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Vitamin D substrate–product relationship in idiopathic hypercalciuria

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ABSTRACT

Absorptive hypercalciuria (AH) is associated with elevated levels of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). While no increase of $1,25(OH)_2D$ after oral administration of 25-hydroxyvitamin D (25OHD) at high doses has been claimed in normal subjects, a substrate–product relationship has been reported in normal children, young people after UV irradiation, older persons, postmenopausal women, primary hyperparathyroidism, renal failure, osteomalacia, and sarcoidosis. No data of this relationship in AH is available. To investigate 25OHD-$1,25(OH)_2D$ substrate–product relationship in AH, 161 AH patients (mean age 60.9 ± 11.7 years) and 110 age- and sex-matched controls (mean age 61.5 ± 12.4 years) were studied. In 57 controls and 52 AH subjects 25OHD-$1,25(OH)_2D$ relationship in basal conditions and after 2-week oral 25OHD (25 μg/day) administration were evaluated. In basal conditions 25OHD and $1,25(OH)_2D$ were correlated in both, controls and AH; 25OHD treatment was followed by an increase in serum 25OHD and $1,25(OH)_2D$ in both groups. However, delta responses of 25OHD and $1,25(OH)_2D$ to 25OHD were higher in AH suggesting an enhanced activity of 1α-hydroxylase. In conclusion, the higher response of $1,25(OH)_2D$ after oral 25OHD in AH patients suggests a differential capacity between both groups in handling the increases in $1,25(OH)_2D$.

1. Introduction

Cholecalciferol (Vitamin D3) and ergocalciferol (Vitamin D2) are the precursors of 25-hydroxyvitamin D (25OHD), which is considered a good marker of vitamin D intake and the precursor of the double hydroxylated active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). When a normal amount of 25OHD is present in the blood, a large increase of this metabolite as achieved by high doses of exogenous 25OHD, is thought not to influence circulating $1,25(OH)_2D$. This has been shown in experimental animals [1,2] and in small numbers of young subjects [3,4], the explanation being that elevated levels of 25OHD increase the metabolic clearance rate of $1,25(OH)_2D$ [2]. Conversely, there are several reports of a significant response of $1,25(OH)_2D$ to 25OHD in normal children [5], postmenopausal women [6,7], normal aging [8], and normal adults who have undergone ultraviolet radiation [9]. In addition, several clinical situations have been associated to a similar positive substrate–product relationship i.e. primary hyperparathyroidism [4], vitamin D deficiency [10], sarcoidosis [11], renal failure [12], and pulmonary tuberculosis [13].

Vitamin D metabolism is strictly controlled in that a reciprocal feedback exists between 25OHD and $1,25(OH)_2D$. Chronic administration of $1,25(OH)_2D$ increases the metabolic clearance rate of 25OHD [14,15] or decreases its production [16] with consequent depletion of 25OHD stores. On the other hand, the administration of elevated doses of vitamin D or 25OHD increases the metabolic clearance rate of $1,25(OH)_2D$ probably in order to adjust the circulating levels to those required for calcium homeostasis [2]. This could be the explanation of the failure to observe elevations in $1,25(OH)_2D$ after high doses of 25OHD [3,4].

We evaluated the relationship between 25OHD and $1,25(OH)_2D$ in otherwise healthy subjects, assessed during wintertime. In these conditions, the administration of 25 μg/day of 25OHD stimulated an increase in 25OHD within the normal range (below 120 ng/ml) and a rise in $1,25(OH)_2D$. We also evaluated the same relationship, i.e. substrate–product relationship of 25OHD-$1,25(OH)_2D$ in subjects with absorptive hypercalciuria (AH), in whom some authors have reported increased $1,25(OH)_2D$ levels [1,17–22], suggesting that this increase, through a positive feedback on vitamin

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D receptors [23,24], may help to explain the enhanced calcium intestinal absorption.

