

# Iron Indices and Vitamin D Receptor Polymorphisms in Hemodialysis Patients

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**Cardiovascular disease caused by accelerated atherosclerosis is the major determinant of morbidity and mortality in chronic kidney disease patients. Vitamin D and its analogs provide survival benefit for hemodialysis (HD) patients. Vitamin D exerts its effects through the vitamin D receptor (VDR) that is coded for by a gene showing several polymorphisms that, in turn, are associated with a variety of diseases and differential responses to vitamin D. In this study, we evaluated the association between 4 VDR polymorphisms (ie, those identified by the restriction enzymes *BsmI*, *ApaI*, *TaqI*, and *FokI*) and iron indices (serum iron, transferrin, transferrin saturation, and ferritin) in 88 hemodialysis patients routinely treated with vitamin D. The absence or presence of the *BsmI*, *ApaI*, *TaqI*, and *FokI* restriction sites were denominated B and b, A and a, T and t, F and f, respectively. Our results show that in HD patients with transferrin saturation <20%, the F allele was more frequent than in HD patients with transferrin saturation >20% ( $P = .03$ ). This relationship may provide a link between VDR alleles and iron and nutritional markers, which are highly predictive variables of cardiovascular morbidity and mortality in hemodialysis patients.**

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**Index Words:** Vitamin D receptor; Gene polymorphism; Chronic kidney disease; Hemodialysis; Iron indices; Transferrin saturation

It is widely recognized that cardiovascular morbidity and mortality in chronic kidney disease (CKD) patients is a major issue in developed countries.<sup>1</sup> Ischemic heart disease, stroke, heart failure, and sudden death represent the main causes of death because of cardiovascular causes in CKD patients. Common risk factors for cardiovascular disease (high blood pressure, diabetes, smoking, and dyslipidemia) are present in CKD subjects as well as risk factors associated with chronic kidney failure, such as anemia, hyperparathyroidism, malnutrition, and inflammation.<sup>2,3</sup> This feature suggests that risk factors other than those commonly associated with cardiovascular disease could be at work in CKD

patients. In the search for these risk factors, genetic-association studies revealed that polymorphisms of the gene coding for the vitamin D receptor (VDR) influence mortality in dialysis patients. One polymorphism in particular (ie, the *BsmI* polymorphism) was associated with survival in a study of 143 hemodialysis (HD) patients.<sup>4</sup> This association makes biological sense because vitamin D is actually involved in several of the cardiovascular risk factors quoted previously. Thus, vitamin D is a negative endocrine regulator of the renin-angiotensin system,<sup>5</sup> affects parathyroid hormone balance and function,<sup>6</sup> influences the development of coronary artery disease,<sup>7</sup> and insulin secretion.<sup>8</sup> Furthermore, it has been recently suggested that the VDR *BsmI* gene polymorphism may predict both erythropoietin need and hemoglobin level in HD patients.<sup>9</sup>

We hypothesized that VDR gene polymorphisms could affect the management of anemia in HD patients. In particular, we sought to determine whether VDR polymorphisms (ie, *BsmI*, *ApaI*, *TaqI*, and *FokI*) were associated with iron indices because absolute or relative iron deficiency is still one of the leading causes of anemia and inadequate response to erythropoietin treatment in HD patients.

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Supported by grants from Foundation "Cassa di Risparmio di Firenze" and the University of Firenze, Italy.

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1548-5595/08/1502-0015\$34.00/0

doi:10.1053/j.ackd.2008.01.013

## Subjects and Methods

Eighty-eight dialysis patients treated at the Department of Nephrology of Prato Hospital (Italy) for at least 12 months were studied. The mean age of the 88 patients (58 men and 30 women) included in this study was  $51 \pm 14$  (range, 30-72) years. None had received steroid therapy or had undergone parathyroidectomy. Erythropoiesis-stimulating agents were administered in an attempt to maintain a target hemoglobin level between 11 and 12 g/dL. Maintenance intravenous sodium ferric gluconate of 62.5 mg was administered weekly unless the ferritin level and transferrin saturation (%) (TSAT) increased to more than 800 ng/mL or 50%, respectively. Water was purified by reverse osmosis; dialysate containing 3.0 mEq/L calcium and 0.5 mEq/L magnesium was used throughout the study. Blood samples were drawn before the beginning of the dialysis to assess biochemical profiles and for DNA extraction. Serum iron, transferrin, ferritin, and albumin were measured by the Beckman CX7 autoanalyzer (Beckman Coulter Inc., Brea, CA). TSAT was calculated as  $([\text{serum iron}/\text{serum transferrin}] \times 70.9)$ . Kt/V was used as an index of the adequacy of treatment. Characteristics of the patients enrolled in this study are shown in Table 1.

Genomic DNA was extracted from peripheral blood leucocytes by using a QIAamp DNA Blood Mini Kit (QIAGEN S.p.A., Milan, Italy) and amplified by polymerase chain reaction (PCR).

