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Research paper

Vitamin D levels in a large Mediterranean cohort: reconsidering normal cut-off values

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ABSTRACT

OBJECTIVE: The determination of the normal range of 25-hydroxyvitamin D [25-(OH)D], though currently based on suppression of PTH levels, still remains a controversial issue. The 25-(OH)D levels exhibit gender and seasonal variability, the latter attributed in part to changes of insolation. DESIGN: The aim of this cross-sectional study was to estimate the levels of 25-(OH)D on the island of Crete and their correlation with metabolic, hormonal and bone turnover parameters. The study was performed over a period of five years and involved 8,183 male and female individuals (8,042 analyzed). RESULTS: Our results are as follows: (1) 25-(OH)D was significantly lower than expected (19.48±9.51 and 18.01±9.01 (ng/mL±SD) in males and females, respectively); (2) seasonal variation of 25-(OH)D was observed in both sexes (females < males), with values peaking in August; (3) a decline of 25-(OH)D was evident with advancing age, with lower levels in females compared to males up to menopause and no apparent difference between the genders thereafter; (4) levels of 25-(OH)D were lower in renal function impairment, diabetes/insulin resistance and inflammation, while no correlation was detectable in thyroid dysfunction; (5) normalization of PTH levels was observed at ~20 ng/mL 25-(OH)D. At the same cut-off level, a significant decrease of all measured bone turnover indices (b-ALP, osteocalcin and CTX) was evident. CONCLUSION: Based on the above data, it appears that a cut-off level of 25-(OH)D close to 20 ng/mL better reflects the physiology of our population.

Key words: Bone metabolic biomarkers, Cut-off values, Vitamin D deficiency, PTH, 25-(OH)vitamin D

INTRODUCTION

The steroid hormone/vitamin D (VitD) exists in two forms, D2 (ergocalciferol) and D3 (cholecalciferol). The former is obtained from food (plant-derived, mainly mushrooms), while the latter is naturally synthesized in the skin, through exposure to ultraviolet light, and also ingested in the diet (animal-derived, mainly fatty fish, eggs, liver).[1,2] Both forms are used for food fortification (dairy products, cereals and juice) and in VitD supplements. In humans, the endogenous

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production by the skin following exposure to sunlight UV radiation represents the main source of VitD.[1]

The principal biological effect of VitD is on growth and remodeling of the skeleton by enhancing intestinal calcium absorption.[3] Recently though, the discovery of the almost ubiquitous distribution of VDR and cytochrome P450 27B1 in the human body[4] suggests that local conversion of 25-hydroxyvitamin D [25-(OH)D] to its hormonally active 1,25-dihydroxy form [1,25-(OH)2D] may have multiple biological roles in almost all tissues, beyond the skeleton. More specifically, VitD appears to have a pivotal role in the regulation of cell growth, proliferation, differentiation and apoptosis, as well as various immunological functions and is thus implicated in several pathophysiological conditions, including cancer, diabetes, cardiovascular disease, multiple sclerosis, other autoimmune diseases and neuropsychiatric disorders.[4-8]

VitD deficiency or insufficiency may be due to reduced intake/bioavailability (obesity or malabsorption), decreased direct sunlight exposure or hepatic and/or renal disease.[9,10] The subsequent decline in intestinal calcium and phosphorus absorption may lead to increased PTH levels, resulting in secondary hyperparathyroidism and consequently depletion of the bones of calcium in order to maintain normocalcemia.[1] Therefore, in addition to the levels of circulating calcium and phosphate, PTH and biomarkers of bone turnover are useful indicators of VitD status, although their levels do not linearly follow 25-(OH) D fluctuations.

The generally accepted normal cut-off levels for 25-(OH)D have been set at >30 ng/mL to ensure optimal effects on calcium economy and skeletal health.[11] This rationale is based on maximal intestinal calcium absorption and nadir concentrations of parathyroid hormone (PTH), reported to be achievable at 25-(OH)D levels of approximately 30-40 ng/mL,[12-15] and is underscored by the updated recommendations of the US Endocrine Society.[1] However, both tenets have recently been challenged:[16-19] The boundary for clinically relevant VitD deficiency, characterized by osteopenia and rickets/ osteomalacia, has traditionally been set at 10 ng/mL 25-(OH)D,[17,20] a point recently disputed by the US Endocrine Society, that has raised the VitD deficiency threshold to 20 ng/mL 25-(OH)D.[1] 25-(OH)D "insufficiency" spans the range between the frank deficiency threshold and the lower normal level (traditionally 10-30 ng/mL), indicating inadequate stores for optimal VitD functions, but generally not associated with overt clinical symptoms.[21]

VitD overall status in vivo is best estimated by the concentration of serum total 25-(OH)D, i.e. the sum of 25-(OH)D2+25-(OH)D3.[1] Fully automated platforms have been developed and concerns stemming from the lack of agreement regarding the methods used to measure VitD are being abated by advances in methodology and availability of accurate calibration materials and quality assessment programs.[8,15]

Abundant evidence exists that VitD deficiency and insufficiency have emerged as a global health threat across all ethnicities and age groups, with approximately 1 billion people estimated to be affected worldwide, the Mediterranean region being no exception in spite of the high insolation (total amount of solar radiation energy received per surface area) of the area.[11,22] The aim of this cross-sectional study was to determine the total serum 25-(OH)D levels on the island of Crete, Greece, an area with high insolation. In addition, we sought to explore 25-(OH)D association with biomarkers of diabetes, metabolic syndrome, inflammation and bone remodeling, as well as the influence of season change and gender. Finally, we attempted to establish a 25-(OH)D threshold for our population and to compare it with the internationally proposed cut-offs for VitD sufficiency.

MATERIALS AND METHODS

Population

The Department of Clinical Chemistry-Biochemistry at the University Hospital of Heraklion is the regional reference center for VitD assays. We therefore retrieved 25-(OH)D measurements blindly from the Laboratory Information System (LIS) of the Hospital, without any patient identification. Our cohort included 8,183 total 25-(OH)D results, measured in our Department during a 5-year time period, between August 2009 and May 2014. Of the 8,183 subjects, 2,789 were hospital inpatients and 5,394 outpatients. The inpatients were hospitalized in diverse departments: endocrinology (n=1398),

paediatrics (n=337), rheumatology (n=201), internal medicine (n=169), nephrology (n=126), orthopaedics (n=109), paediatric oncology (n=91), haematology (n=81), oncology (n=70), neurology (n=57), surgery (n=48), gastroenterology (n=30), pulmonary medicine (n=25), cardiology (n=22), psychiatry (n=10), obstetrics/gynecology (n=10, excluding pregnant women), dermatology (n=5). The outpatients were prescribed routine testing for VitD status. For the purpose of our study, we stratified this population according to age, sex (Table 1) and season of the year when the analysis was performed. Age was not available for 141 subjects (43 males and 98 females, 85 outpatients and 56 inpatients) and their results were discarded from all further analyses.

Laboratory assays

Details of all laboratory methods together with their reference intervals and the number of patients for whom results were available are presented in Table 2. These include routine biochemical workout tests (glucose, urea, creatinine, albumin, calcium, phosphate, triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol), glycated hemoglobin A1c (HbA1c), C-reactive protein (CRP), hormonal determinations (intact PTH, TSH, insulin), bone turnover biomarkers (bone-specific isoform of alkaline phosphatase (b-ALP as activity and ostase as mass), N-Mid osteocalcin (OC), C-terminal telopeptide of type I collagen (CTX), urine free deoxypyridinoline crosslinks [Dpd]), tumor markers (CEA, CA 19-9, CA 125) and virology indices (HBsAg, HIV1/2, HCV). However, as tumor markers and virology indices were available in a very small sub-group of our data, we waived their further analysis. The laboratories performing the above mentioned assays participated in External Quality Assurance Services programs for the entire duration of the study and the observed deviation in Levey-Jennings charts was lower than ±1SD for all analytes measured.

Biochemical monitoring of the rate of bone turnover is assessed by measurement of specific biomarkers in the blood and/or urine, either proteins or enzymes released during bone formation, or degradation products released during bone resorption.[23] The bone-specific

Table 1. This table displays the results of all individuals included in the present study. Subjects were additionally stratified by gender and age groups (see Results for further details). For each parameter, the number of assayed subjects is presented, together with mean±SD and range