2. Methods

All the subjects signed informed consents and the Institutional Review Board approved the study that was conducted in accordance with the Declaration of Helsinki guidelines. Patients were randomly selected during wintertime from November 2005 to March 2006 from those attending our clinic in the context of an ongoing program of evaluation of bone and nutritional status of postmenopausal women in Northern Milan, Italy [25,26] and from men with urolithiasis referred to us for evaluation of related bone and metabolic diseases. One hundred and sixty one subjects with AH and renal stone disease (45–75 years old; mean ± SD age: 60.9 ± 11.7 years) were evaluated as well as 110 age and sex-matched controls (45–76 years old; mean ± SD: 61.5 ± 12.4 years). Clinical characteristics of the studied subjects are shown in Table 1. Control subjects had no evidence of kidney stones and there was no family history of kidney stones in their first- and second-degree relatives. Controls and patients had normal renal function, as evaluated by normal creatinine clearance, and none of them had renal tubular acidosis, as evaluated by the venous pH obtained for the determination of ionized calcium, from a blood sample taken with venipuncture without the use of a tourniquet. Urolithiasis was present in all AH subjects. Control and AH subjects had normal ionized calcium as well as normal intact parathyroid hormone (PTH) levels. Patients with AH recruited in the study had a calcium urinary excretion higher than 250 mg/day (6.25 mmol/day; normal values: 2.5–6.25 mmol/day) on a free diet and not taking diuretics. After one month of dietician-assisted calcium free diet they exhibited a reduction in daily calcium excretion (below 100 mg/day or 2.5 mmol/day) and a decrease in calcium/creatinine ratio in fasting 2-h urine (below 0.35 mmol Ca/mmol creatinine; normal values: 0.10–0.20 mmol Ca/mmol creatinine), fulfilling the conventional criteria for AH diagnosis [27]. Fifty-seven controls and 52 patients with AH, selected randomly, were also evaluated after two-week oral administration of 25 μg/day of 25OHD$_3$ (calcifediol, Didrogyl®, Bruno Farmaceutici, Milan, Italy) while on an unrestricted calcium diet.

2.1. Analytical methods

Blood and urinary calcium, phosphate, and creatinine were measured with the Technicon Autoanalyzer SMA-12/60; plasma ionized calcium was measured by StatProfile M Nova (Milan, Italy); serum immunoreactive 1-84 PTH was evaluated by Nichols kit (Nichols Institute, San Juan Capistrano, CA, USA); vitamin D metabolites were assessed by IDS gamma-B 25-hydroxy Vitamin D kit (Immunodiagnostic System Limited; Boldon, UK), intra-assay CV 6.9%, inter-assay CV 9%, normal range 30–120 ng/mL, and IDS gamma-B 1,25-dihydroxy Vitamin D kit (Immunodiagnostic System Limited, Boldon, UK), intra-assay CV 9.7%, inter-assay CV 12%, normal range 15–65 pg/mL). According to the manufacturer, 25OHD has a cross-reactivity in 1,25(OH)$_2$D assay of 0.001%. In order to verify the possible technical interference for 1,25(OH)$_2$D assay, we have spiked serum of subjects with 200 ng of 25OHD per 1 ml of serum obtaining 25OHD serum levels of ∼250 ng/ml. In these sera, no differences were observed in the levels of 1,25(OH)$_2$D. This was performed in 20 different sera before the study and every time the assay is run, we repeat the same procedure in 5 samples of sera.

Table 1
Clinical basal characteristics of all studied subjects.

	Controls	AH	P
n	110	161	
Age (years)	61.5 ± 12.4	60.9 ± 11.7	ns
Sex (F/M)	58/52	97/63	
PTH (pg/mL)	38.1 ± 5.0	38.2 ± 7.0	ns
Creatinine clearance (mg/min)	103.2 ± 32.0	102.4 ± 50.8	ns
25OHD (ng/mL)	21.9 ± 10.9	22.7 ± 10.4	ns
1,25(OH)$_2$D (pg/mL)	48.7 ± 19.3	64.4 ± 23.7	<0.01
Ca^{++} (mmol/L)	1.15 ± 0.03	1.17 ± 0.03	ns
P (mg/dL)	3.5 ± 0.08	3.1 ± 0.07	<0.01
Urinary Ca (mg/24 h)	110 ± 51	289 ± 86	<0.01
Urinary P (mg/24 h)	712 ± 60	870 ± 75	<0.01

2.2. Statistical analyses

Statistical analyses were performed using GraphPad 4 package version 4.0 (GraphPad Software, Inc. San Diego, CA); t-test (unpaired for the whole group analyses and paired for the treatment groups analyses) was utilized to compare differences in variables between the studied groups. Relationships between vitamin D metabolites before and after treatment were analyzed with linear regression using Pearson correlation coefficients. Comparisons between regression lines were performed using confidence intervals [28]. A p value lower than 0.05 was considered to be statistically significant.

