Nutritional rickets: vitamin D, calcium, and the genetic make-up

Mohamed El Kholy[1], Heba Elsedfy[1], Monica Fernández-Cancio[2], Rasha Tarif Hamza[1], Nermine Hussein Amr[1], Alaa Youssef Ahmed[1], Nadin Nabil Toaima[1] and Laura Audí[2]

BACKGROUND: The prevalence of vitamin D (vitD) deficiency presenting as rickets is increasing worldwide. Insufficient sun exposure, vitD administration, and/or calcium intake are the main causes. However, vitD system-related genes may also have a role.

METHODS: Prospective study: 109 rachitic children completed a 6-mo study period or until rachitic manifestations disappeared. Thirty children were selected as controls. Clinical and biochemical data were evaluated at baseline in patients and controls and biochemistry re-evaluated at radiological healing. Therapy was stratified in three different protocols. Fifty-four single-nucleotide polymorphisms (SNPs) of five vitD system genes (*VDR*, *CP2R1*, *CYP27B1*, *CYP24A1*, and *GC*) were genotyped and their association with clinical and biochemcial data was analyzed.

RESULTS: Therapy response was similar in terms of radiological healing although it was not so in terms of biochemical normalization. Only *VDR* gene (promoter, start-codon, and intronic genotypes) was rickets-associated in terms of serum 25-OH-D, calcium, radiological severity and time needed to heal. Eight patients with sufficient calcium intake and 25-OH-D levels carried a *VDR* genotype lacking minor allele homozygous genotypes at SNPs spread along the gene.

CONCLUSION: Although patients presented epidemiologic factors strongly contributing to rickets, genetic modulation affecting predisposition, severity, and clinical course is exerted, at least in part, by *VDR* gene polymorphic variation.

T he prevalence of vitamin D (vitD) deficiency presenting as rickets in children is increasing worldwide owing to insufficient vitD intake and lack of exposure to sunlight. However, considering that relatively few children with low serum 25-hydroxyvitamin D (25-OH-D) levels have symptoms, genetic factors may additionally be linked to the manifestation of vitD deficiency and rickets (1).

As natural food contains scant amounts of vitD, humans naturally obtain vitD through precursor photosynthesis of UVB-dependent vitamin D3. Sunlight exposure and skin pigmentation are therefore main determinants of circulating vitD levels (2) that can be increased through dietary vitD intake (3). Interindividual differences in aspects of the vitD endocrine system have been well documented. They could be caused by genetic differences in important proteins in the vitD endocrine system, such as VDR (4–6).

Multiple polymorphic variations exist in the *VDR* gene: 5'-promoter variations can affect mRNA expression patterns and levels while 3'-UTR sequence variations can affect mRNA stability and/or protein translation efficiency (7–10). Other genes encoding proteins in the vitD endocrine system (25-hydroxylase (*CP2R1*), 1-α-hydroxylase (*CYP27B1*), 24-hydroxylase (*CYP24A1*), and vitamin D binding globulin (*GC*)) also present polymorphic variations that could influence vitD synthesis efficiency and circulating 25-OH-D circulating levels (9).

It has long been speculated that genetic predisposition may influence the manifestations of vitD deficiency. Accordingly, in a prospective series of rickets patients, we attempted to analyze the role of genotypes of *VDR* and other genes related to the vitD system on rickets predisposition, severity, and response to therapy.

METHODS

Patients and Controls

Rachitic infants and children attending the Pediatric Outpatient Clinic, Ain Shams University, Cairo, Egypt (latitude 30.01N) were included. Participation was voluntary and written informed consent was obtained from parents or guardians. The study protocol was approved by the Ethics Committee of the University.

This was a prospective study: 168 consecutive rachitic patients presenting within a time frame of 12 mo were included (recruitment period included winter and summer, as the sun is shining all the year round in Egypt). Only 109 children either completed the 6-mo follow-up period or continued on treatment until their rachitic manifestations disappeared.

Inclusion criteria included clinical and radiological evidence of rickets and age from 7 mo to 3 y. Children were excluded if they had taken vitD or calcium supplements during the 6 mo prior to the study, if they had a history of kidney disease, tuberculosis, liver disease, prematurity, intestinal, cardiac, central nervous system, or other chronic disease. Bone disease (with the exclusion of rickets) or a history of hereditary forms of rickets together with weight <60% of the mean weight for height were also exclusion criteria.

Thirty age- and sex-matched children were selected as controls from siblings of nonrachitic patients attending the outpatient clinic or children with minor illnesses such as upper respiratory tract infections.