	Total-Age Groups														
	0-20			21-50			51-70			>70			Total		
	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)
Glucose (mg/dl)	699	87.96±18.98	85.00 (49.00-275.00)	841	93.25±21.13	90.00 (44.00-327.00)	1885	102.72±31.36	95.00 (55.00-451.00)	1552	109.96±36.98	99.00 (36.00-389.00)	4977	101.30±31.38	94.00 (36.00-451.00)
Hb_A1c (%)	52	5.41±0.73	5.30 (4.30-9.10)	118	5.81±1.43	5.50 (4.40-14.30)	314	6.33±1.06	6.00 (4.70-14.00)	241	6.81±1.30	6.40 (4.90-12.50)	725	6.34±1.26	6.00 (4.30-14.30)
INS(µU/ml)	26	11.74±5.77	10.35 (1.80-28.50)	45	10.76±10.27	8.20 (2.20-67.50)	31	11.08±6.50	10.20 (1.50-30.70)	11	15.38±10.26	14.10 (3.00-32.80)	113	11.52±8.45	9.30 (1.50-67.50)
Urea (mg/dl)	703	35.60±21.80	32.00 (6.00-269.00)	947	34.05±15.03	32.00 (8.00-158.00)	2177	36.31±17.49	33.00 (8.00-261.00)	1740	42.26±22.68	37.50 (7.00-249.00)	5567	37.70±19.71	34.00 (6.00-269.00)
Creatinine (mg/dl)	705	0.59±0.16	0.60 (0.20-1.20)	959	.87±.32	0.80 (0.10-5.00)	2262	0.87±0.39	0.80 (0.30-9.20)	1787	1.02±0.60	0.90 (0.50-10.50)	5713	0.88±0.46	0.80 (0.10-10.50)
									0.00 (0.00-0.00)						
Albumin (g/dl)	342	4.47±0.40	4.50 (2.80-5.40)	750	4.42±.39	4.50 (2.00-5.30)	1743	4.37±0.35	4.40 (1.60-5.20)	1253	4.13±0.51	4.30 (1.70-5.30)	4088	4.31±0.44	4.40 (1.60-5.40)
CRP (mg/dl)	112	0.92±2.11	0.20 (0.20-16.20)	229	.73±2.41	0.20 (0.20-28.70)	456	0.54±0.97	0.20 (0.20-8.45)	339	1.42±2.91	0.20 (0.20-20.60)	1136	0.88±2.15	0.20 (0.20-28.70)
									0.00 (0.00-0.00)						
Triglycerides (mg/dl)	417	82.20±48.82	69.00 (9.00-315.00)	649	107.75±77.52	87.00 (28.00-880.00)	1506	122.45±61.09	109.00 (23.00-581.00)	1057	129.78±69.44	114.00 (23.00-745.00)	3629	117.33±67.17	103.00 (9.00-880.00)
Cholesterol (mg/dl)	429	166.04±33.05	162.00 (58.00-286.00)	660	198.37±39.02	197.00 (58.00-376.00)	1517	211.94±41.70	211.00 (79.00-613.00)	1062	194.92±39.78	192.00 (66.00-368.00	3668	199.20±42.18	198.00 (58.00-613.00)
HDL Chol (mg/dl)	406	52.51±14.02	51.00 (13.00-103.00)	618	54.57±14.40	53.00 (14.00-111.00)	1461	58.07±14.54	57.00 (19.00-118.00)	1008	55.06±14.50	54.00 (11.00-109.00)	3493	55.94±14.57	55.00 (11.00-118.00)
LDL Chol (mg/dl)	404	110.47±33.11	108.00 (13.00-233.80)	617	121.60±38.07	121.00 (26.00-500.00)	1461	122.31±35.27	121.00 (23.00-249.00)	1007	118.49±34.93	116.00 (17.00-262.00	3489	119.71±35.62	118.00 (13.00-500.00)
Total/HDL Chol	405	3.35±0.96	3.16 (1.37-9.69)	616	3.86±1.18	3.65 (1.75-9.91)	1457	3.83±1.03	3.70 (1.63-9.57)	1003	3.72±0.98	3.58 (1.83-12.46)	3481	3.75±1.05	3.59 (1.37-12.46)
									0.00 (0.00-0.00)						
TSH (µU/ml)	561	2.23±1.49	1.86 (0.01-12.88)	721	1.84±3.81	1.30 (0.00-70.48)	1578	1.83±5.58	1.13 (0.00-100.00)	1065	1.44±1.74	1.11 (0.00-35.44)	3925	1.78±4.05	1.26 (0.00-100.00)
									0.00 (0.00-0.00)						
CEA (ng/ml)	3	0.70±0.07	0.70 (0.63-0.76)	51	2.17±1.73	1.66 (0.40-7.96)	128	3.01±7.77	1.81 (0.40-88.24)	75	2.81±2.17	2.36 (0.62-14.44)	257	2.76±5.66	1.89 (0.40-88.24)
CA_199 (U/ml)	3	5.21±1.05	5.00 (4.28-6.34)	38	9.59±11.46	6.05 (1.50-56.04)	90	25.81±126.27	6.65 (1.50-1201.99)	62	20.49±46.63	8.38 (1.50-335.57)	193	20.58±90.26	6.85 (1.50-1201.99)
CA_125 (U/ml)	3	16.57±11.90	14.40 (5.90-29.40)	36	15.49±11.74	12.60 (6.10-72.30)	80	17.24±35.58	9.80 (1.60-256.10)	51	36.95±122.60	13.50 (3.40-860.00)	170	22.77±71.81	11.05 (1.60-860.00)
									0.00 (0.00-0.00)						
HIV_1,2 (S/CO)	2	0.00	0.00	5	0.00	0.00	4	0.00	0.00 (0.00-0.00)	4	0.00	0.00	15	0.00	0.00
HBsAg (S/CO)	17	0.00	0.00	56	0.13±0.33	0.00	44	0.20±0.41	0.00 (0.00-1.00)	27	0.11±0.32	0.00	144	0.13±0.34	0.00
HCV (S/CO)	17	0.00	0.00	54	0.00	0.00	37	0.03±0.16	0.00 (0.00-1.00)	26	0.00	0.00	134	0.01±0.09	0.00
									0.00 (0.00-0.00)						
25(OH)VitD (ng/ml)	952	23.65±10.25	23.00 (3.90-105.00)	1322	18.61±8.64	18.00 (3.90-55.00)	3350	18.58±8.62	18.00 (3.90-73.50)	2418	15.68±8.54	14.50 (3.90-144.00)	8042	18.31±9.11	18.00 (3.90-144.00)
Calcium (mg/dl)	699	10.81±0.59	10.20 (5.70-12.90)	1033	9.79±.59	9.80 (5.60-13.40)	2747	9.85±0.63	9.90 (3.90-13.40)	2033	9.79±0.83	9.80 (4.60-15.10)	6512	9.86±0.70	9.90 (3.90-15.10)
Phosphate (mg/dl)	607	4.82±0.87	4.80 (1.20-8.70)	838	3.55±.65	3.50 (1.60-6.30)	2242	3.55±0.63	3.50 (0.40-8.20)	1708	3.51±0.70	3.50 (0.80-9.00)	5395	3.68±0.80	3.60 (0.40-9.00)
PTH (pg/ml)	177	49.91±40.80	40.90 (2.10-351.20)	578	76.30±78.79	63.90 (1.20-1205.90)	1774	85.86±62.43	75.10 (1.60-985.60)	1230	103.53±79.92	84.80 (0.50-970.30)	3759	88.48±71.62	74.80 (0.50-1205.90)
b-ALP (U/l)	3	81.83±22.43	72.0 (66.00-107.00)	31	19.82±8.54	19.00 (4.00-55.00)	311	20.79±8.00	19.00 (2.00-77.50)	220	20.01±10.34	18.00 (4.00-87.00)	565	20.75±10.12	19.00 (2.00-107.5)
N-Mid OC (ng/ml)	7	51.71±28.39	50.00 (20.00-99.00)	104	21.19±12.37	19.00 (5.00-82.00)	634	18.71±9.21	17.00 (3.00-92.00)	399	17.44±13.76	15.00 (4.00-173.00)	1144	18.70±11.78	16.00 (3.00-173.00)
CTX (ng/ml)	4	0.94±0.47	1.03 (0.33-1.36)	103	0.38±0.22	0.32 (0.01-1.03)	658	0.34±0.25	0.29 (0.01-3.55)	410	0.28±0.24	0.22 (0.01-1.96)	1175	0.32±0.25	0.27 (0.01-3.55)
D-Pyrilinks (nM Dpd/ mM creatinine)				7	6.16±3.37	4.80 (3.60-13.00)	60	5.99±2.05	5.80 (2.50-14.30)	27	6.66±2.67	6.60 (2.90-13.80)	94	6.19±2.34	5.80 (2.50-14.30)
Ostase (µg/l)	9	168.17±186.89	58.00 (15.00-500.00)	67	12.05±4.53	11.50 (5.00-25.00)	312	13.77±6.48	12.00 (4.00-48.00)	162	12.91±6.98	11.40 (4.00-51.00)	550	15.84±30.60	12.00 (4.00-500.00)

Table 1. (continued)