3. Results

Data is presented as means ± SD. In basal conditions, levels of 25OHD in the whole groups of AH and control subjects were low normal (Table 1). There were 34 (59.6%) subjects with 25OHD circulating levels below 20 ng/ml among controls, and 32 (61.5%), among AH patients. The circulating levels of 1,25(OH)$_2$D in basal conditions were significantly higher in AH subjects compared to control subjects (Table 1). When considering data obtained from the subgroups that received two-week 25OHD treatment (Table 2), circulating levels of 25OHD increased significantly in both controls and AH. The delta increase of 1,25(OH)$_2$D was, however, higher in AH (31.9 ± 35.2 pg/ml) as respect to controls (18.8 ± 18.6 pg/ml; $p < 0.05$).

There was a significant substrate–product relationship of 25OHD-1,25(OH)$_2$D in AH and in controls in basal conditions (Fig. 1). When considering only subjects who received treatment (Fig. 2), there were significant positive substrate–product relationships in both AH and controls subjects considering pre-treatment (Fig. 2, Panel A and B, respectively) and post-treatment values (Fig. 2, Panel C and D, respectively). Analyzing the delta changes of 25OHD versus the corresponding delta changes of 1,25(OH)$_2$D in AH (Fig. 3, Panel B) and controls (Fig. 3, Panel A), the slope of the 25OHD-1,25(OH)$_2$D relationship was higher in AH than in controls ($p < 0.01$), suggesting an increased availability of substrate and/or activity of renal 1α-hydroxylase in hypercalciuric subjects.

No correlation was found between ionized calcium and neither 25OHD nor 1,25(OH)$_2$D in AH and control subjects (data not shown), probably pointing to the complexity of calcium metabolism regulation, in which several other factors may be implicated.

In control subjects, 24-h urinary calcium increased from 98.6 ± 35.2 mg/day to 160.5 ± 25.2 mg/day ($p < 0.05$); ionized calcium did not change after treatment (1.14 ± 0.05 mmol/L vs. 1.14 ± 0.03 mmol/L; p = NS); blood phosphate decreased from 3.6 ± 0.09 mg/dL to 3.0 ± 0.09 mg/dL ($p < 0.001$) and 24-h urinary phosphate increased from 504.0 ± 125.3 mg/day to 646.4 ± 157.1 mg/day ($p < 0.05$). In AH subjects, 24-h urinary cal-

Table 2
Clinical characteristics of control and AH subjects before and after 25OHD treatment (25 μg/day for two weeks).

	Controls	AH	P
n	57	52	
25OHD (ng/mL; before treatment)	21.3 ± 10.9	21.5 ± 12.5	ns
25OHD (ng/mL; after treatment)	40.05 ± 19.5***	47.1 ± 22.9***	ns
$1,25(OH)_2D$ (pg/mL; before treatment)	49.0 ± 19.4	57.3 ± 25.4	<0.05
$1,25(OH)_2D$ (pg/mL; after treatment)	66.9 ± 21.9***	89.2 ± 33.5***	<0.001
Ca^{++} (mmol/L; before treatment)	1.14 ± 0.05	1.15 ± 0.05	ns
Ca^{++} (mmol/L; after treatment)*	1.14 ± 0.03	1.12 ± 0.07	ns
P (mg/dL; before treatment)	3.6 ± 0.09	3.1 ± 0.09	<0.001
P (mg/dL; after treatment)	3.0 ± 0.09**	3.1 ± 0.07	<0.001
Urinary Ca (mg/24 h; before treatment)	98.6 ± 35.2	266.8 ± 70.7	<0.001
Urinary Ca (mg/24 h; after treatment)	160.5 ± 25.2*	271.9 ± 45.0	<0.001
Urinary P (mg/24 h; before treatment)	504.7 ± 125.3	797.5 ± 158.7	<0.001
Urinary P (mg/24 h; after treatment)	646.4 ± 157.1*	625.4 ± 190.2	ns
PTH (pg/ml; before treatment)	32.5 ± 18.4	33.3 ± 15.7	ns
PTH (pg/ml; after treatment)	36.9 ± 16.2	39.5 ± 18.3	ns

