# Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D$_3$

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## Summary

OBJECTIVE In the vitamin D receptor (VDR) gene
a *BsmI* restriction fragment length polymorphism
(RFLP) in intron 8 and a translational start-site poly-
morphism, identified as a *FokI* RFLP, have been
described. Crucial for a proper interpretation of
these polymorphisms in association studies is the
knowledge whether they have direct consequences
for 1,25-(OH)$_2$D$_3$ action at cellular level. The present
study was designed to assess functional significance
of the *FokI* and *BsmI* VDR gene polymorphisms in
peripheral blood mononuclear cells (PBMC) with a
natural occurring VDR genotype for cell growth inhi-
bition by 1,25-(OH)$_2$D$_3$.

DESIGN PBMC of women were isolated, VDR geno-
typed and *in vitro* inhibition by 1,25-(OH)$_2$D$_3$ of Phy-
tohemagglutinin (PHA)-stimulated growth of PBMC
was examined in relation to VDR genotype.

RESULTS PHA-stimulated growth and maximal growth
inhibition were independent of VDR genotype. How-
ever, the FF genotype had a significant lower ED$_{50}$
than the Ff genotype corresponding to an allele dose
effect of 0·32 nM per f allele copy ($P = 0.0036$). For *BsmI*
genotypes no differences in ED$_{50}$ were observed.

CONCLUSION The present study demonstrates for
the first time in cells with a natural VDR genotype a
direct functional consequence of the VDR gene

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translational start-site polymorphism for the action
of 1,25-(OH)$_2$D$_3$. Especially under conditions of vita-
min D insufficiency these findings might have clinical
implications.

Over the last years the genetic basis of osteoporosis has been
intensively studied. In relation to this, several polymorphisms
in the vitamin D receptor (VDR) gene have been identified.
Firstly, the *BsmI* restriction fragment length polymorphism
(RFLP) is located in intron 8 at the 3′ end of the VDR gene.
Several studies have shown an association between this VDR
polymorphism and bone mineral density (Morrison *et al.*, 1994;
Fleet *et al.*, 1995; Spector *et al.*, 1995; Uitterlinden *et al.*, 1996),
but other studies have not found such relationship (Hustmeyer
*et al.*, 1994; Garnero *et al.*, 1995; Kröger *et al.*, 1995). Putting
all these data together, there seems to be a weak association
with bone mass (Cooper & Umbach, 1996). Also associations
with other phenotypes, like primary hyperparathyroidism,
prostate cancer, and radiographic osteoarthritis have been
described (Carling *et al.*, 1995; Taylor *et al.*, 1996; Ingles *et al.*,
1997; Uitterlinden *et al.*, 1997).

Recently, a polymorphism of the translational start-site
has been identified in the VDR gene (Saijo *et al.*, 1991;
Sturzenbecker *et al.*, 1994; Miyamoto *et al.*, 1996; Arai *et al.*,
1997). This polymorphism is characterized by the presence of
either two ATG start codons separated by 6 nucleotides or due
to a T to C substitution in the 5′ATG site only the presence of
the most 3′ATG codon. The presence of the 5′ATG-site results
in recognition sequence for the *FokI* restriction enzyme (Gross
*et al.*, 1996). For this VDR polymorphism also both the
presence and the lack of association with bone mineral density
has been described (Gross *et al.*, 1996; Arai *et al.*, 1997; Harris
*et al.*, 1997; Eccleshall *et al.*, 1998). In contrast to the *BsmI*
polymorphism, the *FokI* polymorphism has distinct structural
consequences for the VDR. The absence of the *FokI* restriction
site, indicated as F, predicts a VDR protein of 424 amino acids,
whereas the presence of the *FokI* site results in a VDR of 427
amino acids.