	Males-Age Groups														
	0-20			21-50			51-70			>70			Total		
	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)
Glucose (mg/dl)	332	89.31±17.36	88.00 (49.00-263.00)	213	97.37±22.80	93.00 (44.00-216.00)	288	113.58±47.09	101.50 (59.00-451.00)	313	119.16±372.00	104.00 (53.00-372.00)	1146	105.06±38.35	95.00 (44.00-451.00)
Hb_A1c (%)	24	5.48±.81	5.30 (4.80-9.10)	30	6.50±2.13	5.65 (4.40-14.30)	61	6.55±1.31	6.30 (4.70-11.40)	52	7.11±11.00	6.75 (5.30-11.00)	167	6.56±1.57	6.10 (4.40-14.30)
INS(µU/ml)	15	12.17±4.46	11.90 (6.10-19.30)	8	12.69±5.16	12.15 (6.00-20.00)	5	13.64±5.17	14.10 (7.50-21.40)	2	11.30±14.10	11.30 (8.50-14.10)	30	12.49±4.54	12.15 (6.00-21.40)
Urea (mg/dl)	328	35.15±16.17	32.00 (6.00-190.00)	228	37.78±15.12	35.00 (8.00-117.00)	332	38.23±16.49	35.00 (10.00-150.00)	347	46.99±248.00	40.00 (8.00-248.00)	1235	39.79±20.79	35.00 (6.00-248.00)
Creatinine (mg/dl)	329	.60± 17	0.60 (0.20-1.20)	235	1.09±0.50	1.00 (0.50-5.00)	340	1.12±0.77	1.00 (0.30-9.20)	352	1.34±10.50	1.10 (0.50-10.50)	1256	1.04±0.76	0.90 (0.20-10.50)
Albumin (g/dl)	160	4.44±.40	4.50 (2.80-5.20)	188	4.51±0.48	4.60 (2.40-5.20)	305	4.27±0.48	4.40 (2.00-5.20)	279	3.91±5.00	4.10 (1.70-5.00)	932	4.24±0.58	4.40 (1.70-5.20)
CRP (mg/dl)	41	1.47±3.13	0.20 (0.20-16.20)	75	1.17±3.91	0.20 (0.20-28.70)	61	0.86±1.57	0.20 (0.20-8.14)	74	2.16±20.60	0.49 (0.20-20.60)	251	1.44±3.31	0.20 (0.20-28.70)
Triglycerides (mg/dl)	201	78.77±46.95	66.00 (9.00-315.00)	161	140.81±112.36	117.00 (37.00-880.00)	207	139.85±77.76	121.00 (36.00-546.00)	190	141.51±745.00	118.00 (31.00-745.00)	759	124.29±88.94	100.00 (9.00-880.00)
Cholesterol (mg/dl)	206	160.27±33.20	158.00 (58.00-257.00)	163	199.67±45.43	198.00 (72.00-376.00)	211	196.67±49.15	196.00 (84.00-463.00)	190	171.99±331.00	168.00 (72.00-331.00)	770	181.48±46.15	176.00 (58.00-463.00)
HDL Chol (mg/dl)	198	51.19±13.70	50.50 (18.00-96.00)	152	45.75±10.17	45.00 (21.00-71.00)	197	46.66±12.55	46.00 (19.00-84.00)	174	43.87±82.00	44.00 (13.00-82.00)	721	47.04±12.78	46.00 (13.00-96.00)
LDL Chol (mg/dl)	198	108.47±30.17	108.00 (17.00-204.00)	152	121.01±37.70	120.00 (26.00-283.00)	197	123.10±35.92	120.00 (27.00-249.00)	174	114.88±212.00	113.50 (22.00-212.00)	721	116.66±34.80	115.00 (17.00-283.00)
Total/HDL Chol	197	3.29±.85	3.14 (1.37-6.81)	152	4.58±1.34	4.41 (2.24-9.91)	196	4.38±1.17	4.33 (2.00-8.12)	172	4.15±12.46	3.95 (2.07-12.46)	717	4.07±1.27	3.90 (1.37-12.46)
TSH (µU/ml)	261	2.13±1.25	1.83 (0.02-8.64)	148	1.98±2.66	1.39 (0.01-25.89)	235	2.56±11.16	1.08 (0.00-100.00)	172	1.30±5.73	1.03 (0.02-5.73)	816	2.05±6.16	1.39 (0.00-100.00)
CEA (ng/ml)				5	2.11±0.43	1.94 (1.62-2.67)	30	5.02±15.78	1.80 (0.40-88.24)	24	2.63±5.66	2.60 (0.62-5.66)	59	3.80±11.26	2.03 (0.40-88.24)
CA_199 (U/ml)				4	12.89±16.29	7.00 (1.50-36.05)	20	71.46±266.78	6.09 (1.50-1201.99)	22	10.70±31.10	6.24 (1.50-31.10)	46	37.31±176.16	6.09 (1.50-1201.99)
CA_125 (U/ml)				1	17.60±0.00	17.60 (17.60-17.60)	4	52.80±94.84	7.30 (1.60-195.00)	7	18.19±58.40	9.60 (6.60-58.40)	12	29.68±54.13	9.45 (1.60-195.00)
HIV_1,2 (S/CO)	1	0.00	0.00	2	0.00	0.00	2	0.00	0.00				5	0.00±0.00	0.000
HBsAg (S/CO)	6	0.00	0.00	27	0.11±0.32	0.00	22	0.27±0.46	0.00	11	0.09±1.00	0.00	66	0.15±0.36	0.000
HCV (S/CO)	5	0.00	0.00	26	0.00	0.00	16	0.06±0.25	0.00	10	0.00	0.00	57	0.02±0.13	0.000
														0.00±0.00	
25(OH)VitD (ng/ml)	454	24.20±9.75	23.25 (3.90-105.00)	315	19.91±9.30	19.00 (3.90-54.00)	471	18.40±8.73	18.00 (3.90-55.00)	423	15.29±48.50	14.00 (3.90-48.50)	1663	19.48±9.51	19.00 (3.90-105.00)
Calcium (mg/dl)	332	10.21±.57	10.20 (7.20-12.90)	236	9.94±0.67	10.00 (5.60-11.90)	379	9.70±0.81	9.80 (6.20-13.40)	343	9.56±15.10	9.60 (5.00-15.10)	1290	9.84±0.86	9.90 (5.00-15.10)
Phosphate (mg/dl)	303	4.89±.86	4.90 (1.20-7.50)	194	3.59±0.74	3.55 (1.60-5.90)	322	3.27±0.80	3.20 (0.40-8.20)	302	3.34±7.50	3.20 (1.20-7.50)	1121	3.76±1.06	3.60 (0.40-8.20)
PTH (pg/ml)	89	43.41±25.17	38.20 (2.10-135.10)	117	84.64±90.83	63.70 (21.30-693.90)	258	91.94±101.01	76.05 (4.40-985.60)	204	115.84±970.30	87.25 (7.90-970.30)	668	91.49±99.34	71.40 (2.10-985.60)
b-ALP (U/l)				3	23.67±5.77	27.00 (17.00-27.00)	26	21.19±5.88	20.25 (12.00-32.00)	10	20.70±37.00	17.75 (13.00-37.00)	39	21.26±6.12	20.00 (12.00-37.00)
N-Mid OC (ng/ml)	2	63.00±18.38	63.00 (50.00-76.00)	15	25.33±18.16	20.00 (9.00-82.00)	44	16.86±8.75	15.00 (6.00-55.80)	24	16.79±44.00	15.00 (5.00-44.00)	85	19.42±13.23	16.00 (5.00-82.00)
CTX (ng/ml)	1	0.80±.00	0.80 (0.80-0.80)	17	0.49±0.24	0.49 (0.09-0.84)	46	0.35±0.23	0.33 (0.04-1.24)	24	0.35±0.91	0.33 (0.04-0.91)	88	0.38±0.24	0.34 (0.04-1.24)
D-Pyrilinks (nM Dpd/ mM creatinine)				2	9.05±5.59	9.05 (5.10-13.00)	12	5.46±1.38	4.95 (3.90-7.90)	1	3.70±3.70	3.70 (3.70-3.70)	15	5.82±2.38	5.10 (3.70-13.00)
Ostase (µg/l)	5	183.10±167.40	120.00 (32.00-400.00)	13	12.88±5.15	11.50 (7.00-24.00)	21	12.67±8.21	11.00 (6.00-44.50)	13	9.70±13.00	9.50 (6.00-13.00)	52	28.37±69.51	11.50 (6.00-400.00)

	Females-Age Groups														
	0-20			21-50			51-70			>70			Total		
	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)	N	Mean±SD	Median (Range)
Glucose (mg/dl)	367	86.73±20.27	84.00 (55.00-275.00)	628	91.85±20.36	90.00 (48.00-327.00)	1597	100.76±27.15	94.00 (55.00-351.00)	1239	107.64±33.97	98.00 (36.00-389.00)	3831	100.18±28.88	94.00 (36.00-389.00)
Hb_A1c (%)	28	5.36±0.65	5.30 (4.30-7.30)	88	5.58±1.01	5.40 (4.60-14.00)	253	6.27±0.99	6.00 (4.80-14.00)	189	6.73±1.23	6.40 (4.90-12.50)	558	6.27±1.15	6.00 (4.30-14.00)
INS(µU/ml)	11	11.15±7.39	9.80 (1.80-28.50)	37	10.35±11.08	7.10 (2.20-67.50)	26	10.59±6.70	9.20 (1.50-30.70)	9	16.29±11.16	16.40 (3.00-32.80)	83	11.17±9.47	8.30 (1.50-67.50)
Urea (mg/dl)	375	35.99±25.75	31.00 (10.00-269.00)	719	32.87±14.82	30.00 (11.00-158.00)	1845	35.97±17.64	33.00 (8.00-261.00)	1393	41.08±20.85	37.00 (7.00-249.00)	4332	37.10±19.36	33.00 (7.00-269.00)
Creatinine (mg/dl)	376	0.57±0.15	0.60 (0.20-1.10)	724	0.80±0.20	0.80 (0.10-3.00)	1922	0.82±0.25	0.80 (0.50-6.10)	1435	0.94±0.41	0.90 (0.50-7.50)	4457	0.84±0.31	0.80 (0.10-7.50)
Albumin (g/dl)	182	4.49±0.41	4.50 (2.90-5.40)	562	4.39±0.35	4.40 (2.00-5.30)	1438	4.39±0.31	4.40 (1.60-5.20)	974	4.19±0.45	4.30 (2.10-5.30)	3156	4.33±0.38	4.40 (1.60-5.40)
CRP (mg/dl)	71	0.60±1.08	0.20 (0.20-6.07)	154	0.52±1.05	0.20 (0.20-10.10)	395	0.49±0.83	0.20 (0.20-8.45)	265	1.21±2.61	0.20 (0.20-17.20)	885	0.72±1.65	0.20 (0.20-17.20)
Triglycerides (mg/dl)	216	85.39±50.39	71.00 (19.00-313.00)	488	96.84±58.03	83.50 (28.00-546.00)	1299	119.67±57.55	108.00 (23.00-581.00)	867	127.20±62.08	113.00 (23.00-634.00)	2870	115.48±60.00	103.00 (19.00-634.00)
Cholesterol (mg/dl)	223	171.37±32.08	166.00 (97.00-286.00)	497	197.95±36.72	196.00 (58.00-339.00)	1306	214.40±39.84	213.00 (79.00-613.00)	872	199.92±37.11	198.00 (66.00-368.00)	2898	203.91±39.76	202.00 (58.00-613.00)
HDL Chol (mg/dl)	208	53.76±14.24	54.00 (13.00-103.00)	466	57.45±14.41	56.00 (14.00-111.00)	1264	59.85±14.02	58.00 (23.00-118.00)	834	57.40±13.73	56.00 (11.00-109.00)	2772	58.25±14.12	57.00 (11.00-118.00)
LDL Chol (mg/dl)	206	112.39±35.67	108.50 (13.00-233.80)	465	121.80±38.23	121.00 (34.00-500.00)	1264	122.18±35.18	121.00 (23.00-233.00)	833	119.24±35.09	116.00 (17.00-262.00)	2768	120.50±35.80	119.00 (13.00-500.00)
Total/HDL Chol	208	3.41±1.05	3.21 (1.75-9.69)	464	3.62±1.02	3.45 (1.75-8.80)	1261	3.75±0.98	3.63 (1.63-9.57)	831	3.63±0.88	3.51 (1.83-8.52)	2764	3.66±0.97	3.54 (1.63-9.69)
TSH (µU/ml)	300	2.32±1.67	1.90 (0.01-12.88)	573	1.81±4.06	1.23 (0.00-70.48)	1343	1.70±3.84	1.14 (0.00-100.00)	893	1.47±1.84	1.12 (0.00-35.44)	3109	1.71±3.27	1.22 (0.00-100.00)
CEA (ng/ml)	3	0.70±0.07	0.70 (0.63-0.76)	46	2.18±1.82	1.53 (0.40-7.96)	98	2.40±1.72	1.83 (0.40-9.21)	51	2.89±2.50	2.21 (0.68-14.44)	198	2.45±1.98	1.85 (0.40-14.44)
CA_199 (U/ml)	3	5.21±1.05	5.00 (4.28-6.34)	34	9.20±11.03	6.05 (1.50-56.04)	70	12.76±13.87	7.13 (1.50-63.03)	40	25.88±57.12	9.58 (1.50-335.57)	147	15.35±32.16	7.61 (1.50-335.57)
CA_125 (U/ml)	3	16.57±11.90	14.40 (5.90-29.40)	35	15.43±11.91	12.30 (6.10-72.30)	76	15.37±30.04	9.85 (2.80-256.10)	44	39.94±131.77	13.55 (3.40-860.00)	158	22.24±73.08	11.10 (2.80-860.00)
HIV_1,2 (S/CO)	1	0.00±0.00	0.00	3	0.00	0.00	2	0.00	0.00	4	0.00	0.00	10	0.00	0.00
HBsAg (S/CO)	11	0.00±0.00	0.00	29	0.14±0.35	0.00	22	0.14±0.35	0.00	16	0.13±0.34	0.00	78	0.12±0.32	0.00
HCV (S/CO)	12	0.00±0.00	0.00	28	0.00	0.00	21	0.00±0.00	0.00	16	0.00	0.00	77	0.00	0.00
25(OH)VitD (ng/ml)	498	23.15±10.68	22.00 (3.90-102.00)	1007	18.21±8.38	17.50 (3.90-55.00)	2879	18.60±8.60	18.00 (3.90-73.50)	1995	15.77±8.67	15.00 (3.90-144.00)	6379	18.01±8.98	17.50 (3.90-144.00)
Calcium (mg/dl)	367	10.15±0.61	10.20 (5.70-12.00)	797	9.75±0.56	9.70 (6.60-13.40)	2368	9.88±0.59	9.90 (3.90-13.00)	1690	9.84±0.76	9.90 (4.60-15.00)	5222	9.87±0.65	9.90 (3.90-15.00)
Phosphate (mg/dl)	304	4.75±0.88	4.70 (1.80-8.70)	644	3.54±0.63	3.50 (1.70-6.30)	1920	3.60±0.59	3.60 (1.30-7.90)	1406	3.55±0.66	3.50 (0.80-9.00)	4274	3.66±0.71	3.60 (0.80-9.00)
PTH (pg/ml)	88	56.48±51.39	43.30 (11.00-351.20)	461	74.18±75.39	63.90 (1.20-1205.90)	1516	84.83±53.14	75.05 (1.60-610.50)	1026	101.08±71.20	84.50 (0.50-837.40)	3091	87.83±64.07	75.20 (0.50-1205.90)
b-ALP (U/l)	3	488.50±596.48	72.00 (66.00-107.00)	28	19.41±8.76	18.75 (4.00-55.00)	285	20.75±8.17	19.00 (2.00-77.50)	210	19.97±10.48	18.00 (4.00-87.00)	526	20.72±10.36	18.75 (2.00-107.50)
N-Mid OC (ng/ml)	5	47.20±32.18	34.00 (20.00-99.00)	89	20.49±11.10	19.00 (5.00-79.00)	590	18.85±9.24	17.00 (3.00-92.00)	375	17.49±14.04	14.10 (4.00-173.00)	1059	18.64±11.66	16.00 (3.00-173.00)
CTX (ng/ml)	3	0.98±0.57	1.26 (0.33-1.36)	86	0.36±0.22	0.29 (0.01-1.03)	612	0.34±0.25	0.29 (0.01-3.55)	386	0.27±0.24	0.22 (0.01-1.96)	1087	0.32±0.25	0.27 (0.01-3.55)
D-Pyrilinks (nM Dpd/ mM creatinine)		0.00±0.00		5	5.00±1.83	4.80 (3.60-8.10)	48	6.12±2.18	5.85 (2.50-14.30)	26	6.77±2.66	6.90 (2.90-13.80)	79	6.26±2.35	5.80 (2.50-14.30)
Ostase (µg/l)	4	615.20±1060.94	41.50 (15.00-500.00)	54	11.85±4.40	11.25 (5.00-25.00)	291	13.85±6.35	12.00 (4.00-48.00)	149	13.19±7.15	11.50 (4.00-51.00)	498	14.53±22.82	12.00 (4.00-500.00)