Data Collection

Nutritional data collection included: type of feeding, whether breast fed or not, duration of breast feeding, and weaning. Dietary calcium and caloric intake were calculated according to a food frequency questionnaire based on the pattern of diet in Egypt and compiled by the National Nutrition Institute (11). Calcium insufficiency was arbitrarily defined as an intake <50% of the recommended dietary allowance (RDI) (12). Parents were asked to report the whole dietary intake in detail on the preceding day on two separate occasions.

Nutritional status was determined according to the Wellcome Trust Classification (13).

Sunlight exposure evaluation included children's type of clothing and time spent outdoors every day. Sun exposure of 2 h per week on the face and hands was considered sufficient to maintain normal 25-OH-D concentrations (14). The amount of sun exposure was measured by calculating the total body surface area exposed to the sun while wearing usual clothes using the Lund and Browder charts (15). Exposure duration was calculated by finding the average time spent outdoors per week (in hours) during the period of direct sunlight (0900–1500 h), where sun exposure index = hours of sun exposure per week × fraction of body surface area exposed to sunlight (16).

Assessment of socioeconomic status was done according to the criteria of El-Bohy (17), based on: paternal profession, educational level, type of housing, family income, income source (regular or irregular).

Patient Classifications and Therapeutic Groups

Patients were stratified from the start in three groups according to adequacy of sun exposure and calcium intake. The aim was to determine whether by history alone, the cause of nutritional rickets could be established and the appropriate treatment chosen.

Group 1: Thirty patients with adequate sun exposure (sun exposure ≥2 h/week) and inadequate calcium intake (<50% RDI). They received calcium supplementation (calcium carbonate) alone (40 mg elemental calcium/kg/day (if less than 1 y old) and 50 mg/kg/d (if older than 1 y) divided into two daily doses, PO) for 6 mo or till healing occurred.

Group 2: Forty-one patients with inadequate sun exposure (sun exposure <2 h/week) and inadequate calcium intake (<50% RDI). These patients received vitD (vitamin D3) as Stoss therapy (600,000 units IM) once, plus calcium supplementation (40 mg elemental calcium/kg/day (if less than 1 y old) and 50 mg/kg/d (if older than 1 y) divided into two daily doses, PO) for 6 mo or till healing occurred.

Group 3: Thirty-eight patients with inadequate sun exposure (sun exposure <2 h/week) and adequate calcium intake (≥50% RDI). These patients received vitD as Stoss therapy (600,000 units IM) once, plus calcium supplementation (40 mg elemental calcium/kg/day (if less than 1 y old) and 50 mg/kg/d (if older than 1 y) divided into two daily doses, PO) for 2 wk.

A subgroup of eight patients from Group 3 was characterized as having adequate calcium intake and sufficient 25-OH-D levels (≥20 ng/ml) despite inadequate sun exposure. This subgroup was separately analyzed for genotype characteristics.

Auxology

Length or height and weight were measured and the SD scores (SDS) for length or height and for weight for length/height were calculated (18).

Biochemical Measurements

In cases and controls, serum calcium, phosphorus, and alkaline phosphatase (ALP) were measured photometrically using Hitachi 917 autoanalyzer and Roche reagents. In patients, they were determined at diagnosis, 2 wk after the start of treatment and then monthly till healing (an X-ray score of zero). ALP level was considered normal if ≤471 IU/l (according to levels in age-matched controls with 99.1% sensitivity and 100% specificity).

Serum 25-OH-D was measured by an enzyme-linked immuno-assay method (Immunodiagnostik AG, Bensheim, Germany). The detection limit of the assay was 2.24 ng/ml.

Intact PTH was assayed by enzyme-linked immunoassay using reagents supplied by Biosource (Biosource Europe SA, Nivelles, Belgium). Assay sensitivity was 2 pg/ml (range 15–1,040 pg/ml).

In patients, 25-OH-D and intact PTH were measured in baseline samples and again at the end of the study (X-ray score of zero). In controls, 25-OH-D and intact PTH were measured in baseline samples.

Radiological Evaluation

Assessment of rickets severity was made using standard X-rays of both wrists and knees. A 10-point scoring system was used (zero "normal") to a maximum score of 10 (19). Radiographs were examined by two independent observers and the score was taken as the mean of their scores. X-ray scores were determined at baseline, 2 wk after the start of treatment and then monthly till radiological healing. The criterion for healing was an X-ray score of zero and time to healing was calculated as months needed to achieve a null X-ray score.

Vitamin D System Gene Polymorphisms

Genotyping of 28 single-nucleotide polymorphisms (SNP) of *VDR* (Table 1), 4 SNPs of *CP2R1* (25-hydroxylase: rs10741657, rs12794714, rs10500804 and rs1993116), 1 SNP of *CYP27B1* (1-α-hydroxylase: rs4646536), 10 SNPs of *CYP24A1* (24-hydroxylase: rs4809960, rs2181874, rs6022994, rs6022993, rs912505, rs751089, rs1570670, rs927651, rs927650, and rs4809957), and 11 SNPs of *GC* (vitamin D binding globulin: rs222020, rs222016, rs1491719, rs4364228, rs4752, rs222035, rs7041, rs4588, rs2298850, rs3755967, and rs2282679), was performed using Applied Biosystems TaqMan and OpenArray technologies (Life Technologies, Carlsbad, CA).