Crucial for proper interpretation of genetic association
studies is the demonstration of functional consequences of
these polymorphisms. For the *BsmI* polymorphism some *in vivo*
studies have been performed showing potential association with

parameters related to bone turnover and bone mineral density (Howard *et al.*, 1995; Krall *et al.*, 1995; Matsuyama *et al.*, 1995; Graafmans *et al.*, 1997; Kiel *et al.*, 1997). However, these *in vivo* studies are complex and do not provide direct insights into the consequences for $1,25\text{-}(OH)_2D_3$ action at cellular level. A few *in vitro* studies have been performed to address functionality of VDR gene polymorphisms. In these studies cells were transfected with either one of the VDR genotypes (Morrison *et al.*, 1994; Arai *et al.*, 1997). A potential disadvantage of transfecting cells with VDR genotypes is the absence of control over subtle differences in expression of the gene of interest. Especially, these differences may be very important for the differential phenotypic effect of natural occurring gene polymorphisms. The present study was designed to investigate the functional consequences of the *BsmI* and *FokI* RFLP of the VDR gene for the action of $1,25\text{-}(OH)_2D_3$ in cells, peripheral blood mononuclear cells (PBMC) expressing the natural VDR genotypes. PBMC form a readily accessible target of vitamin D which is used as a model for studying genotype-dependent $1,25\text{-}(OH)_2D_3$ effects.

## Materials and methods

### Subjects

Fasting blood samples from 72 healthy postmenopausal women, aged 59–75 years (mean age $\pm$ SD, $65.7 \pm 4.3$ years) were taken. Women were randomly selected from independently living subjects of the Rotterdam Study (Hofman *et al.*, 1991) whereby those using hormonal replacement therapy, cytostatics, vitamin D, thyroid hormone or known to have diabetes were excluded. Four women were excluded from analysis as no growth dose–response curves were present because no genotype data were available. Written informed consent was obtained from each participant. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam.

### Cell culture

PBMC were prepared from heparinized blood by Ficoll-Hypaque (Pharmacia, Sweden) density gradient centrifugation (Böyum, 1968). The cells were suspended in phenol red-free RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO) supplemented with 10% charcoal-treated FCS, 2 mM glutamine, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin (Life Technologies, Breda, The Netherlands) and 24 mM sodium bicarbonate. Immediately after isolation the cells were used for the proliferation analysis. For this purpose cells ($4,4 \times 10^6$ cells per ml) were cultured for 96 h in 24-wells plates (300 $\mu$l/well) in the presence of 3 $\mu$g/ml phytohemagglutinin (PHA) and $10^{-10}$

M to $10^{-7}$ M $1,25\text{-}(OH)_2D_3$ (kindly donated by Dr L. Binderup of LEO Pharmaceutical Products, Ballerup, Denmark). At the end of the incubation, DNA content of adherent and nonadherent cells was measured according to the ethidium bromide method of Karsten & Wollenberger (1977). For each individual two PBMC cultures were performed each consisting of two DNA measurements.

Due to ethical reasons it was not possible to perform both PBMC growth analyses and VDR measurements in one and the same individual. For the PBMC growth study 3–4 tubes of blood were needed (8–10 ml). In addition to this 3–4 other tubes had to be taken for clinical analyses. This would mean that over 10 tubes of blood from volunteers were needed in order to have at least the possibility of measuring VDR. For measurement of VDR in monocytes only we have calculated that even 21 tubes would be necessary.

### Genotyping procedure

The *BsmI* RFLP at the 3′ end of the VDR gene was assessed by a direct haplotyping PCR procedure as previously described (Uitterlinden *et al.*, 1996). The *FokI* RFLP was analysed by PCR with the primers described previously (Gross *et al.*, 1996). The amplification protocol consisted of 28 cycles of 94, 60 and 72°C for 1 minute each. Ten microliters of the PCR products were digested with 10 U *FokI* and 1.2 $\mu$l of a 10x buffer (containing 150 mM Tris-HCl, pH 7.5, 250 mM NaCl and 35 mM $MgCl_2$) by incubating for 30 minutes at 37°C. Digestion products were analysed on a 3% NuSieve agarose gel run in 0.5X TBE. Capital letters denote absence and lowercase letters the presence of the site for the restriction enzyme *BsmI* (*B/b*) or *FokI* (*F/f*).