isoform of alkaline phosphatase is a biomarker of bone formation not affected by diet and renal function and exhibiting low inter-individual variability. Its main disadvantage is that changes can lag by several weeks, thus making it unsuitable for initial follow-up of antiresorptive therapy. Osteocalcin (OC), produced by

Table 2. Assay characteristics of methods used in the present study, together with reference range and units of measurement

TESTS	COMPANY	ANALYZER	METHOD	REF No	UNITS	REFERENCE RANGE	NUMBER OF CASES	MALES	FEMALES
25-OH vitamin D total	DiaSorin Inc	LIAISON	DIRECT CHEMILUMINESCENCE	310600	ng/ml	>30*	8183	1706	6477
Glucose	Medicon SA	Olympus AU5400	HEXOKINASE	1418-0017	mg/dl	70-115	5079	1175	3904
Urea	Medicon SA	Olympus AU5400	UREASE UV	1418-0027	mg/dl	15-55	5675	1265	4410
Creatinine	Medicon SA	Olympus AU5400	JAFFE	1418-0037	mg/dl	1.2	5821	1286	4535
Albumin	Medicon SA	Olympus AU5400	BCG	1418-0197	g/dl	3,5-5,0	4172	957	3215
Calcium	Medicon SA	Olympus AU5400	ARSENAZO	1418-0207	mg/dl	8,2-10,6	6622	1322	5300
Phosphate	Medicon SA	Olympus AU5400	MOLYBDATE UV	1418-0217	mg/dl	1,9-2,5	5495	1150	4345
Triglycerides	Medicon SA	Olympus AU5400	GPO-PAP	1418-0067	mg/dl	40-160	3713	779	2934
Cholesterol	Medicon SA	Olympus AU5400	CHOD-POD	1418-0047	mg/dl	<200	3753	791	2962
HDL Cholesterol	Medicon SA	Olympus AU5400	IMMUNOINHIBITION	1418-0247	mg/dl	>35	3773	741	3032
LDL Cholesterol	Friedewald equation		CALCULATED		mg/dl	<130	3569	744	2825
Hb A1c	MENARINI diagnostics	HA-8160	HPLC	03176,03177,03178,03179	%	4-6	739	173	566
CRP	SIEMENS diagnostics	BN II	NEPHELOMETRY	OQIY21	mg/dl	0,08-0,8	1148	253	895
intact PTH	Abbott Laboratories	Architect i2000SR	CHEMILUMINESCENCE MICROPARTICLES IMMUNOASSAY(CMIA)	8K25-25	pg/ml	12-65	3785	675	3110
TSH	Abbott Laboratories	Architect i2000SR	CMIA	7K62-25	μU/ml	0,25-3,43	3965	825	3140
b-ALP	Quidel Corporation	MAGO	ELISA	8012	U/l	11-41**	584	43	541
N-Mid Osteocalcin	Roche diagnostics	ELECSYS 2010	ELECTROCHEMILUMINESCENCE	12149133	ng/ml	11-70***	1173	90	1083
CTX	Roche diagnostics	ELECSYS 2010	ELECTROCHEMILUMINESCENCE	11972308	ng/ml	0,02-1,0****	1206	93	1113
D-Pyrilinks	SIEMENS diagnostics	Immulite 1000	SOLID PHASE ENZYME- LINKED CHEMILUMINESCENCE	LKPD1	nM DPD/mM creatinine	2,5-7,4*****	94	15	79
Ostase	DiaSorin Inc	LIAISON	DIRECT CHEMILUMINESCENCE	310970	μg/l	3-30******	564	54	510
INS	Abbott Laboratories	Architect i2000SR	CMIA	8K4127	μU/ml	3-17	115	31	84
HBsAg	Abbott Laboratories	Architect i2000SR	CMIA	2G22-01	S/CO	<1	147	???	???
HIV1,2	Abbott Laboratories	Architect i2000SR	CMIA	4J27-03	S/CO	<1	16	???	???
HCV	Abbott Laboratories	Architect i2000SR	CMIA	6C37-10	S/CO	<1	136	???	???
CEA	Abbott Laboratories	Architect i2000SR	CMIA	7K68-27	ng/ml	0-5	260	59	201
CA19-9	Abbott Laboratories	Architect i2000SR	CMIA	2K91-28	U/ml	0-37	194	46	148
CA125	Abbott Laboratories	Architect i2000SR	CMIA	2K45-28	U/ml	0-35	174	12	162

*25-OH vitamin D total: deficiency <10; insuffiency 10-30; sufficiency 30-100; toxicity >100
**b-ALP: premenopausal women 11-30; postmenopausal women 14-43; men (>25y) 15-41
***N-Mid Osteocalcin: premenopausal women (>20y) 11-43; postmenopausal women (without replacement therapy) 15-46; osteoporotic women 13-48; men (18-30y) 24-70; men (30-50y)14-42; men (50-70y) 14-46
****CTX: premenopausal women 0,03-0,57; postmenopausal women 0,1-1,0; men (30-50y) 0,02-0,6; men (50-70y) 0,10-0,70; men (>70y) 0,16-0,85
*****D-Pyrilinks: women 3,0-7,4; men 2,3-5,4
******Ostase: premenopausal women 3-19; postmenopausal women 6-26; men (>25y) 6-30

osteoblasts and incorporated into the bone matrix during bone formation and also released into the circulation from the matrix during bone resorption, is considered a marker of bone turnover. Its main advantages are tissue-specificity and relatively low within-individual variation, while its principal disadvantage concerns the instability of the intact molecule, which is rapidly degraded. The large N-terminal/midregion fragment (N-Mid, aa 1-43) appears to be considerably more stable.[24] The C-terminal crosslinked telopeptide of type I collagen (CTX) is the bone resorption marker of choice, recommended by the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).[25] β-CTX levels are indicative of the degree of the breakdown of mature type I collagen and are the preferred marker for monitoring the start of antiresorptive therapy. The main disadvantage stems from its large circadian variation, necessitating a morning fasting sample for accurate interpretation of results. The urine free deoxypyridinoline cross-links (Dpd), released when mature type I collagen is degraded, are tissue-specific and not influenced by the diet. The main disadvantage is the necessity of a 24-hour urine sample and the need to correct results for creatinine excretion.

As a general procedure, a blood sample was collected from each subject; the blood was allowed to clot and serum was separated by centrifugation, with the exception of HbA1c which was determined in whole blood. Testing was performed on the day of blood collection for routine biochemistry, HbA1c, CRP and hormonal measurements. Serum and 24-hour urine samples for 25-(OH)D and bone turnover biomarkers measurements were frozen at -20°C after collection and tested later, as assays for these parameters are performed on a weekly basis. All assays were performed according to the manufacturers' instructions.

Validation of the serum total 25-(OH)D assay used in our study

Prior to analysis, we confirmed that our assay actually measured the total amount of 25-(OH)D. For this purpose, we collected and pooled samples in which measured values were >30 ng/mL. One volume of saturated trichloroacetic acid (TCA) was added to four volumes of sample and the resulting mixture was vortexed and centrifuged. The addition of TCA causes protein precipitation and release of any bound (micro)molecules, including 25-(OH)D. The resulting supernatant was buffered with the 25-(OH)D assay buffer (200 μL supernatant plus 500

μL of buffer) in order to restore pH, necessary for the correct performance of the immunoassay and assayed again for 25-(OH)D. Taking all dilutions into account, the recovery was 109±8%, suggesting that in our assay the majority of protein-bound 25-(OH)D was released from proteins and hence our data should represent total 25-(OH)D.

