in a number of markers of immunity. The difference in results between these trials and our study may be explained by the follow-up time points documented, which included early response only in our trial.

Our results support the threshold recommendation of 30 ng/mL of the IOF and the Endocrine Society as they show that among participants in the HyD group who all reach the threshold within 3 to 5 weeks, greater benefits are observed with regard to lower extremity function, blood pressure, and innate immunity compared with participants of the vitamin D₃ group

who reach the 30 ng/mL threshold only in about 50% of cases after 16 weeks.^(2,3)

Our trial has several strengths despite its small size. We had complete adherence to the study protocol and 100% of participants attended 14 clinical visits. The trial contributes new data on HyD effects on blood pressure, lower extremity function, and markers of immunity compared to the current standard dose of vitamin D₃. The trial was sufficiently powered for the primary endpoint, but had limited power for secondary endpoints, which were included to provide pilot data. However, multiple repeated measurements compensate a small sample size by reducing measurement error to some extent, and allowed for adequate power for some of the secondary endpoints; ie, systolic blood pressure. Notably, as we did not test an equivalent dose of HyD and vitamin D₃ with respect to 25(OH)D level increase, benefits documented by HyD may likely be caused by its rapid increase in 25(OH)D level and higher achieved 25(OH)D level compared with the standard dose of vitamin D₃ tested. Alternatively, HyD may have additional benefits superior to vitamin D₃, which will need further investigation.

In conclusion, our results show that oral supplementation with HyD (25(OH)D₃ metabolite) at a dose of 20 μ g per day resulted in a safe, immediate, and sustained increase in 25(OH)D levels in all participants. The higher and faster increase in circulating level of 25(OH)D reached with HyD compared with vitamin D₃ may explain the benefit of HyD on systolic blood pressure reduction, improvement in lower extremity function, and the more pronounced reduction in several markers of innate immunity among healthy postmenopausal women.

Disclosures

The collaborating authors from DSM Research & Development performed the analysis of 25(OH)D levels blinded to treatment allocation. DSM also performed the biomarker analysis for innate immunity (the authors are grateful to Nathalie Richard), also blinded to treatment. All other measurements and analyses were performed at the Centre on Aging and Mobility at the University of Zurich and the Institute of Clinical Chemistry at the University Hospital in Zurich. DSM had no influence on the design of the study, the content of the publication or the underlying statistical analyses. The principal investigator (Heike Bischoff-Ferrari) was supported exclusively by a Swiss National Foundations Professorship Grant (PP00B-114864). None of the university affiliated co-authors have a conflict to report.

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Authors' roles: HBF designed the study with input by BDH, JOE, WCW, HBS, AJ, and ES. Innate immune markers and 25(OH)D were measured at DSM under supervision by ES, SW, and JS; all other biomarkers were measured by AvE. Data collection was performed by AE, JH, and HBF.

Appendix 1. Exclusion Criteria

- Suspect lack of compliance
- Subjects who are enrolled in any interventional study or have received an investigational new drug or product within the last 60 days prior to screening
- Serum calcium >2.6 nmol/L
- Fasting glucose >100 mg/dL
- Use of HRT within the previous 6 months
- Use of dietary supplements while on study, except multi-vitamins, must stop before entering the study and refrain from using until the end of the study
- Use of high-dose vitamin D supplements >400 IU
- Use of high-dose calcium supplements >600 mg
- Hypertension: higher than 145/95 mmHg measured in resting position
- Treatment for hypertension
- Subjects with a history of a psychological illness or condition that is likely to interfere with the subject's ability to understand requirements of the study
- Systematic practice of high-intensity exercise (high-intensity sports more than 3 times per week)
- Any condition that might interfere with absorption of the investigational product (intestinal malabsorption syndrome, sprue, colitis, gastrointestinal surgery)
- Diseases that carry a risk for hypercalcemia: sarcoidosis, tuberculosis, lymphoma, primary hyperparathyroidism
- Kidney stones
- Creatinine clearance of less than 30 mL/min (severe kidney insufficiency)
- Known hypersensitivity or allergy to dairy or milk products
- Co-medications: anticoagulants, parathyroid hormone, corticosteroids, thiazide diuretic (Herzglykoside: digoxin), anticonvulsants
- Use of any drug that might interfere with bone metabolism (bisphosphonate, hormone replacement therapy, estrogen receptor modulators, calcitonin) within the previous 12 months
- Nonfat diets or other extreme dietary habits
- Subjects on a weight reduction program or a medically supervised diet
- Signs of acute or severe illness: unintentional weight loss, night sweats, treatment for cancer
- Fracture in the last year or a fall in the last 3 months
- Alcohol abuse: ≥ 21 units/week
- Current smokers
- Those who over the 4-month course of supplementation are planning a vacation to a "sunny" location (for example a mountain ski resort or a winter sun resort).

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