* Means $p < 0.05$ vs. before treatment.
** Means $p < 0.001$ vs. before treatment.
*** Means $p < 0.0001$ before treatment.

cium (266.8 ± 70.7 mg/day vs. 271.9 ± 45.0 mg/day; p = NS), ionized calcium (1.15 ± 0.05 mmol/L vs. 1.12 ± 0.07 mmol/L; p = NS), blood phosphate (3.1 ± 0.09 mg/dL vs. 3.1 ± 0.07 mg/dL; p = NS), and urinary phosphate (797.5 ± 158.7 mg/day vs. 625.4 ± 190.2 mg/day; p = NS), were similar before and after treatment (Table 2).

PTH values did not significantly change after treatment either in AH subjects or in controls (controls: n = 57, baseline: 32.5 ± 18.4 pg/ml; after 25OHDtreatment: 36.9 ± 16.2 pg/ml, p = NS; AH: n = 52, baseline: 33.3 ± 15.7 pg/ml; after 25OHD treatment: 39.5 ± 18.3 pg/ml, p = NS). PTH values were not significantly different between AH and controls, either before or after 25OHD oral treatment (Table 2).

4. Discussion

The novel findings of the present study are two: i) exploring the substrate–product of 25OHD-$1,25(OH)_2D$ in basal condition in a large number of controls and AH, achieving 25OHD circulating concentrations within the normal range, there is a significant positive relationship; ii) this relationship differs in AH in that a higher level of $1,25(OH)_2D$ is found for any correspondent level of 25OHD when patients and controls were treated with 25 μg/day of 25OHD.

Some authors have reported a lack of significant substrate–product relationship for 25OHD and $1,25(OH)_2D$ in experimental animals [1,2]. It is noteworthy that in all of these studies high doses of 25OHD were used, which may have induced an elevation of the metabolic clearance rate of $1,25(OH)_2D$ to its final products [2]. However, when more physiological doses of 25OHD were administered to rats [2] the resulting $1,25(OH)_2D$ concentrations in serum correlated with 25OHD demonstrating a close substrate–product relationship for these vitamin D metabolites. Similar results have been reported in human subjects using elevated doses of 25OHD (above 50 μg/day) achieving abnormally elevated circulating levels of 25OHD [3,4]. Conversely, when 25OHD serum levels were increased but still remaining within normal range by ultraviolet administration in normal subjects [9] or by giving a more "physiological" dose [5,7,8], a correspondent increase of $1,25(OH)_2D$ has been reported. The direct substrate–product relationship between 25OHD and $1,25(OH)_2D$ was not statistically evident in these studies probably because they included a low number of subjects. Exploring this relationship maintaining "normal" levels for both metabolites in a large population of normal and AH subjects we observed a significant positive relationship, with similar results as those reported by Need et al. [6] in 262 postmenopausal women. A recent study [29] reports a direct relationship between 25OHD and $1,25(OH)_2D$ in 560 Norwegians, men and women (age 45–60) and in 161 immigrants from Pakistan. Interestingly, the slope of the relationship between 25OHD and $1,25(OH)_2D$ found in Norwegians was very similar to that observed in our population i.e. a 0.5 pg/ml increase of $1,25(OH)_2D$ per 1 ng/ml increase of 25OHD. The effect of 25OHD oral administration may be different in young compared to older people. In a careful work, Barger-Lux et al. reported that the administration of 10 μg of 25OHD was the only way to increase (even if mildly, by 11%) the levels of $1,25(OH)_2D$ in healthy men 20–37 years old [30]. On the other hand, in elderly people and in post menopausal women Lawoyin et al. [7] demonstrated that oral long-term administration of 20–30 μg of 25OHD was followed by an increase of about 90% of $1,25(OH)_2D$, an increase that may be