Statistical analysis

The analysis was performed with the SPSS V21 program. All data were normalized by z-transformation ((parameter value-mean)/SD) prior to any statistical test. However, for the readers' convenience, non-transformed values are reported in the Tables and Figures. For the comparison of the two groups, we used a t-test with unequal variance; for the comparison of multiple groups, we used one or two ways ANOVA, as appropriate, with Bonferroni-correction for post-hoc analyses. Multivariate analysis was used for group comparisons, the confounders being detailed in the Results section. Subjects were stratified by decade of age or by classification into four age groups (0-20, 21-50, 51-70, >70 y), for some analyses, as detailed in the Results section. Menopausal age for female individuals was arbitrarily set at 50 years. 25(OH)VitD values were stratified into the following groups: <10, 10-15, 15-20, 20-25, 25-30, >30 ng/mL. Correlations were analyzed by extracting the Pearson coefficient. For logistic regression, all variables were coded as low (0), normal (1) or high (2), the cut-offs being presented in Table 2. Stepwise backward parameter elimination was performed, with $p<0.05$ for entry and $p>0.1$ for the removal of parameters. In all tests a $p<0.05$ was considered as significant.

RESULTS

Distribution of total 25-(OH)D values

Effect of age and gender

Of the 8,042 individuals included in the study, 1,663 were males (20.7%) and 6,379 females (79.3%). In both groups, mean 25-(OH)D levels were unexpectedly low for a population living in a Mediterranean region. Mean 25-(OH)D concentration was 19.48±9.51 (ng/mL±SD) in males and 18.01±9.01 (ng/mL±SD) in females. A comparison of inpatients and outpatients yielded similar results; therefore we combined these two groups and performed the analysis on the total population of our study. Age distribution of mean total 25-(OH)D values (Figure 1A) showed that the highest values were present in children and adolescents of both genders (mean values for males=24.2±9.75, females=23.15±10.67 ng/mL; mean±SD), while a progressive and significant ($p<0.001$) decrease with age was evident. The mean concentration of serum 25-(OH)D in the elderly (>70 years) was significantly lower compared to the other age groups (mean±SD values for males=15.29±48.50, females=15.77±8.67 ng/mL, $p<0.001$ in both cases) and the lowest observed in our subjects. An inverse linear trend was apparent with advancing age, being more evident in subjects over 70 years.

An interesting observation was that despite the high percentage of sunny days on the island of Crete, 21% of our population was severely VitD deficient according to the standard criteria of our method, exhibiting levels below 10 ng/mL 25-(OH)D. Additionally, a significant ($p=0.02$) difference between sexes was evident in that the levels of 25-(OH)D were significantly lower in females compared to males, up to the age of 50 years. Thereafter, 25-(OH)D levels did not differ between genders (Figure 1A).

Seasonal variation

25-(OH)D levels depend on sun exposure, which fluctuates with the seasons.[2,10,26-28] In our cohort also, a significant seasonal variation of 25-(OH)D was evident ($p<0.001$) (Figure 1B, Table 3). Interestingly, there was one major peak throughout the year, with values significantly increasing from April to August and linearly decreasing thereafter, roughly corresponding to maximum insolation and UV index on the island of Crete (source http://www.bing.com/search?q=weather). This profile remained unchanged throughout the five years of the study. It is of note that 25-(OH)D levels in December were still significantly higher compared to those in January-March ($p=0.002$). Restricting the analysis to subjects aged <50 years, in which the gender difference was significant, resulted in two parallel month-distribution curves, this suggesting a similar variation of 25-(OH)D concentration between sexes and confirming the major effect of sunshine on endogenous VitD production (Figure 1C).

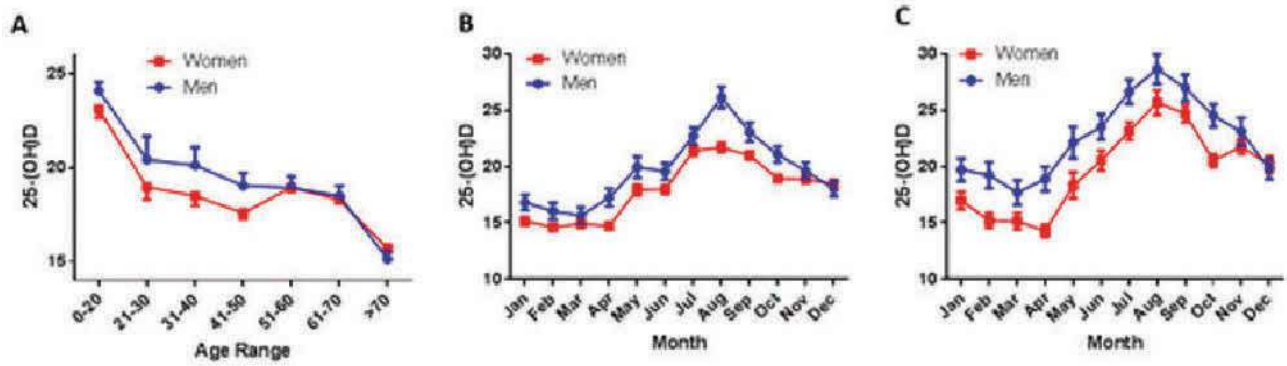

Figure 1. *25-(OH)D significantly varies with age, season and gender.* **A.** Mean ± SE variation of 25-(OH)D concentration in male (blue curve) and female individuals (red curve), as a function of age. Subjects have been divided by decades of age, except for individuals between 0 and 20 and over 70 years. Values were significantly different ($p<0.001$) among different age-groups. Females always had lower values than males ($p<0.001$), until menopause, arbitrarily set at 50 years of age. According to these results, we have further constructed four different age-groups (see Table 1), representing children and adolescents (age 0-20), adults (age 20-50), middle-aged (age 50-70) and elderly (>70 years). **B.** and **C.** Monthly fluctuation of mean±SE values of 20-(OH)D variations in the whole population (B) and in individuals ≤ 50 years (premenopausal females and corresponding males) (C). Month values were significantly different ($p<0.001$), with females always lower than corresponding males.

Correlation of total 25-(OH)D levels with PTH and bone turnover biomarkers

As a second step, we explored possible univariate correlations between 25-(OH)D and parameters of bone metabolism and turnover (Ca, P, PTH, b-ALP, Ostase, N-Mid-Osteocalcin and D-Pyrilinks; Figure 2A). We confirmed the well-established[10,29,30] strong positive correlation between circulating 25-(OH)D levels and Ca and P, together with a significant inverse correlation with PTH[16,31] (ANOVA $p<0.001$, for all three) (Figure 2B, Table 3). In addition, we observed a weak but significant correlation between 25-(OH)D and osteocalcin and CTX ($p=0.03$ and 0.003, respectively) (Table 3).

Table 3. Univariate correlation between 25-(OH)D and bone metabolism parameters. Pearson's correlation coefficient of normalized (z-transformed) values is presented, together with the number of subjects in parenthesis and statistical significance. Significant correlations are denoted in **bold** characters

		Ca	P	PTH	bALP	OC	CTX	DPyrilinks	Ostase
25(OH)VitD	Correlation Coefficient (n)	**0.092 (6622)**	**0.151 (5495)**	**-0.274 (3785)**	-0.001 (584)	**-0.063 (1173)**	**-0.086 (1206)**	-0.087 (95)	0.063 (564)
	p	**.001**	**.001**	**.001**	0.979	0.032	0.003	0.403	0.132
Ca	Correlation Coefficient (n)		0.002 (5338)	-0.014 (3298)	**0.161 (526)**	**0.110 (1030)**	-0.086 (1206)	-0.016 (81)	**0.163 (489)**
	p		0.902	0.411	**.001**	**.001**	0.272	0.884	**.001**
P	Correlation Coefficient (n)			**-0.155 (2659)**	**0.213 (518)**	0.045 (985)	0.057 (1012)	**0.269 (79)**	**0.208 (469)**
	p			**.001**	**.001**	0.163	0.067	**0.016**	**.001**
PTH	Correlation Coefficient (n)				**0.218 (143)**	**0.148 (445)**	**0.161 (469)**	-0.164 (33)	-0.044 (272)
	p				**0.009**	0.002	**0.001**	0.363	0.471
bALP	Correlation Coefficient (n)					**0.609 (537)**	**0.496 (548)**	0.106 (40)	
	p					**.001**	**.001**	0.516	
OC	Correlation Coefficient (n)						**0.669 (1060)**	**0.465 (85)**	**0.488 (486)**
	p						**.001**	**.001**	**.001**
CTX	Correlation Coefficient (n)							**0.482 (84)**	**0.556 (494)**
	p							**.001**	**.001**
DPyrilinks	Correlation Coefficient (n)								**0.396 (49)**
	p								**.005**

Since significant differences in circulating 25-(OH)D levels were observed between sexes and age-groups, we used these two factors as confounders in multivariate analysis, with 25-(OH)D groups as the grouping variable. Ca, P and PTH varied significantly compared to with 25-(OH)D levels ($p<0.001$ for all three parameters, Figure 2B). PTH levels decreased sharply ($p<0.001$) until 25-(OH)D levels reached ~20 ng/mL, at which threshold normalization was attained (dotted line) and further decreased in a slow, not statistically significant, manner thereafter. From ~20 ng/mL 25-(OH)D, a plateau in Ca levels was also observed, while P gradually increased with increasing 25-(OH)D levels. A mean 25-(OH)D concentration of 19.854 ng/mL (confidence interval 19.292-20.416 ng/mL, corrected for gender and age differences) was sufficient to normalize PTH levels ($p<0.001$), with Ca levels of 9.86 mg/dL (CI 8.01-8.91) and P concentrations of 3.70 mg/dL (CI 3.66-3.75).

Levels of 25-(OH)D were inversely correlated with b-ALP, ostase, CTX and osteocalcin ($p<0.001$ for all analytes) in a multivariate model with age and gender as confounders. No significant changes for D-pyrilinks were found, most probably due to the extremely small number of available results (n=94). Nevertheless, it should be stressed that all bone turnover biomarkers varied within their normal reference interval (Figure 2C).