### Statistical analysis

For each individual a best fitted growth curve and maximal inhibition and $ED_{50}$ values were calculated and analysed using GraphPad Prism software (GraphPad Software, Inc., San Diego, USA). Next, per genotype the mean maximal inhibition and $ED_{50}$ ($\pm$ SE) were calculated. Analysis of variance (ANOVA) was performed and for pairwise comparison student's *t*-test was used. The relation between allele dose and $ED_{50}$ was quantified by linear regression analysis.

## Results

As shown in Table 1 incubation of PBMC with PHA resulted in an almost 2.3 times stimulation of growth compared to control cells. PHA-stimulated cell growth was strongly dose-dependently inhibited by $1,25\text{-}(OH)_2D_3$ (Fig. 1a) with a maximal inhibition of about 75% at $10^{-8}$–$10^{-7}$ M (Table 1). The half-maximal
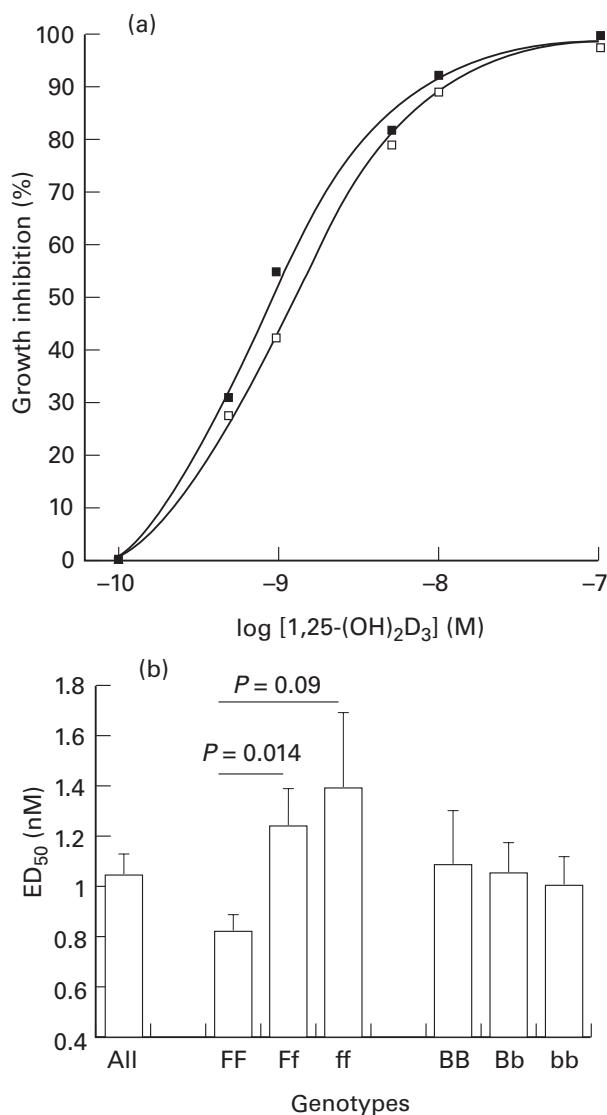
**Fig. 1** Dose-dependent inhibition of PHA-stimulated growth by 1,25-(OH)$_2$D$_3$ (a) and calculated ED$_{50}$ of PMBC growth inhibition according to the *FokI* and *BsmI* VDR genotypes (b). ■ FF; □ Ff/ff. For clarity reasons the dose–response curves of Ff and ff are combined. Data are transformed according to a maximal inhibition of 100% (a). Data are presented as mean ± SEM (b). FF: $n = 34$; Ff: $n = 25$; ff: $n = 9$; BB: $n = 10$; Bb: $n = 35$; bb: $n = 21$. All = ED$_{50}$ independent of genotypes.