Statistical Analysis

JMP 7.01 (SAS Institute, Cary, NC) was used for data entry and analysis. Numeric variables were expressed as mean ± SD when normally distributed and as median and interquartile range when nonparametric. Comparison of variables between groups was made using Student's *t*-test and Mann Whitney *U*-test for normal and nonparametric variables, respectively. Multiple subgroups were compared using Kruskall Wallis test. Chi-square (χ^2) test was used to compare the frequency of qualitative variables among the different groups. Spearman's correlation test was used to correlate nonparametric variables. Genotype frequencies were compared among groups by χ^2 Pearson test. In the entire rickets population, a stepwise regression analysis was applied to predict the contribution of *VDR* genotypes to baseline serum 25-OH-D and calcium levels, baseline X-ray score and time to radiologic healing, and ALP normalization. A *P* value < 0.05 was considered statistically significant for all tests.

RESULTS

Demographic and Biochemical Characteristics of the Three Groups

The demographic, anthropometric, and biochemical characteristics of patients (at diagnosis and after healing) and controls are shown in Table 2.

At diagnosis, in the whole rickets population, serum calcium correlated positively with height-SDS ($r^2 = 0.02$; $P = 0.03$), sun exposure ($r^2 = 0.13$; $P < 0.0001$), calcium intake ($r^2 = 0.11$; $P < 0.0001$) and serum phosphorus ($r^2 = 0.03$; $P = 0.01$), and negatively with serum PTH ($r^2 = -0.04$; $P = 0.008$) and ALP ($r^2 = -0.06$; $P = 0.001$); serum 25-OH-D correlated positively with sun exposure ($r^2 = 0.25$; $P < 0.0001$), calcium intake ($r^2 = 0.04$; $P = 0.008$) and serum phosphorus ($r^2 = 0.03$; $P = 0.0003$) and negatively with serum PTH ($r^2 = -0.13$; $P < 0.0001$) and ALP ($r^2 = -0.08$; $P = 0.03$); radiological score correlated positively with serum ALP ($r^2 = 0.11$; $P = 0.0002$).

Table 1. SNPs analyzed using TaqMan assays on OpenArray platform (Applied Biosystems)

Gene	TaqMan assay name	Number dbSNP	Nucleotides	Position	Gene location
VDR-1	C__27909097_10	rs4237856	G/T	48,338,050	Promoter
VDR-2	C__27909106_10	rs4073729	C/T	48,337,069	Promoter
VDR-3	C___2880808_10	rs11568820	G/A	48,302,545	Promoter (Cdx2)
VDR-4	C__27904554_10	rs4760658	T/C	48,296,486	Promoter (Intron 1a)
VDR-5	C___2880803_10	rs10783219	T/A	48,295,488	Promoter (Intron 1a)
VDR-6	C__44841112_10	rs11168293	C/A	48,293,716	Promoter (Intron 1a)
VDR-7	C__12060037_10	rs10875695	G/T	48,293,037	Promoter (Intron 1a)
VDR-8	C__12060038_10	rs7302235	A/G	48,292,838	Promoter (Intron 1a)
VDR-9	C__15823889_10	rs2853564	C/T	48,278,487	Promoter (Intron 1a)
VDR-10	C__12060044_1_	rs1989969	T/C	48,278,010	Promoter (Intron 1a)
VDR-11	C___3290655_1_	rs2238136	G/A	48,277,713	Promoter (Intron 1a)
VDR-12	C__12060045_20	rs2228570	T/C	48,272,895	Exon 2 (Met1Thr) [FokI]
VDR-13	C___3290647_10	rs3782905	C/G	48,266,167	Intron 2
VDR-14	C___3290638_1_	rs2238138	C/T	48,264,493	Intron 2
VDR-15	C__30742507_10	rs11574070	C/T	48,257,895	Intron 3
VDR-16	C___3290631_1_	rs2239180	G/C	48,256,046	Intron 3
VDR-17	C___3290630_1_	rs2248098	T/C	48,253,356	Intron 3
VDR-18	C__30742483_10	rs7305032	C/T	48,249,860	Intron 5
VDR-19	C__16174104_10	rs2239183	G/T	48,244,660	Intron 6
VDR-20	C__30742462_10	rs12314197	T/C	48,242,722	Intron 6
VDR-21	C__16031766_10	rs2525044	T/C	48,242,256	Intron 6
VDR-22	C___8716062_10	rs1544410	G/A	48,239,835	Intron 8 [BsmI]
VDR-23	C__30742443_10	rs11574114	G/A	48,238,883	Intron 8
VDR-24	C__28977635_10	rs7975232	G/T	48,238,837	Intron 8 [ApaI]
VDR-25	C___2404008_10	rs731236	T/C	48,238,757	Exon 9 (Ile352Ile) [TaqI]
VDR-26	C___2404007_10	rs739837	C/A	48,238,221	3'UTR
VDR-27	C___2404006_10	rs3847987	G/T	48,238,068	3'UTR
VDR-28	C__15823846_10	rs2853563	G/A	48,235,738	3'UTR

