Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 209–214
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Allele-frequency determination of *Bsm*I and *Fok*I polymorphisms of the *VDR* gene by quantitative real-time PCR (QRT-PCR) in pooled genomic DNA samples☆

J.M. Quesada[a,b,*], A. Casado[b], C. Díaz[a], L. Barrios[a], R. Cuenca-Acevedo[a], G. Dorado[c,*]

[a] *Unidad Metabolismo Mineral, Hospital Reina Sofía, Avda Menéndez Pidal s/n Córdoba, Spain*
[b] *SANYRES XXI (Grupo PRASA) Córdoba, Spain*
[c] *Dep. Bioquímica y Biología Molecular, Universidad de Córdoba, Córdoba, Spain*

Abstract

The *vdr* gene is a candidate for osteoporosis susceptibility, with conflicting results in association studies. We have designed and optimized an individual allele-specific and DNA pooling PCR-based methodology to quantitate *Bsm*I and *Fok*I polymorphisms of the *vdr* gene and studied single-nucleotide polymorphisms (SNPs) from pooled DNA samples. The allele frequency in DNA pooling experiments has been analyzed by kinetic PCR: quantitative real-time PCR (QRT-PCR). A Spanish cohort of 225 healthy postmenopausal women was studied. Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DEXA) and quantitative ultrasound calcaneous densitometry. Allele-specific PCR amplification of *Bsm*I and *Fok*I genes showed full concordance with the PCR-RFLP approach. The prevalence of the three *Bsm*I VDR genotypes was 19.1, 44.9 and 36.0% for *BB*, *Bb* and *bb*, respectively. In the case of the *Fok*I locus, the prevalence of genotypes was 40.4, 48.0 and 11.6% for *FF*, *Ff* and *ff*, respectively. No positive correlation was found between polymorphism and BMD. The DNA pooling procedure was validated. No differences were found *in* allele frequencies and *T*-score data obtained using the high throughput DNA pooling approach, as compared to known individual frequencies. In our hands, this is a very useful approach to study quantitative (thus polygenic) traits like osteoporosis susceptibility.

Keywords: Genetic; VDR; Polymorphism; Bone mineral density (BMD); Osteoporosis; Postmenopausal women

1. Introduction

The Vitamin D endocrine system plays a central role in calcium and phosphate homeostasis as well as bone mineralization. This is accomplished by promoting the dietary absorption of calcium and by facilitating the bone resorption and mineralization. Moreover, other multiple activities have been recently reported for Vitamin D throughout several other organs and systems of the body. The non-classic actions of the hormonally active form of the Vitamin D or calcitriol [1,25$(OH)_2$D] include regulation of cellular proliferation and differentiation, regulation of hormone secretion, and regulation of immune function. Most of the Vitamin D actions are mediated through the Vitamin D receptor (VDR), which is not restricted to the classic target tissues of Vitamin D, but rather widely distributed [1].

The gene encoding the VDR is located on chromosome 12q. Several allelic variants have been reported [2], including a *Fok*I restriction fragment length polymorphism in exon II, *Bsm*I and *Apa*I polymorphisms in the intron VIII (between exons VIII and IX), a synonymous *Taq*I variant in exon IX, and a poly(A) mononucleotide [$(A)_n$] repeat polymorphism in the 3′ untranslated region.

The possible functional consequences of the common allelic variants of the VDR alleles remain unclear. Nevertheless, since genetic factors play an important role in bone mass, the VDR gene (*vdr*) is a candidate for osteoporosis susceptibility, with conflicting results in association studies [3,4]. In addition, recent studies suggest a possible role of the *vdr* polymorphisms in the development of other diseases such as primary hyperparathyroidism [5], osteoarthritis [6], atherosclerotic coronary artery disease [7], colon, breast, prostate and other cancers [8,9], diabetes mellitus [10], psoriasis [11] and other autoimmune diseases.

DNA pooling is a practical way to reduce the cost of large-scale association studies to identify susceptibility loci

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

* Corresponding authors.
E-mail addresses: md1qugoj@uco.es (J.M. Quesada), bb1dopeg@uco.es (G. Dorado).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.03.085

for common diseases. Pooling allows allele frequencies in groups of individuals to be measured using far fewer PCR reactions and genotyping assays than are required when genotyping individuals [12,13]. The allele frequency in DNA pooling experiments has been quantitated by different approaches [13], being the quantitative real-time PCR (QRT-PCR) a promising one. This is a kinetic PCR methodology that has been already validated in several studies, showing a high degree of accuracy and reproducibility [14–16].