inhibition was achieved at 1·05 nM 1,25-(OH)$_2$D$_3$ (Fig. 1b). Neither PHA-stimulated cell growth nor maximal inhibition by 1,25-(OH)$_2$D$_3$ appeared to be VDR genotype dependent. However, the ED$_{50}$ showed differences between *FokI* genotypes in a gene-dose dependent manner (Fig. 1a,b). The PMBCs with the *FF*-genotype had the lowest ED$_{50}$ value (0·82 ± 0·072) *Ff* heterozygotes had an intermediate ED$_{50}$ value (1·24 ± 0·15),

while *ff* homozygotes had the highest ED$_{50}$ value (1·39 ± 0·3). Linear regression analysis demonstrated an allele dose effect of 0·32 nM per *f* allele copy ($P = 0.0036$). No significant differences were observed when the 3′end of the VDR was genotyped by the direct haplotyping procedure (Uitterlinden *et al.*, 1996), which may be due to too low numbers per genotype group to allow analysis (data not shown). Also when the analysis was restricted to the single *BsmI* polymorphism no with differences in ED$_{50}$ (Fig. 1b) and maximal inhibition were observed (Table 1). The *ApaI* and *TaqI* polymorphisms were also not associated with differences in maximal inhibition by 1,25-(OH)$_2$D$_3$ and ED$_{50}$ (data not shown).

## Discussion

The current study demonstrates direct functional consequences of natural occurring VDR gene polymorphisms for the cellular action of 1,25-(OH)$_2$D$_3$ in PBMC of healthy postmenopausal women. In most previous studies the functional consequences of VDR genotypes at the cellular level were only studied in cells transfected with different VDR alleles (Morrison *et al.*, 1994; Arai *et al.*, 1997; Gross *et al.*, 1998a). Our study demonstrates that the half maximal concentration for 1,25-(OH)$_2$D$_3$ inhibition of PHA-stimulated growth is significantly different between PBMC characterized by different alleles of the VDR translational start-site while the maximal inhibition is similar for all genotypes. The ED$_{50}$ for cells homozygous for the 424 amino acids long VDR, i.e. the *FF* genotype, is lower than the ED$_{50}$ for cells containing the 427 amino acids long VDR, i.e. the *Ff and ff* genotypes. A significant allele dose effect was observed which means that PBMC containing both forms of the VDR, the heterozygotes, have an intermediate ED$_{50}$. Arai *et al.* (1997) have also shown a more potent effect by the short VDR using HeLa cells transfected with a VDR *FokI* genotype and a 24-hydroxylase VDRE-reporter construct. They tested only one concentration of 1,25-(OH)$_2$D$_3$ and it is not clear whether this reflects the maximal concentration or approaches the half-maximal effective dose for the response analysed by Arai *et al.* (1997). Also others showed a more active 424 amino acids VDR (Jurutka *et al.*, 1998; Remus *et al.*, 1998) while one other study did not observe differences between VDR *FokI* genotypes (Gross *et al.*, 1998a). Together, our data support the hypothesis that the 424 amino acids-long VDR is more efficient in exerting 1,25-(OH)$_2$D$_3$ effects than the 427 amino acids-long VDR.

Although we were not able to measure VDR levels in the same PMBC of the individuals used in the growth study (see Materials and Methods), it is unlikely that the differences in effect are due to increased VDR expression by the *FF* genotypes (Arai *et al.*, 1997). Arai *et al.* (1997), even reported that the *FF* genotype appears to have a somewhat (± 20%) reduced VDR expression. Maybe the numbers of ff are too low

| Genotype | $n$ (%)* | PHA-stimulated growth§ (fold-stimulation) | Maximal 1,25-$(OH)_2D_3$ effect (% inhibition) |
|---|---|---|---|
| All† | | $2·25 \pm 0·07$ | $72·8 \pm 1·46$ |
| FF | 34 (50·0) | $2·18 \pm 0·10$ | $71·0 \pm 2·15$ |
| Ff | 25 (36·8) | $2·28 \pm 0·12$ | $71·6 \pm 2·18$ |
| ff | 9 (13·2) | $2·31 \pm 0·19$ | $79·5 \pm 3·73$ |
| BB | 10 (15·2) | $2·31 \pm 0·14$ | $72·4 \pm 4·55$ |
| Bb | 35 (53·0) | $2·21 \pm 0·11$ | $73·7 \pm 5·91$ |
| bb | 21 (34·9) | $2·30 \pm 0·13$ | $71·5 \pm 2·33$ |

* $n$ = the number of subjects. § PHA growth is expressed as fold stimulation over cell growth in the absence of PHA. † All = Effect independent of genotypes. Data are presented as means $\pm$ SEM.

to show significance, but the somewhat higher maximal inhibition by the ff genotypes may reflect an increased VDR level to compensate for the lower affinity.