SNP positions are indicated according to Genome Build 37.1.
SNP, single-nucleotide polymorphisms.

The time elapsed to achieve an X-ray score of zero (healing) did not differ among the three therapeutic groups. At healing, serum calcium, phosphorus, and 25-OH-D were significantly higher and ALP significantly lower in groups 2 and 3 compared to group 1; PTH did not differ among groups (**Table 2**). However, time to ALP normalization differed significantly among groups, with group 1 needing the longest time (**Table 2**).

Vitamin D System Gene Frequencies
Only two *VDR* genotype frequencies showed statistically significant differences among patient and control groups: in group 1 compared to groups 2 and 3 and controls, major allele homozygous genotypes at VDR-18 (rs7305032) and VDR-21 (rs2525044) were increased and minor allele homozygous genotypes were absent (**Figure 1a,c**). VDR-18 and VDR-21 were in linkage disequilibrium (96.7%). Allele frequencies were significantly different (**Figure 1b,d**), with group 1 presenting increased frequency of the *T* and *C* alleles at VDR-18 and VDR-21, respectively, compared to groups 2 and 3 and controls.

Vitamin D System Genotype Association With Clinical Data
Only *VDR* genotypes showed several significant associations with clinical data.

In the entire rickets population, stepwise regression analysis between *VDR* genotypes and baseline 25-OH-D, calcium and X-ray score, and time to radiologic healing and ALP normalization showed that:

- Baseline 25-OH-D was significantly associated with genotypes at three *VDR* promoter SNPs (VDR-3, VDR-4, and VDR-9), explaining 29% of variation with the minor allele *AA* genotype at VDR-3 and the major allele *TT* genotypes at VDR-4 and VDR-9 presenting the lowest 25-OH-D levels (**Table 3**).

Table 2. Demographic and biochemical characteristics of the four groups

	Controls (*n* = 30)	Group 1 (*n* = 30)	Group 2 (*n* = 41)	Group 3 (*n* = 38)	*P*a
At diagnosis					
Age (years)	1.8 ± 0.8	1.5 ± 0.7	1.5 ± 0.6†	1.2 ± 0.6†,*	0.0007
Height-SDS	−0.08 ± 0.81	−2.15 ± 1.17†,*	−1.75 ± 1.51†	−1.18 ± 1.68†,*	<0.0001
Weight for height SDS	−0.2 (0.95)	0.345 (2.19)†,*	−0.3 (1.86)	−0.66 (1.62)*	0.03
Sun (hours/week)	11 (8.25)	3.4 (3.69)†,*	1 (1.3)†	0.75 (1.32)†	<0.0001
Sun exposure index	316 (279)	104 (178)†,*	23 (44.9)†	23.3 (30.4)†	<0.0001
Calcium intake (mg/d)	581 (128)	127 (69)†	149 (97)†	352 (180)†,*	<0.0001
RDIb (%)	118 (31)	30 (15)†	30 (24)†	90 (36)†,*	<0.0001
SEL (L/M/H) (%)	3/80/17	100/0/0†	90/10/0†	75/21/4†	< 0.0001
Serum calcium (mg/dl)	9.6 ± 0.4	8.1 ± 1.0†	8.1 ± 1.4†	8.3 ± 1.1†,*	<0.0001
Serum phosphorus (mg/dl)	4.2 ± 0.6	3.0 ± 1.0†	2.9 ± 0.9†	3.2 ± 1.1†	<0.0001
Serum ALP (IU/L)	371 (114)	961 (909)†	1,087 (625)†	1,081 (643)†	<0.0001
Serum 25-OH-D (ng/ml)	42.5 (12)	24.0 (12)†	20.0 (18)†	7.8 (6.5)†,*	<0.0001
Serum PTH (pg/ml)	29.5 (10.2)	145.0 (142.5)†	200.0 (115.0)†	175.0 (122.5)†	<0.0001
Radiologic score	–	8.0 (0.6)	8.0 (1)	8.0 (3)	0.5
At healing (X ray 0)					
Time to X ray 0 (months)	–	3.0 (1)	3.0 (2)	3.0 (2)	0.3
Time to normal ALP (months)	–	3.5 (3)*	3.0 (1)	2.0 (2)	0.0004
Serum calcium (mg/dl)	–	9.0 (0.5)*	9.5 (0.9)	9.7 (0.6)	0.001
Serum phosphorus (mg/dl)	–	4.6 ± 0.8*	5.3 ± 0.7	5.4 ± 0.6	<0.0001
Serum ALP (IU/l)	–	411 (96)*	327 (118)	371 (147)	0.05
Serum 25-OH- D (ng/ml)	–	32.0 (12.5)*	100.0 (66.0)	140.0 (72.5)	<0.0001
Serum PTH (pg/ml)	–	22 (20)	12 (25)	10 (22)	0.08