PCR was performed in a final volume of 50  $\mu$ L containing 200 ng of genomic DNA, 240 ng of each primer (Genenco M-medical S.r.l., Milan, Italy), and 25  $\mu$ L of HotStarTaq Master Mix Kit (QIAGEN S.p.a) by using standard conditions on a Mini Cycler (Mj Research Inc, Genenco M-medical S.r.l.). Each sample was

subjected to 32 cycles, each composed of 3 steps: 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. Before the first cycle, initial DNA denaturation was performed at 95°C for 15 minutes; the last cycle was followed by an extension step of 10 minutes at 72°C.

For the detection of the polymorphic BsmI restriction enzyme site, 2 primers were used: downstream primer 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA and upstream primer 5'-AAC CAG CGG GAA GAG GTC AAG GG. For detection of the polymorphic ApaI and TaqI restriction enzyme sites, the following primers were used: downstream primer 5'-CAG AGC ATG GAC AGG GAG CAA G and upstream primer 5'-GCA ACT CCT CAT GGC TGA GGT CTC A. For detection of the polymorphic FokI restriction enzyme site, 2 primers were used: downstream primer 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT and upstream primer 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC.

The PCR products were digested with the respective restriction enzymes according to the manufacturer's instructions as follows: at 65°C for 1 hour 30 minutes with BsmI (New England Biolabs, Herts, United Kingdom), at 25°C for 2 hours with ApaI (New England Biolabs, Herts, United Kingdom), at 65°C for 2 hours with TaqI (Fermentas M-Medical S.r.l.), and at 55°C for 3 hours with FokI (Fermentas M-medical S.r.l.).

The digested samples were analyzed on 2% agarose gels (Shelton Scientific Corporation, Peosta, IA). The absence or presence of the BsmI, ApaI, TaqI, and FokI restriction sites were denominated B and b, A and a, T and t, and F and f, respectively.

The chi-square test was used to calculate whether allele and genotype frequencies in HD patients deviated from the expected Hardy-Weinberg equilibrium observed in the control population. Differences in the frequency of the allele and genotype between HD patients with normal and low TSAT were tested by using the chi-square test. Differences were considered significant when P was <.05.

## Results

The FokI polymorphism site is unlinked with ApaI, BsmI, and TaqI polymorphisms; it is

**Table 1. Patients' Characteristics (n = 88)**

Age (y)	51 $\pm$ 14
Age range (y)	30-72
Period of dialysis (mo)	60 $\pm$ 25
Kt/V	1.32 $\pm$ 0.08
Serum iron ( $\mu$ g/dL)	69 $\pm$ 25
Transferrin (mg/dL)	174 $\pm$ 47
Transferrin saturation (%)	30 $\pm$ 16
Ferritin (ng/mL)	385 $\pm$ 108
Albumin (g/dL)	4.1 $\pm$ 0.5

located in a coding exon at the start site of the gene and affects the length and the structure of the VDR. The distribution of the allele and genotype frequencies of FokI polymorphism in HD patients and in normal subjects is shown in Table 2. The f allele and the ff genotype were less frequent in HD patients compared with normal controls. The differences were statistically significant ( $P < .05$ ).

ApaI, BsmI, and TaqI polymorphisms are located in a regulatory site at the 3' end of the VDR gene; these polymorphic sites are in linkage disequilibrium. None of them affects the structure of the VDR. The distribution of the allele and genotype frequencies of BsmI, ApaI, and TaqI polymorphisms in HD patients and in normal subjects is shown in Table 2. There were no statistically significant differences, as shown by  $P$  values  $>.05$  between HD patients and normal individuals in the distribution of allele and genotype frequencies of BsmI, ApaI, and TaqI polymorphisms.

Iron, transferrin, ferritin, and albumin serum levels did not show any significant association with VDR polymorphisms, regardless of low, normal, or high plasma concentrations. No correlation could be established between iron dosages, albumin, and any polymorphism. When TSAT was analyzed, there was no statistically significant difference in the distribution of allele and genotype frequencies of BsmI, ApaI, and TaqI polymorphism between patients with TSAT  $> 20$  and patients with

TSAT  $< 20\%$  (Table 3). On the contrary, in HD patients with TSAT  $< 20\%$ , the F allele was more frequent than in HD patients with TSAT  $> 20\%$  ( $P = .03$ ) (Table 3).

## Discussion

The allele and genotype distribution of the translation initiation codon polymorphism FokI in our control population was analogous to the distributions observed in other white populations.<sup>10</sup> HD patients and normal controls showed a different FokI allele and genotype distribution. These results are consistent with those recently reported by Vigo Gago et al<sup>11</sup> in a study in 64 Spanish patients with chronic kidney failure in which the FF genotype was overrepresented in CKD patients.