Correlation of total 25-(OH)D with metabolic parameters

25-(OH)D serum concentrations were correlated with a number of metabolic parameters, after correct-

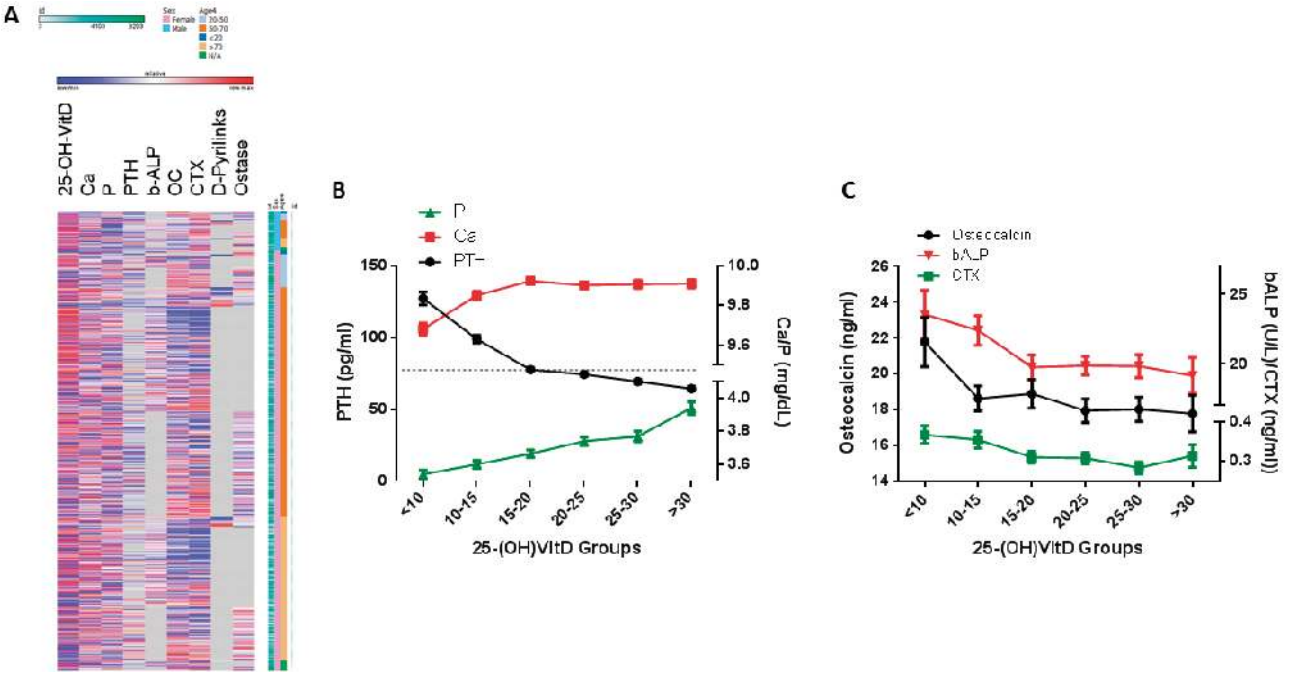

Figure 2. *25-(OH)D correlates with bone-metabolic indices.* **A.** Heatmap of normalized (z-transformed) values of bone metabolism-related parameters. In the vertical axes, individuals were sorted by gender and age groups. **B.** Variation (mean±SE) of intact PTH (black line), calcium and phosphate (red and green line respectively), as a function of 25-(OH)D groups, as detailed in the ordinate. Dotted line shows the upper normal cut-off value for PTH. All three parameters vary significantly among groups ($p<0.001$). As detailed in Table 1 and the Results section, PTH decreases significantly up to a 25-(OH)D concentration of 20 ng/mL, and non-significantly thereafter. At the same threshold, calcium serum concentrations reach a plateau. **C.** Bone metabolic indices, as a function of 25-(OH)D concentration (mean±SE). Osteocalcin, bone-specific alkaline phosphatase activity (b-ALP) and C-terminal telopeptide of type I collagen (CTX), bone turnover, formation and resorption markers (black, red and green lines) are significantly ($p<0.001$) decreased as 25-(OH)D increases, in multivariate analysis with age and gender as confounders. As presented, at 25-(OH)D levels ~20 ng/mL, the maximum significant decrease is observed, for all parameters. It is noteworthy, however, that all three indices vary within their normal range, at all 25-(OH)D concentrations.

ing for gender, age and seasonal variations (Figure 3, Table 4).

A significant negative correlation between 25-(OH)D and glucose, as well as HbA1c, was observed ($p<0.001$ for both). The absence of any correlation with insulin was probably due to the extremely small number of available insulin data (n=115).

A significant negative correlation between 25-(OH)D and biochemical markers of renal function was evidenced (urea and creatinine, $p<0.001$).

A significant negative correlation was noted between 25-(OH)D and both circulating triglycerides and total cholesterol ($p<0.001$ for both), but no associations were apparent with either HDL or LDL cholesterol. However, a significant negative correlation between 25-(OH)D and the total/HDL cholesterol ratio (cut-off value 5,[32] $p<0.001$) was observed.

No correlation between 25-(OH)VitD and TSH was found.

Finally, a strong positive correlation ($p<0.001$) was evident between 25-(OH)D and albumin.

Taken together, these data suggest that 25-(OH)D is decreased: (i) in patients with impaired renal function (reflected by elevated urea and creatinine levels), (ii) in patients with a biochemical profile indicative of metabolic syndrome (increased glucose, triglycerides and total cholesterol levels), (iii) in patients with overt diabetes (reflected by increased HbA1c).

Lastly, we performed a logistic regression of 25-(OH)D with a number of serum metabolic analytes. The cut-off level for 25-(OH)D was set at 20 ng/mL, based on our results on PTH and bone turnover indices. Only the significant analytes retained by the model, after backward elimination, are represented in Figure 4A. We conclude that a significant probability for decreased total 25-(OH)D, as expressed by odds ratios, is expected in overt diabetic fasting glucose levels (>126 mg/dL), and elevated cholesterol and creatinine levels, compatible with metabolic disease

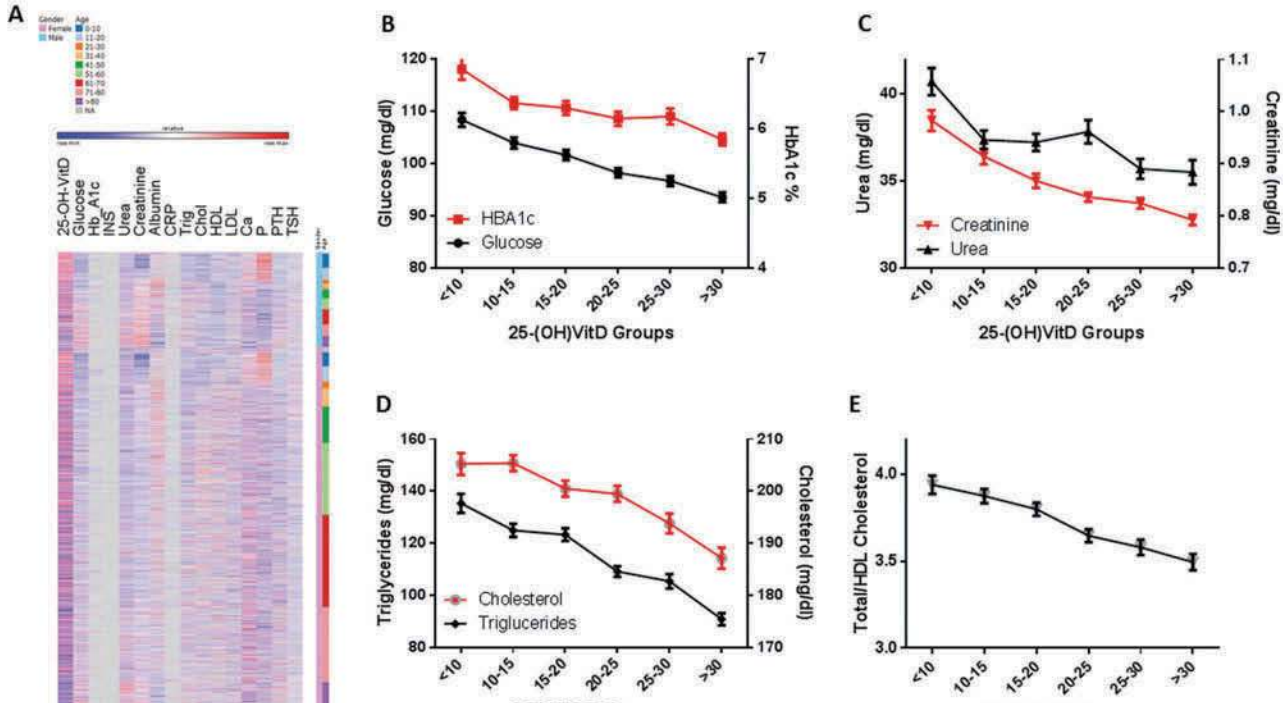

Figure 3. *Metabolic parameters vary significantly with 25-(OH)D concentration.* **A.** Heatmap of normalized (z-transformed) values of metabolic parameters. In the vertical axes, individuals were sorted by gender and age groups. Glucose and HbA1c **(B)**, urea and creatinine (**C**), cholesterol and triglycerides (**D**) are presented (mean±SE) as a function of 25-(OH)D groups (concentration ranges are presented in the ordinate). All changes are significant in a multivariate analysis ($p<0.001$), with age and gender as confounders. **E.** Total/HDL cholesterol ratio (cut-off level <5 [32]) also varies significantly ($p<0.001$) with circulating 25-(OH)D, in a multivariate analysis, with the same confounders.

Table 4. Univariate correlation between 25-(OH)D and metabolic parameters. Pearson's correlation coefficient of normalized (z-transformed) values is presented, together with the number of subjects in parenthesis and statistical significance. Significant correlations are denoted in bold characters.