Mean ± SD for normally distributed and median (interquartile range) for nonparametric variables.
ALP, alkaline phosphatase; SEL, socioeconomic level: L = low, M = medium, H = high.
aKruskal-Wallis test among groups. bRDI = Recommended Dietary Intake. † significantly different from controls. * significantly different from other rickets groups.

- Baseline calcium was significantly associated with genotypes at eight *VDR* SNPs (two in the promoter region and six intronic), explaining 54% of variation (**Table 3**). VDR-9 and -10 (77.2%), VDR-18 and -21 (96.7%), and VDR-23 and -24 (57.1%) were in linkage disequilibrium. Homozygous minor allele combined genotypes at VDR-18 and VDR-21 were associated with the lowest calcium (*CCTT* = 6.5 ± 1.5 mg/dl; *n* = 6), the heterozygotes had intermediate concentrations (*TCTC* = 8.0 ± 1.1 mg/dl; *n* = 42) (*P* = 0.0026) and the major allele homozygous presented the highest values (*TTCC* = 8.4 ± 1.0 mg/dl; *n* = 56) (*P* = 0.0001).
- X-ray score was associated (37%) with genotypes at VDR-12 (rs2228570, start codon, *Fok1*) and VDR-18 (rs7305032, intron 5). The lowest scores were associated with the VDR-12 minor allele homozygous *TT* and the VDR-18 minor allele homozygous *CC* genotypes (**Table 3**).
- Time to radiologic healing was only associated (12%) with genotype at intron 8 VDR-24 SNP (rs7975232, *Apa1*), with the minor allele homozygous genotype *GG* showing the shortest time (**Table 3**).
- Time to ALP normalization was associated (22%) with genotypes at *VDR* promoter (VDR-7) and intron

8 (VDR-24, rs7975232, *Apa1*), with the minor allele homozygous genotype *GG* showing the shortest time (**Table 3**).

The subgroup of patients from Group 3 with sufficient calcium intake (>50% RDI) and 25-OH-D levels (≥20 ng/ml) consisted of eight patients. They only differed from all other patients by age (0.8 ± 0.3 vs. 1.4 ± 0.6 y; *P* = 0.002), calcium intake (128.4 ± 71.6 vs. 49.7 ± 40.2%RDI; *P* = 0.0002), and 25-OH-D serum levels (27.9 ± 5.9 vs. 19.1 ± 13.3; *P* = 0.019). This subgroup was genetically different from the rest because all lacked the minor allele homozygous genotypes at VDR-3 (*AA*), -4 (*CC*), -6 (*AA*), -9 (*CC*), -10 (*TT*), -12 (*TT*), -13 (*CC*), -14 (*TT*), -17 (*CC*), and -27 (*TT*).

DISCUSSION
The present study was designed to ascertain the epidemiological and genetic factors involved in rickets development and therapy response in young Egyptian children.