In view of the localization of the polymorphism in the N-terminal A/*B* region of the VDR it is unlikely that it will have an effect on 1,25-$(OH)_2D_3$ binding to the ligand binding domain in C-terminal E/*F* region. It is tempting to speculate that the difference in amino terminal sequence between the VDR genotypes directly affects binding of the VDR to its target genes. In this respect it is noteworthy that the VDR has a very short A/*B* region and therefore the sequence variation is located close to the DNA binding domain (C-region). Interesting in this respect are the observations by Jurutka *et al*. (1998) that the 424 amino acids VDR interacts more efficiently with the transcription cofactor TFIIB and possesses elevated transcriptional activity compared to the 427 amino acids VDR.

The VDR *FokI* genotype effect on $ED_{50}$ and not on maximal inhibition has potential clinical significance. In this way it is conceivable that dependent on the vitamin D status a VDR genotype effect is present or absent. So, especially under conditions of vitamin D insufficiency, for instance in elderly subjects, the biological consequences may become apparent, while at sufficient 1,25-$(OH)_2D_3$ levels the genotype consequences will be absent.

In contrast to the *FokI* RFLP, no relationship was observed between the VDR *BsmI* RFLP and 1,25-$(OH)_2D_3$ action which is in line with earlier findings (Gross *et al*., 1998b). An explanation for the dissociation with some *in vivo* data showing an association between *BsmI* VDR genotypes and response to 1,25-$(OH)_2D_3$ (Howard *et al*., 1995; Krall *et al*., 1995; Matsuyama *et al*., 1995; Graafmans *et al*., 1997; Eccleshall *et al*., 1998) is unknown and yet purely speculative. Considering the localization of the *BsmI* polymorphism it is unlikely that it will have a direct structural effect on the VDR. The *BsmI* RFLP can be in linkage with polymorphisms in the 3′ UTR (Morrison *et al*., 1994; Ingles *et al*., 1997) and in this way be related to stability of VDR mRNA and VDR expression (Nesic *et al*.,

1993). Maybe the present experimental system is not sensitive sufficient to observe effects of potential differences in VDR mRNA stability between *BsmI* genotypes. Some data showed that the *BsmI* RFLP does not affect the abundance of the VDR mRNA (Mocharla *et al*., 1997), while other studies indicated a difference in abundance of VDR mRNA between TaqI genotypes without an effect on VDR mRNA stability (Verbeek *et al*., 1997). Unfortunately as mentioned above, it was not possible to relate our present observations directly to VDR levels in PBMCs of the individuals in this study. Although the 3′ polymorphisms are not in linkage with the 5′start site polymorphism analysis of combinations VDR genotypes may reveal further diversity in VDR activity (Remus *et al*., 1998). Preliminary analysis of the various combinations of *FokI* and *BsmI* genotypes in the current study didn't provide additional information over the *FokI* analysis alone. It must, however, be noted that the number of individuals per genotype combination group is low and limits statistical analysis.

In conclusion, the current study provides evidence for direct functional consequences of the vitamin D receptor translational start-site polymorphism for the cellular action of 1,25-$(OH)_2D_3$ and provides insights into the vitamin D receptor genotype–phenotype association studies. The relationship with 1,25-$(OH)_2D_3$ concentrations is interesting and potentially important under conditions related to low 1,25-$(OH)_2D_3$ levels such as vitamin D deficiency and renal impairment. More generally, the present data demonstrate that differences in responsiveness between individuals may occur on the basis of naturally occurring variants of a single gene. Finally, it should be noted that in the present study one specific response of one specific target cell of 1,25-$(OH)_2D_3$ has been examined. For a full 1,25-$(OH)_2D_3$ response interaction of the vitamin D receptor with cofactors (e.g. transcription factor IIB, see above) is essential and these cofactors may vary between responses and cell types. Therefore, additional analyses of other responses and cell types are needed to further assess the generality of the concept that the 424 amino acid vitamin D receptor is the more active form.

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