Unlike FokI polymorphism, the BsmI, ApaI, and TaqI polymorphisms were not distributed differently among HD patients and normal controls. When individually assessed, serum iron, transferrin, ferritin, and albumin were not associated with VDR polymorphisms. When we compared the allele frequencies pertaining to FokI polymorphism in HD patients with normal or low TSAT, we found a statistically significant increase of the F allele in the group of HD patients with TSAT lower than 20%. TSAT combines the serum transferrin level, which is an established nutritional marker<sup>3,12</sup> with serum iron concentration. TSAT represents the percentage of the

**Table 2. Allele and Genotype Frequencies of FokI, BsmI, ApaI, and TaqI Polymorphisms in Normal Subjects and Hemodialysis Patients**

VDR polymorphism	Alleles	Allele Frequency		$P$	Genotypes	Genotype Frequency		$P$
		Normal (n = 214)	HD Patients (n = 88)			Normal (n = 214)	HD Patients (n = 88)	
FokI	F	0.54	0.65	.010	FF	0.32	0.39	.011
	f	0.46	0.35		Ff	0.44	0.52	
					ff	0.24	0.09*	
BsmI	B	0.45	0.43	.803	BB	0.20	0.18	.958
	b	0.55	0.57		Bb	0.50	0.51	
ApaI	A	0.53	0.60	.151	bb	0.30	0.31	.318
	a	0.47	0.40		AA	0.26	0.34	
					Aa	0.54	0.51	
TaqI	T	0.63	0.59	.426	aa	0.20	0.15	.732
	t	0.37	0.41		TT	0.40	0.35	
					Tt	0.46	0.48	
				tt	0.14	0.17		

\*Statistically significant genotype.

Abbreviations: VDR, vitamin D receptor; HD, hemodialysis.

Table 3. Allele and Genotype Frequencies of FokI, BsmI, ApaI, and TaqI Polymorphisms in Hemodialysis Patients With TSAT &lt; 20% and TSAT &gt; 20%

VDR Polymorphism	Allele Frequency		Genotype Frequency		P	P
	Alleles	HD Patients	Genotypes	HD Patients		
		With TSAT > 20% (n = 40)		With TSAT < 20% (n = 48)		
FokI	F	0.57	0.72	FF	0.28	0.48
	f	0.43	0.28	Ff	0.57	0.48
BsmI	B	0.49	0.40	BB	0.15	0.04
	b	0.51	0.60	Bb	0.18	0.19
ApaI	A	0.60	0.59	bb	0.62	0.42
	a	0.40	0.41	BB	0.20	0.39
TaqI	T	0.57	0.62	AA	0.30	0.37
	t	0.43	0.38	Aa	0.60	0.44
				aa	0.10	0.19
				TT	0.28	0.42
			Tt	0.57	0.39	
			tt	0.15	0.19	

Abbreviations: NS, not significant; TSAT, transferrin saturation; VDR, vitamin D receptor; HD, hemodialysis.

transferrin iron-binding capacity actually occupied by iron, and it mirrors iron status but it may be altered by factors that are unrelated to iron metabolism.<sup>13</sup> The combination of 2 unrelated values giving origin to a new and significant factor is not unusual in biomedical analysis, body mass index being the clearest example.

The FokI polymorphism alters the length and the structure of the VDR, but it is not determined whether this polymorphism has any effect on the function of the VDR. Gross et al<sup>14</sup> reported that the 2 different receptors showed the same affinity for 1,25 (OH)<sub>2</sub> vitamin D<sub>3</sub>, the same DNA-binding affinity, and the same transactivation activity, whereas in other studies this polymorphism was associated with altered vitamin D-dependent gene transcription.<sup>15</sup> However, a recent study has linked Fok-I polymorphism with cardiovascular risk factor in the general population,<sup>16</sup> and the present results and those of Vigo Gago et al<sup>11</sup> suggest that this polymorphism might indeed alter the function of the VDR in a way that it could influence the development and/or progression of CKD and its cardiovascular complications.

As discussed in recent reviews<sup>17,18</sup> in HD patients, vitamin D treatment may provide beneficial and protective effects that seem to be unrelated to the classical vitamin D influence on mineral metabolism parameters. Vitamin D and its analog therapy improve kidney and cardiovascular functions in patients with CKD.<sup>19-22</sup> Vitamin D has several important activities that may contribute to its renoprotective property, such as the inhibition of profibrotic growth factors and inflammatory cytokines and the suppression of the renin-angiotensin system. A sizable body of animal data show a mitigating effect on CKD progression with vitamin D treatment.<sup>23,24</sup> A recent study<sup>25</sup> showed that VDR knockout mice develop more severe diabetic nephropathy compared with wild-type mice, supporting previous reports showing that vitamin D can prevent the loss of podocytes and reduce podocyte injury in nephrectomized rats.<sup>26</sup> These results suggest a protective role of VDR, at least in diabetic nephropathy.

The BsmI polymorphism of the VDR gene may affect PTH levels and predict mortality, hemoglobin level, and erythropoietin need in

HD patients, with the BB genotype showing lower vitamin D–dependent reduction in parathyroid hormone–circulating levels, greater mortality rate, lower hemoglobin level, and greater erythropoietin need.<sup>2,4,6,9</sup> In the present study, FokI polymorphism of the VDR gene was associated with transferrin saturation; this relationship may provide a link with iron and nutritional markers, which are highly predictive variables of cardiovascular morbidity and mortality in CKD patients.<sup>3,17,18</sup> Because cardiovascular complications are the largest cause of mortality in chronic kidney failure, the determination of VDR polymorphisms could help identify those HD patients with a greater risk of cardiovascular morbidity and mortality.

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