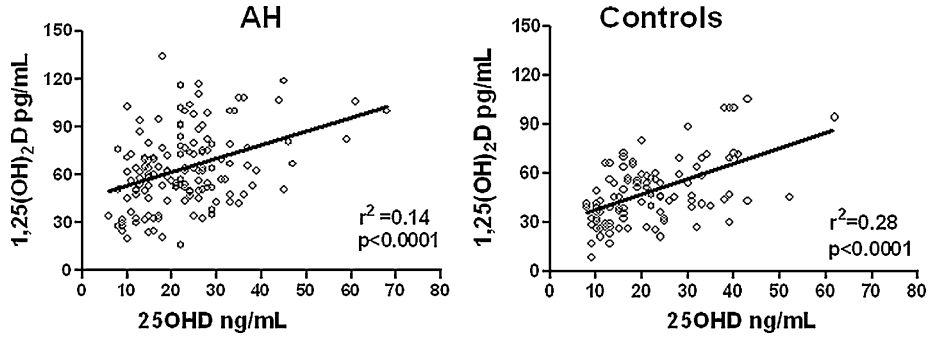

Fig. 1. Substrate–product relationship for 25OHD and $1,25(OH)_2D$ during winter-time in 161 patients with AH (Panel A) and in 110 normal controls (Panel B). To convert mg/dL in mmol/L multiply by 2.496 for 25OHD and by 2.4 for $1,25(OH)_2D$.