The therapeutic results in terms of time to healing according to the X-ray score were similar in the three treatment groups, indicating that elucidating the cause of rickets by history-taking and the subsequent treatment were similarly effective.
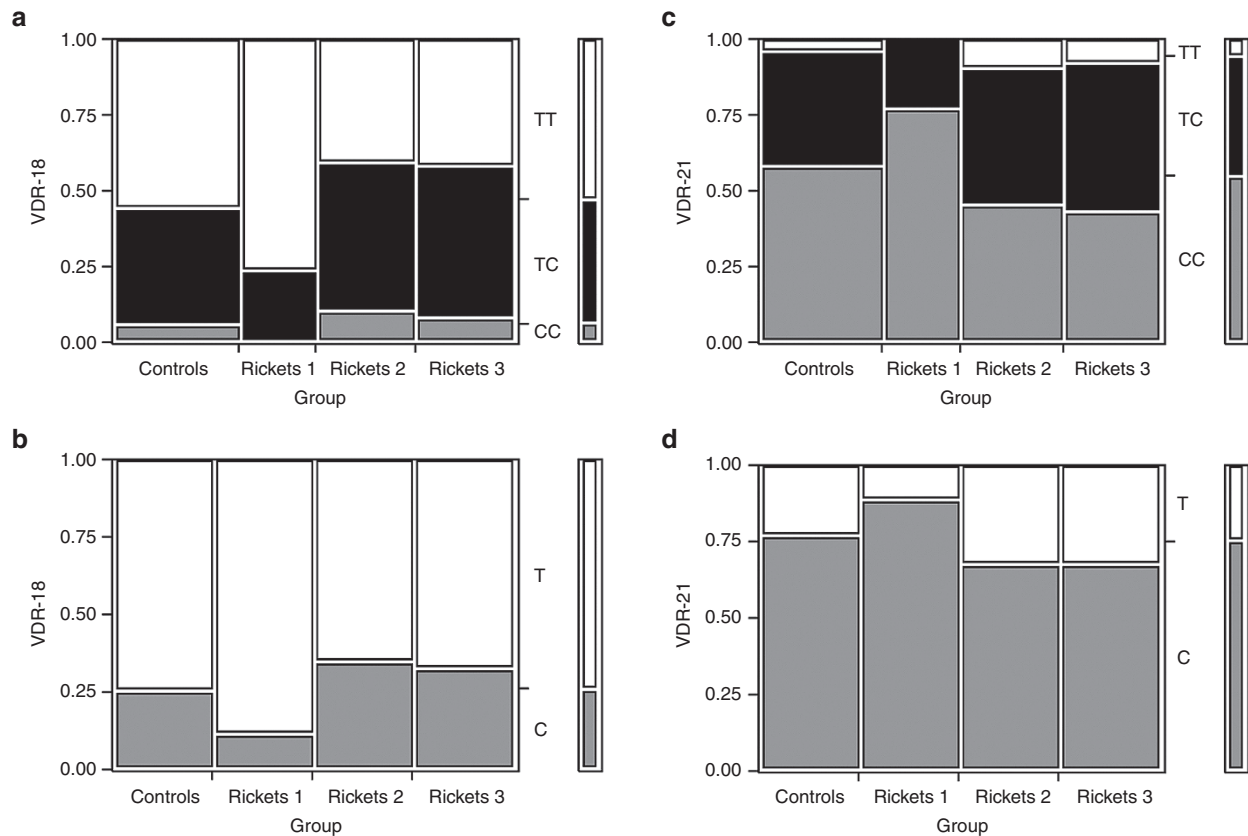
Figure 1. VDR-18 and VDR-21 genotype and allele frequencies in control and the three rickets patient groups. (**a**) VDR-18 genotype frequencies in control and the three rickets patient groups. In rickets 1 group, the major allele homozygous genotype (*TT*) is significantly increased (*P* = 0.02) and the minor allele homozygous genotype (*CC*) is absent. (**b**) Allele frequencies at VDR-18 in rickets and control groups. Major allele *T* is significantly increased in rickets 1 group (*P* < 0.0001). (**c**) VDR-21 genotype frequencies in control and the three rickets patient groups. In rickets 1 group, the major allele homozygous genotype (*CC*) is significantly increased (*P* = 0.03) and the minor allele homozygous genotype (*TT*) is absent. (**d**) Allele frequencies at VDR-21 in rickets and control groups. Major allele *C* is significantly increased in rickets 1 group (*P* < 0.0001).

However, it was not so in terms of time to ALP normalization, which was longer in group 1, and clinical data at X-ray score normalization demonstrated a poorer biochemical profile in terms of serum calcium, phosphorus, ALP and 25-OH-D (although only 3 of 30 patients in group 1, treated with calcium alone, presented 25-OH-D levels between 14 and 15 ng/ml; data not shown). In groups 2 and 3, 25-OH-D levels reached a maximum concentration of 140 ng/ml with no signs of toxicity.

With a view to establishing whether socioeconomic and nutritional factors accounted fully for the development of rickets in Egyptian children, we also aimed to ascertain whether genetic variations in several genes of the vitD system could predispose to or modify the clinical course.

Several epidemiological studies have analyzed the impact of genetic variation on vitD insufficiency determination: Suaini *et al.* (20) described increased frequency of vitD insufficiency in carrier children of a minor allele in 6 vitamin D binding globulin gene (*GC*) SNPs, comprising 4 of the SNPs analyzed in the present study. However, our study detected no genotype frequency difference among groups or any association with clinical and biochemical data for the *CPR2, CYP27B1, CYP24A1*, and *GC* SNPs analyzed. Only *VDR* proved to be associated with rickets and several promoter and intronic *VDR* genotypes were associated

with baseline serum 25-OH-D and calcium, X-ray score and time to radiologic healing and ALP normalization.