		Glucose	Hb_A1c	INS	Urea	Creatinine	Albumin	CRP	Trig	Chol	HDL	LDL	Total/HDL Chol	TSH
25-(OH)VitD	Pearson Correlation (n)	**-0.144 (5079)**	**-0.184 (739)**	-0.176 (115)	**-0.070 (5675)**	**-0.121 (5821)**	**0.200 (4172)**	**-0.116 (1148)**	**-0.187 (3712)**	**-0.115 (3752)**	0.0220 (3573)	-0.010 (3569)	**-0.136 (3561)**	-0.022 (3965)
	p	**0.001**	**0.001**	0.059	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	0.189	0.559	**0.001**	0.171
Glucose	Pearson Correlation (n)		**0.681 (655)**	0.132 (107)	**0.072 (4886)**	**0.145 (4901)**	**-0.187 (3234)**	**0.082 (966)**	**0.243 (3470)**	-0.024 (3503)	**-0.151 (3349)**	0.004 (3344)	**0.160 (3340)**	-0.006 (2869)
	p		**0.001**	0.175	**0.001**	**0.001**	**0.001**	**0.011**	**0.001**	0.172	**0.001**	0.839	**0.001**	0.733
Hb_A1c	Pearson Correlation (n)			**0.372 (54)**	**0.115 (630)**	**0.133 (641)**	**-0.209 (516)**	0.076 (153)	**0.252 (566)**	0.0003 (563)	**-0.163 (547)**	0.0219 (546)	**0.181 (541)**	0.043 (482)
	p			**0.006**	**0.004**	**0.001**	**0.001**	0.344	**0.001**	0.994	**0.001**	0.610	**0.001**	0.345
INS	Pearson Correlation (n)				-0.105 (92)	0.0154 (92)	0.057 (73)	0.2968 (17)	**0.230 (79)**	-0.173 (80)	**-0.330 (79)**	0.0484 (79)	**0.240 (79)**	0.104 (76)
	p				0.319	0.884	0.632	0.247	**0.041**	0.124	**0.003**	0.672	**0.033**	0.372
Urea	Pearson Correlation (n)					**0.331 (5616)**	**-0.161 (3714)**	**0.111 (981)**	**0.044 (3485)**	**-0.050 (3518)**	**-0.058 (3359)**	-0.012 (3355)	**0.038 (3348)**	-0.018 (3158)
	p					**0.001**	**0.001**	**0.001**	**0.001**	**0.003**	**0.001**	0.504	**0.028**	0.303
Creatinine	Pearson Correlation (n)						**-0.221 (3774)**	**0.135 (998)**	**0.147 (3514)**	**-0.074 (3548)**	**-0.147 (3389)**	0.0024 (3385)	**0.133 (3378)**	0.025 (3238)
	p						**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	0.889	**0.001**	0.160
Albumin	Pearson Correlation (n)							**-0.503 (631)**	**-0.055 (2357)**	**0.225 (2379)**	**0.252 (2282)**	**0.054 (2282)**	**-0.106 (2277)**	0.018 (2232)
	p							**0.001**	**0.008**	**0.001**	**0.001**	**0.010**	**0.001**	0.408
CRP	Pearson Correlation (n)								**0.256 (667)**	-0.043 (672)	**-0.121 (626)**	-0.001 (625)	**0.163 (624)**	-0.027 (661)
	p								**0.001**	0.265	**0.003**	0.984	**0.001**	0.493
Trig	Pearson Correlation (n)									**0.233 (3686)**	**-0.389 (3565)**	-0.016 (3557)	**0.612 (3556)**	**0.072 (2294)**
	p									**0.001**	**0.001**	0.347	**0.001**	**0.001**
Chol	Pearson Correlation (n)										**0.389 (3561)**	**0.109 (3553)**	**0.378 (3561)**	**0.089 (2314)**
	p										**0.001**	**0.001**	**0.001**	**0.001**
HDL	Pearson Correlation (n)											0.015 (3565)	**-0.648 (3561)**	0.018 (2210)
	p											0.376	**0.001**	0.410
LDL	Pearson Correlation (n)												**0.051 (3553)**	0.022 (2206)
	p												**0.002**	0.304
Total/HDL Chol	Pearson Correlation (n)													**0.066 (2200)**
	p													**0.002**

and impaired renal function. It is of interest to note that borderline glucose levels (>100 mg/dL) were not associated with a probability of decreased 25-(OH)D concentration (OR 0.983, CI 0.793-1.218). Finally, a decreased albumin concentration (indicative of liver damage or inflammation) also predicted low levels of 25-(OH)D (see below and Figure 4B).

Total 25-(OH)D and inflammation

The levels of albumin correlated positively with 25-(OH)D. In order to decipher whether this was the result of inflammation (albumin reduction is an acute phase marker of inflammation), we examined the relationship between 25-(OH)D and albumin against the prototype biomarker of inflammation, C-reactive protein (CRP) (Figure 4B, Table 4). In our dataset, a CRP value was available in 1,148 subjects. A significant correlation of 25-(OH)D with albumin and CRP was evident ($p<0.001$ for both). These data were also found in our logistic regression analysis, with odds ratios 0.090 (95% CI 0.031-0.260) and 0.697 (95% CI 0.451-1.076) for CRP**,** indicating a probability of

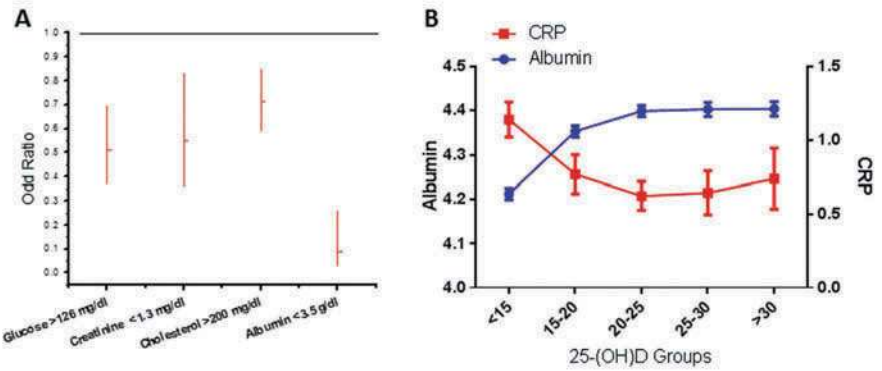

Figure 4. A. *Results of binary logistic regression between 25-(OH)D high/low levels (cut-off set at 20 ng/mL) and all biological parameters included in our study.* For all parameters low/normal/high coding was used, based on cut-off values presented in Table 2. Backward stepwise elimination of parameters (p to exclude= 0.10) was performed, and only significant parameters are shown (as Odd Ratios and 95% confidence intervals). **B.** *Correlation of 25-(OH)D levels and indices of inflammation.* Figure depicts the variation of albumin (blue curve) and CRP (red curve) as a function of 25-(OH)D groups, presented in the ordinate (mean±SE).

decreased 25-(OH)D when albumin levels were <3.5 g/dL and CRP levels were >0.8 mg/dL, confirming the association of decreased 25-(OH)D with inflammation. Notably, in a univariate analysis (with gender, age and season as confounders), a significant ($p<0.05$) correlation of 25-(OH)D with normal CRP variation (cut-off <0.8) was also evidenced.

DISCUSSION

In the present study, we performed a cross-sectional analysis of 8,042 single measurements of serum total 25-(OH)D over a 5-year period on the island of Crete. We investigated the effects of seasonal fluctuation, age and gender on circulating levels of 25-(OH)D and attempted to establish correlations with a number of biochemical indices of inflammation, metabolic syndrome and diabetes, as well as with PTH and several biomarkers of bone turnover. Furthermore, as no dietary products are fortified with calcium and VitD in Greece (with the exception of infants' and children's milk) and 66% of our subjects were outpatients, our data represent a valid estimate of the general population of a large Mediterranean island adhering, in principle, to the traditional Mediterranean diet.

The globally reported seasonal fluctuation of 25-(OH)D levels,[28,33-41] attributed to cutaneous synthesis of VitD being affected by geographical location and season,[2,26,27,42] was also observed in our cohort. Consequently, given this universally observed seasonal variation of VitD levels, caution should be exercised when single "snapshot" measurements of 25-(OH)D form the basis for recommendations on VitD supplementation and associations with various health outcomes.[43] Furthermore, in agreement with other large population-based studies,[44-46] our data revealed a linear decline of circulating 25-(OH)D levels with advancing age. The highest values, observed in children and adolescents of both genders, probably reflect VitD and calcium-fortified milk consumption in the 0-20 years age category. The progressive decrease thereafter may be explained by either avoidance of exposure to direct sunlight (or use of sunscreens) with advancing age because of skin cancer concerns, or drastic decrease of mobility due to orthopedic or other health problems and the well-documented reduction of the ability of the ageing skin to synthesize VitD precursors,[11,27] coupled with dietary habits that seldom include VitD-rich foods and the absence of fortified products in Greece. One should also be aware of the age-related PTH increase, together with the physiological loss of bone mass with age, which might confound correlations between VitD status and bone remodeling laboratory indices.[47-49] The reverse phenomenon has been reported in Finland where advancing age has been associated with higher 25-(OH)D values in both sexes up to the age of 80 and was attributed to an "active pensioner" outdoor lifestyle, increased consumption of fatty fish, as well as dairy product fortification and VitD supplement use.[20,50]

Consistent with previous studies,[20,38,40,51-54] 25-(OH)D levels in females were found to be lower compared to males, up to the age of 50 years; thereafter the levels of 25-(OH)D were not different from those of males. A plausible explanation might include VitD and calcium supplementation in post-menopausal women together with changes in circulating estrogen levels, as well as lifestyle factors not addressed in this study (e.g. differences in sun exposure, diet and BMI between sexes).[52,55] Indeed, 25-(OH)D has been significantly and positively associated with total testosterone and SHBG levels before and after adjustment for age and ethnicity,[56] although VitD supplementation *per se* does not appear to influence testosterone levels.[57] In addition, short-term administration of testosterone does not appear to alter 25-(OH)D levels.[58]

The most intriguing finding of our study was the suboptimal 25-(OH)D values observed in both genders (males=19.48±9.51 and females=18.01±9.01, ng/mL±SD), contrary to what would be expected in an area with abundant sunshine and a population prone to outdoor activities. This is in line with other studies indicating that 25-(OH)D levels in Mediterranean countries,[12,55,59-64] and in Greece specifically,[28,64] are often surprisingly low, with reported levels as low as 9.4 ng/mL in males and 8.5 ng/mL in females in autumn.[65] Our results are also in agreement with published reports suggesting that insolation in a certain region may not be the sole, or even an accurate, predictor of the VitD status.[28,59,66,67] Along the same lines, an unexpected inverse north-south gradient for VitD levels in Europe is often reported,[41,59] with 25-(OH)D levels positively correlated with latitude, and is primarily attributed to dietary habits (higher

consumption of VitD-rich foods in the north), supplementation (Greece presented the lowest mean supplement intake, 2% in men and 6.7% in women),[68] and differences in the population's skin pigmentation.[55] The opposite has also been observed in the "SUpplémentation en VItamines et Minéraux AntioXydants" study (SUVIMAX, 1994) in 35-65 year-old French adults, with reported mean serum levels of 17.2 ng/mL in the north versus 37.6 ng/mL in the south.[12] The above discrepancies could be attributed to differences in methodologies employed, populations tested, season of sample collection and, certainly, nutritional, anthropometric, sociodemographic and genetic factors, known to influence VitD status.[52,55]

The well-documented inverse relationship between PTH and 25-(OH)D[16,31] was also evident in our dataset and the intersection point, where PTH levels normalized, was at approximately 20 ng/mL of 25-(OH)D in both sexes. Of course, low levels of VitD cause secondary hyperparathyroidism, which preserves normocalcemia at the expense of bone health;[69] on the other hand, the 25-(OH)D concentration below which high levels of PTH ensue is not well established and a widely discrepant range (10-49 ng/mL) has been reported.[52,70] It is worthy of note that other studies, one of them from Greece,[28] using a VitD loading test and the locally weighed regression scatterplot smoothing (LOWESS) method between PTH and 25-(OH)D, concluded that the lower normal levels for 25-(OH)D in their population were 22[28] and 20 ng/mL,[71] comparable with those reported in the present study. The interrelationship between PTH and VitD levels is always interwoven with calcium intake and absorption, parameters that are difficult to measure in everyday clinical practice.[29,52] Therefore, while the major biological effect of PTH in our cohort was manifested at 25-(OH)D levels below 20 ng/mL, we recognize that potentially harmful non-skeletal effects at 25-(OH)D concentrations between 20-30 ng/mL cannot be excluded, albeit below the sensitivity of bone metabolic indices, which always varied within their normal range.