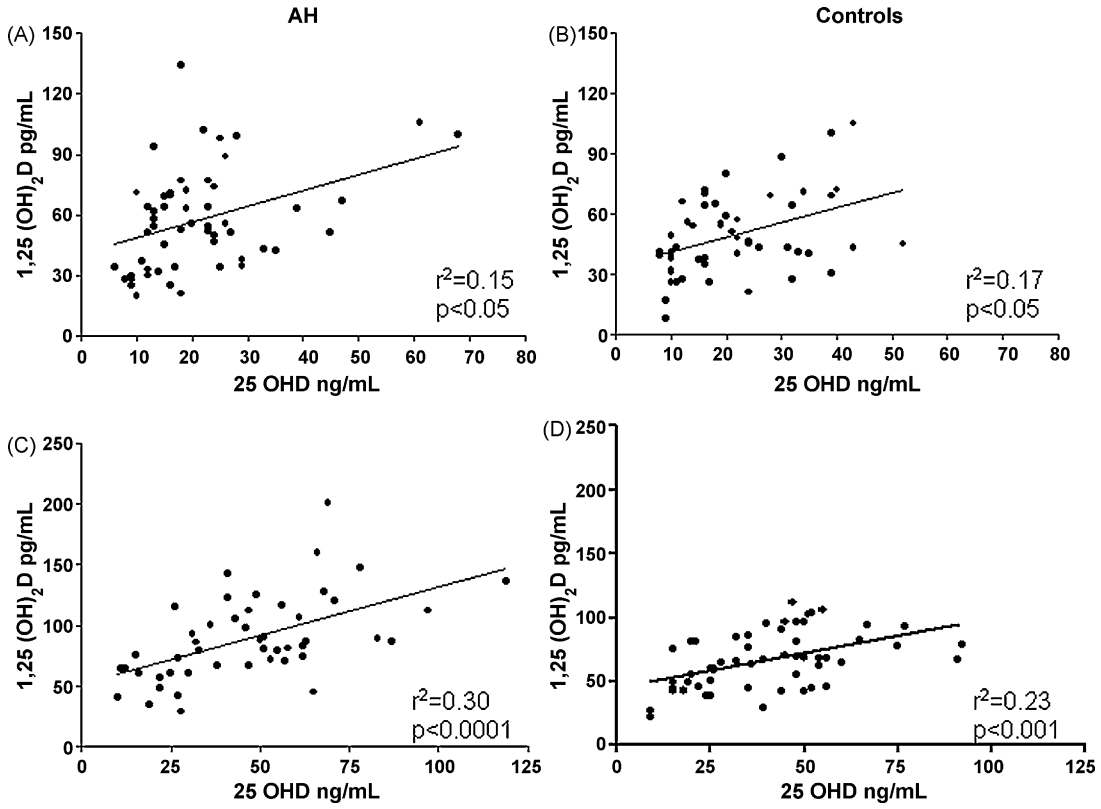

Fig. 2. Substrate–product relationship for 25OHD and 1,25(OH)$_2$D during winter-time before and after two-week administration of 25 μg/day of 25OHD in 52 patients with AH (Panel A and C) and 57 normal controls (Panel B and D). To convert mg/dL in mmol/L multiply by 2.496 for 25OHD and by 2.4 for 1,25(OH)$_2$D.

comparable to that observed in our patients (34% in controls and 56% in AH) after a less lasting treatment. Similarly, Francis et al. demonstrated an increased 1,25(OH)$_2$D after 25OHD administration in older subjects [8]. The fact that a relatively large number of subjects in our study had low levels of 25OHD at baseline in both groups may have enhanced 1,25(OH)$_2$D response after 25OHD oral treatment.

The elevation of 1,25(OH)$_2$D may entail pathogenic consequences. In fact, renal and intestinal vitamin D receptor (VDR) undergoes homologous up-regulation by 1,25(OH)$_2$D [23]. It is possible that elevated levels of 1,25(OH)$_2$D achieved during summer-time after sun exposure, in the presence of an adequate amount of substrate, may trigger an elevation of VDR levels that remain high during fall and winter, since VDR's half-life is prolonged upon 1,25(OH)$_2$D binding. This is in accordance with recent data showing that VDR levels are always increased in AH independently of seasonal variability of 1,25(OH)$_2$D [22,24]. From an evolutionary point of view, this may be a calcium preservation mechanism involving maximization of the vitamin D pathways during summer-time that would remain up-regulated for the following months, allowing to contrast the natural sun exposure decrease during fall- and winter-time with consequent decrease in 1,25(OH)$_2$D synthesis. In turn, the increased VDR concentrations [22,24] may stimulate the renal and extra renal activity of calcium-sensing receptor [31] that may explain the hypercalciuria and some other endocrine abnormalities in AH [26]. The seasonality of urinary calcium excretion and the increase of renal stones episodes during summer and fall have been studied by several authors with contrasting results, with some demonstrating the seasonality of renal stones [32,33] and others reporting negative findings [34,35].

The final cause of the increased levels of 1,25(OH)$_2$D in AH has not yet been completely elucidated. Our findings showing a similar slope of the substrate–product relationship between 25OHD and 1,25(OH)$_2$D in normal and AH subjects suggest that the affinity of 25OHD and 1α-hydroxylase is similar in both groups of subjects. However, the amount of 1α-hydroxylase and/or its activity may be increased in AH patients. The activity of renal 1α-hydroxylase is under a complex regulation by PTH, calcium, phosphate, and 1,25(OH)$_2$D itself [36]. P450 1α-hydroxylase has been cloned [37–39] and studies on the molecular mechanisms involved in this regulation have been demonstrated in that PTH and calcitonin administration, restriction of dietary calcium, and vitamin D deficiency increase P450 1-α mRNA. Conversely, administration of 1,25(OH)$_2$D induces a decrease in P450 1-α hydroxylase expression and prevents the increased expression induced by PTH and calcitonin [36]. In this regard, in our study an increased 1α-hydroxylase activity/amount in AH is not related to PTH since levels of PTH were found normal. Low levels of serum phosphate have been often implicated as the cause of the increased 1α-hydroxylase [40]. In the present study, levels of phosphate in AH were low-to-normal (Table 1) perhaps explaining the activation of 1α-hydroxylase. Higher levels of 1,25(OH)$_2$D in AH at all levels of 25OHD could be explained also by a lower rate of metabolic clearance of the metabolite in AH. However, the metabolic clearance rate of 1,25(OH)$_2$D has been studied in AH and was found normal. Thus, elevated levels of 1,25(OH)$_2$D in AH may be more probably related to an enhanced production of the metabolite [20].