At diagnosis, up to 29% of serum 25-OH-D levels could be explained by genotypes at three *VDR* promoter SNPs: VDR-3 (rs11568820, Cdx2), VDR-4 (rs4760658), and VDR-9 (rs2853564). VDR-3 is located at the site interacting with the intestinal-specific transcription factor Cdx2, which has been investigated for its association with BMD and fracture risk: individuals that are homozygous *GG* have lower BMD than homozygous *AA* individuals (21), the *A* allele has a protective effect on fracture risk (22,23) and the *G* allele has diminished transcriptional activity (21). In our rickets patients, the minor allele homozygous *AA* (*n* = 7) presented the lowest 25-OH-D levels, although they normalized ALP levels in the shortest time (*AA* = 1.9 ± 1.1; *GA* = 2.5 ± 1.3; *GG* = 3.1 ± 1.4 mo; *P* = 0.025). These results concur with recently published data in Chinese women in whom serum 25-OH-D was highest in *GG* carriers and lowest in the *AA* (24). VDR-4 and VDR-9 minor allele homozygous *CC* genotypes showed the highest 25-OH-D levels. The three *VDR* promoter genotypes had been included together with several skin color-genes in two multilocus networks identified as displaying strong latitudinal clines (6). VDR-3 was included together with two other *VDR*

Table 3. Association between *VDR* genotypes and baseline data in rickets patients

VDR genotypes associations	25-OH-D[a]	Calcium[b]	X-ray score[a]	Time to radiologic healing	Time to ALP normalization
Stepwise r^2 adjusted (*P*)	0.29 (<0.0001)	0.54 (<0.0001)	0.37 (<0.0001)	0.12 (0.0071)	0.22 (0.0003)
Intercept	4.7	7.4	7.0	4.1	2.8
VDR-3 (rs 11568820) (promoter, Cdx2) (*AA/GA&GG*): Estimate ± SE (*P*)	−5.75 ± 2.62 (0.0315)				
VDR-4 (rs 4760658) (promoter) (*TT&TC/CC*): Estimate ± SE (*P*)	−6.69 ± 2.05 (0.0018)				
VDR-7 (rs 10875695) (promoter) (*TT&TG/GG*): Estimate ± SE (*P*)				−0.42 ± 0.13 (0.0020)	
VDR-9 (rs 2853564) (promoter) (*TT&TC/CC*): Estimate ± SE (*P*)	−2.42 ± 1.16 (0.0417)	−0.32 ± 0.10 (0.0026)			
VDR-10 (rs 1989969) (promoter) (*CC&TT/TT*): Estimate ± SE (*P*)		−0.69 ± 0.22 (0.0026)			
VDR-12 (rs2228570 (start codon: *Fok1*) (TT/CC&TC): Estimate ± SE (*P*)			−0.59 ± 0.16 (0.0006)		
VDR-14 (rs 2238138) (intron 2) (*TT/TC&CC*): Estimate ± SE (*P*)		−0.38 ± 0.13 (0.0045)			
VDR-18 (rs 7305032) (intron 5) (*CC/TC&TT*): Estimate ± SE (*P*)		+1.80 ± 0.44 (0.0001)	−1.05 ± 0.4 (0.0109)		
VDR-20 (rs 12314197) (intron 6) (*TT*): Estimate ± SE (*P*)		+0.57 ± 0.20 (0.0049)			
VDR-21 (rs 2525044) (intron 6) (*TT/TC&CC*): Estimate ± SE (*P*)		−2.38 ± 0.48 (<0.0001)			
VDR-23 (rs 11574114) (intron 8) (*GG*): Estimate ± SE (*P*)		−0.57 ± 0.28 (0.0428)			
VDR-24 (rs 7975232) (intron 8: *Apa1*) (*GG/TG&TT*): Estimate ± SE (*P*)		−0.49 ± 0.23 (0.0415)		−0.32 ± 0.12 (0.0107)	−0.52 ± 0.21 (0.0165)

[a]Weighted by Sun Exposure Index. [b]Weighted by calcium intake (% RDI).

promoter (VDR-7 and -8) and *MC1R, SLC24A5*, and *SLC45A2* genotypes in a network showing the strongest cline with population geographical origin latitudes, and the three *VDR* promoter SNP haplotype showed a dramatic in- vs. out-of Africa frequency pattern, with a frequency of 0.2 in sub-Saharan Africa, and an average frequency of 0.9 with no latitudinal association outside of Africa. The haplotype containing VDR-3 *G* allele (linked to higher 25-OH-D in our present study) showed the lowest frequency in dark skin Sub-Saharan populations, highest in light skin European populations and intermediate in Egyptian. VDR-4 and -9 were included with two other skin color-genes (*TYR* and *TYRP1*) in another network showing a significant linear regression against latitude (6). The haplotype containing the VDR-4 and VDR-9 *T* allele (linked to lower 25-OH-D levels in our present study) presented the highest frequency in dark skin populations and the lowest in the light skin ones. These previously published results demonstrated that *VDR* promoter adapted with skin color genes to the need to use ultraviolet (UVR) for vitamin D synthesis or to protect from intense UVR radiation (6). Egyptian rickets children presenting the lowest 25-OH-D levels had VDR-3, -4, and -9 promoter genotypes that are more frequent in dark skin populations, although Egyptian rickets patients and controls in the present study had similar skin colors (light brown).