We further observed weak significant negative correlations with two biochemical indices of bone metabolism, osteocalcin (bone turnover) and CTX (bone resorption), by applying the 25-(OH)D level corresponding to PTH normalization in our cohort (20 ng/mL) as a cut-off. These correlations were maintained in multivariate analysis, with age and sex as confounders. Our results are in agreement with those reported in the Longitudinal Aging Study Amsterdam (LASA), that serum osteocalcin and urinary deoxypyridinoline, another resorption marker, decreased with increasing serum 25-(OH)D up to 16 ng/mL. Other groups have also observed that markers of bone resorption show the biggest rise when 25-(OH)D levels fall below 20 ng/mL[41,72-74] and acceleration of bone turnover due to secondary hyperparathyroidism has been reported at 25-(OH)D levels of less than 6 ng/mL.[17] The documented inverse relationship between bone-specific alkaline phosphatase and 25-(OH)D levels[33,41,73,75] was also evident in our cohort, although variations were strictly within the normal reference range. It is noteworthy that significant changes in biomarkers of bone turnover are not always evident, despite an observed increase in 25-(OH)D levels and concomitant decrease in PTH levels when administering supplementation with oral VitD.[76,77]

As has been reported by others,[78,79] a negative correlation between 25-(OH)D levels and glucose, triglycerides and total cholesterol was apparent in our population, confirming that higher serum concentrations of 25-(OH)D tend to be associated with a more favorable lipid profile. Literature reports on the association between VitD and total cholesterol are often discrepant, even when extensive adjustments for confounding factors (particularly BMI) have been applied,[45,80,81] while the association between VitD and triglycerides is consistently reported as negative,[82] with two exceptions concerning adolescent girls.[83,84] Interestingly, no significant correlation between 25-(OH)D levels and HDL cholesterol was observed in our cohort, consistent with existing literature.[79,85] However, this element is debated in the majority of cross-sectional studies,[82,86] while interventional studies demonstrated minor effects of VitD supplementation on lipid variables,[82,87] particularly on serum HDL.[88] It is of interest that the total/HDL cholesterol atherosclerotic ratio (normally <5)[32] was negatively correlated with 25-(OH)D concentrations, further confirming the protective effects of VitD on vascular health.[82,89,90]

Another finding, which further corroborated the in-

verse correlation of 25-(OH)D with metabolic disease/diabetes in our analysis, was the negative association between 25-(OH)D levels and HbA1c values. This inverse association is supported by other published reports[20,91-93] and the seasonal variation of 25-(OH)D levels is inversely mirrored by HbA1c, with the lowest levels observed in the summer.[94] Indeed, low levels of 25-(OH)D are considered a risk factor for the development of insulin resistance and diabetes,[4,95] although the preventive effect of VitD supplementation has not yet been fully established.[96-98] One should bear in mind that although low levels of 25-(OH)D have been linked to the metabolic syndrome, obesity, diabetes, myocardial infarction and other cardiovascular diseases and despite the fact that overall cardiovascular death and manifestations of cardiovascular events are more frequent in the winter, when 25-(OH)D levels are at their lowest, associations have not been proven to be causal.[78,94,99-103]

No correlation was evident between TSH levels and 25-(OH)D groups in our population, in spite of reported associations between 25-(OH)D and thyroid function.[104]

Decreased levels of 25-(OH)D are a common finding in patients with impaired kidney function,[105,106] which was also obvious in our dataset. Chronic kidney disease (CKD) is characterized by secondary hyperparathyroidism, which progresses with gradual deterioration of renal function and is partially attributed to the derangement of VitD metabolism.[107] VitD has an expanded role in renal physiology, through both classical and non-classical actions, and appears to affect disease progression and mortality in CKD patients.[108,109]

The inverse association between VitD status and inflammation has been described in published reports[110,111] and was also evident in our population, through correlations of 25-(OH)D with albumin and CRP levels, even within the normal reference range for CRP. The immunomodulatory and anti-inflammatory properties of VitD are among its earliest and best described non-classical actions.[112-117] Indeed, the acute-phase response has been linked to an early and sharp decrease in circulating 25-(OH)D levels, which correlates with inverse changes in CRP and persists even after resolution of the inflammatory reaction.[110,118,119] Practically all studies evaluating the association between 25-(OH)D levels and acute-phase response have reported a concomitant decrease in serum albumin.[110,111] This observed decline in serum 25-(OH)D concentration has been attributed to several mechanisms, including decreased levels of carrier proteins, increased conversion of 25-(OH)D to its active 1,25 form and acute fluid shift.[111] Inflammation-related hypovitaminosis D is difficult to interpret since the causality/consequence is unclear and both the biological implications and clinical relevance of this correlation are presently not known. A recent hypothesis proposes that low VitD levels (coupled with increased 1,25-(OH)2D concentrations) are a consequence rather than the cause of chronic inflammation, provoked by occult intracellular infection.[120,121] Finally, the association of decreased 25-(OH)D levels with the various components of the metabolic syndrome and, as a result, low-grade metabolic inflammation could be yet another confounder,[20,122] further supporting the proposed anti-inflammatory actions of VitD.[123]

Several variables are known to affect VitD status in a given population, including sun exposure, use of sunscreens, nutritional habits, anthropometric indices and lifestyle features, such as outdoor activity, smoking and alcohol consumption. There are no cultural restrictions, as in the Arab world for females, on sun exposure or excessive clothing in Crete, and moderate sun exposure through daily-life activities appears to adequately improve VitD status.[67] Food sources rich in VitD are not routinely consumed in Greece, fortified products are absent (with the exception of infants' and children's milk) and vitamin supplementation is uncommon. On the other hand, adequate calcium intake protects from the development of hyperparathyroidism when VitD status is suboptimal and in this situation low 25-(OH)D levels are associated with lower than expected PTH levels.[16,124] We can assume that calcium intake is adequate in our population due to high availability and consumption of dairy products.[125,126] Regarding adiposity indices, a negative association between levels of 25-(OH)D and overweight/obesity is well established[44,67,127-131] and more pronounced with percentage of body fat rather than body mass index (BMI). Underweight, though not as extensively explored, may also be negatively related to VitD status.[51,67] In overweight/

obese subjects, the upper PTH value has also been reported to be higher than in lean individuals[124] and obesity may also possibly blunt 25-(OH)D seasonal variation.[132] Physical activity per se, and not as a surrogate marker for sun exposure, may also be linked to higher VitD status.[67,133] Smoking is inversely related to 25-(OH)D levels in most studies,[134,135] although no association has also been reported.[136,137] Smoking is also associated with lower PTH levels,[138] blunted 1,25-(OH)2D-PTH axis and impairment of calcium absorption.[139] Excessive alcohol consumption is linked to lower 25-(OH)D concentrations,[140] but moderate intake is consistently reported to relate to better VitD status.[30,67,131,141,142]

Strengths of our study include the large number of subjects for whom 25-(OH)D measurements were available and the wide age distribution, which lead us to consider our cohort as being representative of the general population of a Mediterranean island. The inherent limitations of our work lie in its retrospective design and the mixed population studied (in- and outpatients), although no significant differences between the two groups were found. In addition, since inpatients represented various hospital departments, our data are not skewed by the results of a certain in-hospital unit and do not promote any given pathology. All data were extracted from the Hospital LIS system and as a consequence we cannot provide information on additional parameters influencing 25-(OH)D circulating levels, as detailed above, or on the medical history of our subjects. The demographic group of our study was primarily composed of women (79%), which could account for lower VitD status.[20,38,40,51-53] However, 25-(OH)D levels were below 20 ng/mL for both sexes and no sex-related differences in PTH concentrations were observed.

It is worth mentioning that even when most of the determinants known to influence VitD status are taken into account, the individual variability of 25-(OH)D levels is difficult to explain.[137] The genetic component is emerging as a strong predictor, as polymorphisms within various genes involved in the metabolic pathway of VitD (VitD-binding protein, megalin, cubilin, CYP27B1, CYP24A1, VDR) may explain not only a substantial percentage of the interindividual variability of 25-(OH)D, but also its association with major clinical outcomes[5,18,67,143,144]

Several recently published reports challenge the established "normal" cut-off point for 25-(OH)D sufficiency.[19,21] According to the NHANES 2005-2006 findings, the mean 25-(OH)D concentration is approximately 24 ng/mL in most age groups. Furthermore, the US Institute of Medicine (IOM) in 2011 concluded that serum 25-(OH)D levels >20 ng/mL are sufficient for almost the entire (97.5%) population, at least as regards Caucasians.[15]

In conclusion, our data lead us to adopt the Institute of Medicine's 2011 guidelines regarding the normal reference range for serum total 25-(OH)D and to propose setting the normal low level for 25-(OH)D to indicate sufficiency at 20 ng/mL, at least for the population of Crete. In addition, our study also raises questions as to the use of "snapshot" single-measurements of 25-(OH)D levels when attempting to demonstrate health risks associated with VitD deficiency and their outcomes, consistent with the recommendation of Anthony Norman that "normal 25-(OH)D serum levels" are those that support all VDR-containing target organs in all the world's population groups.[145]

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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