When extra 25OHD is given to normal subjects, the increase of 1,25(OH)$_2$D and consequent rise in ionized calcium, lead to a decrease of the main regulator of 1-(hydroxylase, i.e. PTH. This is likely to occur in relatively long-term experiments (two-three months of 25OHD administration). In our control subjects, we observed a significant decrease of serum phosphate and an increase in urinary phosphate. Such changes were not observed in AH. The

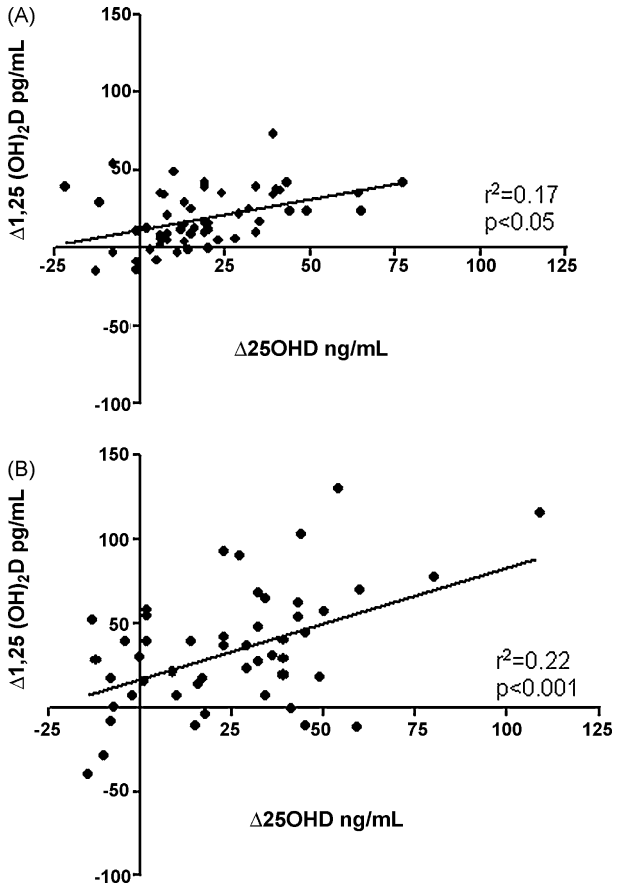

Fig. 3. Scatterplots describing the relations of changes in 25OHD and correspondent changes in 1,25$(OH)_2$D after two-week administration of 25 μg/day of 25OHD in 57 normal controls (Panel A) and 52 patients with AH (Panel B).

biochemical changes seen in 25OHD-treated controls in levels of serum/urinary phosphate/calcium are not dissimilar to, or less severe than, those seen in untreated AH subjects–i.e. the mechanisms responsible for these changes in controls may already have been saturated in AH, precluding further changes in response to 25OHD treatment in the AH population. In AH subjects, 1-(hydroxylase is not so tightly regulated as shown by the lack of 1,25$(OH)_2$D suppression by elevation of dietary calcium [19]. An inappropriate phosphate excretion in AH has been as well observed previously by others [41].

It is worth mentioning that the present is a short-term study, and lengthy studies are needed to clarify what is the response of 1,25$(OH)_2$D after 25OHD treatment in AH subjects after a longer period of time, since after 2 weeks it its probable that a new steady state is not yet established.

Limitations of our study may include the fact that there was a high number of mildly D deficient subjects and the responses we observed may have been altered by this deficiency. It is also noteworthy that the acute changes we observed may not represent chronic steady state levels.

In conclusion, the analyses of the substrate–product relationship between 25OHD and 1,25$(OH)_2$D maintaining normal levels of 25OHD disclosed a highly significant relationship in both controls and patients with AH. In AH, a higher level of 1,25$(OH)_2$D for each correspondent 25OHD level suggests an increase 1α-hydroxylase availability and/or activity. Further studies are needed to elucidate whether the increase in 1α-hydroxylase and consequent elevation of 1,25$(OH)_2$D levels may help to explain the elevation of VDR expression that seems to be a hallmark and a good explanation for the clinical findings in AH, such as hypercalciuria, increased calcium intestinal absorption, and perhaps bone demineralization [27].

Disclosure statement

The authors have nothing to disclose.

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