At diagnosis, 54% of serum calcium could be explained by genotypes at eight *VDR* SNPs, two in the promoter region and six intronic. Promoter VDR-9 and VDR-10 (77% linked) genotypes showed the lowest calcium in parallel with the lowest 25-OH-D levels and these haplotypes were included, as previously indicated, together with several skin color genes in a network showing a significant linear regression against latitude (6). Intronic VDR-14 (rs2238138) and VDR-24 (rs7975232, *Apa1* site) were also included in a haplotype network not associated with latitude, with the minor allele homozygous genotypes showing the lowest calcium levels. VDR-18 (rs7305032) and VDR-21 (rs2525044) (96.7% linked) were also included in another network with the minor allele homozygous genotypes carrying the lowest serum calcium levels, and this genotype was absent in rickets patients Group 1. This genotype may predispose patients to rickets and was absent in Group 1 patients who combined adequate sun exposure and insufficient calcium intake.

At diagnosis, X-ray score was associated to VDR-18 (see above) and VDR-12 (rs2228570, start codon, *Fok1* site) with the minor allele homozygous *TT* genotype (*ff* according to *Fok1* restriction enzyme site) at VDR-12 showing the lowest score. The *TT* genotype encodes a longer VDR protein which has been demonstrated to be transcriptionally less active (25),

has been shown to be associated with higher concentrations of 25-OH-D (26) and was less frequent than in controls in our previously studied rickets population (27).

VDR-24 (rs7975232, *Apa1* site) genotype was also significantly linked to time to radiologic healing and ALP normalization and the minor allele homozygous genotype (*AA* according to the *Apa1* restriction enzyme site) presented the shortest time to healing. Intron 8 VDR-22 (*BsmI*) and VDR-24 (*ApaI*) genotypes were the first to be described as associated with bone mineral density (BMD) variation and their *bb* (*GG* nucleotides) and *AA* (*GG* nucleotides) genotypes had been shown to be associated with higher BMD values (28–30). The present study confirms that VDR-24 modulate serum calcium levels as well as bone involvement in a situation of clinical rickets. Other studies reported similar or different associations between *VDR* intronic genotypes and haplotype frequencies or clinical data: Kitanaka *et al.* (1) showed that some haplotype frequencies at VDR-22, -24, and -25 (among which was the VDR-22 *B* allele associated to the highest X-ray scores and the lowest 25-OH-D levels in our previous (27) and present studies) were increased in rickets patients compared to controls.

When different patient classification criteria were applied, in particular according to calcium intake adequacy and sufficiency of 25-OH-D serum levels, a subgroup of 8 patients emerged with the striking characteristics of presenting sufficient calcium intake and 25-OH-D levels despite inadequate sun exposure. In terms of *VDR* genotypes, they were the only ones to lack several of the minor allele homozygous genotypes at SNPs spread along the gene: five in the promoter region, one in the start codon, three intronic, and one in the 3'UTR region. The lacking genotype at VDR-3 (*AA*) encodes the most active protein in terms of intestinal calcium absorption and protection against fracture risk (21–23) and was associated with increased risk of T2DM (31), with lower 25-OH-D levels (24) and the lowest 25-OH-D levels in the present study. The lacking promoter genotypes at VDR-4, VDR-6, VDR-9, and VDR-10 are associated with the highest 25-OH-D and calcium levels in the present study. The lacking minor allele homozygous genotype (*TT*) at the start codon VDR-12 (rs2228570, *FokI*) encodes a longer and transcriptionally less active VDR (25), which has been shown to be associated with higher concentrations of 25-OH-D (26), was less frequent in our previously studied rickets population (27) and is associated with lower X-ray score in the present study.

These patients were the only ones in our study to present a lack of each of these minor allele homozygous genotypes, thus suggesting that their *VDR* genotype could have predisposed them to rickets in terms of low calcium, elevated PTH and bone disease, despite adequate calcium intake and 25-OH-D serum levels.

In summary, although Egyptian patients showed epidemiological factors strongly contributing to rickets such as low SEL and inadequate sun exposure and calcium intake, genetic modulation affecting predisposition and/or severity is exerted, at least in part, by *VDR* gene polymorphic variation.

Our study further strengthens the conclusion that rickets development, severity, and response to therapy in children depend not only on epidemiologic factors but also on genetic conditioning.

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