Most reports have focused on the *Bsm*I site, yet the *Fok*I variant remains a candidate for functional polymorphism assessment. We designed and optimized both an allele-specific as well as a DNA pooling PCR-based methodology to quantitate the *Bsm*I and *Fok*I polymorphisms of the *vdr* gene and studied single-nucleotide polymorphisms (SNPs) from pooled DNA samples.

1.1. Study subjects

The study was performed in a cohort of 225 postmenopausal Spanish women who were referred by general practitioners to the Mineral Metabolism Unit at Hospital Universitario "Reina Sofía" of Córdoba (Spain) during the course of a local osteoporosis screening program. Detailed medical histories and biochemical studies were performed. All were white Caucasian in good health. None of them had been treated with hormone-replacement therapy or receiving other drugs that potentially could affect bone mass or Vitamin D metabolism.

1.2. Bone density measurements

The bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DEXA) with an Hologic QDR_{1000} from Hologic (Bedford, MA, USA) at both the lumbar spine (LS) and femoral neck (FN) to estimate mainly trabecular and cortical bone, respectively. For the lumbar BMD evaluation, the LS densitometry of subjects with vertebral fractures, calcifications or other significant degenerative changes (assessed by X-rays) was excluded. The coefficients of variation (CV) at LS and FN were 1.3 and 1.8%, respectively. The long-term daily quality control was followed by a lumbar spine phantom, with CV of $0.0 \pm 0.1\%$. A total of 1331 healthy females served to establish the mean BMD in this healthy Spanish population and to calculate the *T*-score: standard deviations (S.D.) of the patient's value from the mean of young control population. The characteristics of this reference population have been described previously [17].

According to the World Health Organization (WHO) criteria, we considered osteoporosis when the LS and/or the FN *T*-score was equal or below −2.5 S.D. [18]. Yet, since there are several limitations to this approach to identifying patients with osteoporosis, we also used the treatment criteria for osteoporosis based on guidelines from the National Osteoporosis Foundation (NOF), and thus considered a *T*-score below −2.0 S.D. as cut-off point [19].

1.3. Quantitative ultrasound calcaneous measurements

Quantitative ultrasound calcaneous assessments were performed using the Sahara Clinical Sonometer from Hologic. Broadband ultrasound attenuation (BUA), speed of sound (SOS), quantitative ultrasound index (QUI) and estimated bone mineral density were determined. The CV was 4.8% for BUA, 0.36% for SOS, 3.45% for QUI and 4.15% for BMD. In vitro precision was 0.4% for SOS and 2.67% for BUA [20].

1.4. Genotyping

The analysis were performed from 3 ml of total blood on Venoject vials from Terumo (Leuven, Belgium) with Na_2-EDTA and kept frozen at −80 °C until processed. Blood samples from each women were obtained and the DNA was purified following a "salting out" procedure.

1.5. Individual allele-specific genotyping by PCR

The population was genotyped for the *Bsm*I and *Fok*I polymorphisms of the *vdr* gene using an individual allele-specific genotyping by PCR that we have setup and optimized in our laboratories. This procedure performs two PCR amplifications with each DNA sample. The amplicons were then subjected to agarose gel electrophoresis (AGE) and visualized under UV light. Each amplification was carried out with a common primer and a specific primer for each allele. The allele-specific primers differ in the first 3′-end base, thus differentially amplifying the appropriate allele. To increase or gain such specificity, the second 3′-end base of the allele-specific primers was changed, thus the two 3′-end bases were mismatched for the non-amplified alleles.

The *Bsm*I polymorphism is due to a G → A transition, being differentially amplified by the following primers: 5′-GCAAGAGCAGAGCCTGAGTATTGGGAAT**A**-3′ and 5′-GCAAGAGCAGAGCCTGAGTATTGGGAAT**T**-3′. The allele-specific 3′-end base is shown in boldface; the changed base to guarantee the differential specificity is underlined. The standard common primer to both alleles is 5′-GGAGAGAAGGGCGAGGGTCAGACCCAT-3′.

The *Fok*I polymorphism is due to a T → C transition, being differentially amplified by the following primers: 5′-CGTGGCCTGCTTGCTGTTCTTACAGGG**T**-3′ and 5′-CGTGGCCTGCTTGCTGTTCTTACAGGG**C**-3′. The allele-specific 3′-end base is shown in boldface; the changed base to guarantee the differential specificity is underlined. The standard common primer to both alleles is 5′-CACAGCAACCTCAGGAAAGCGATTTCCAAG-3′.

As a positive control, all PCR amplifications were performed in the presence of an extra primer that was common for both alleles. Such primer (together with the standard

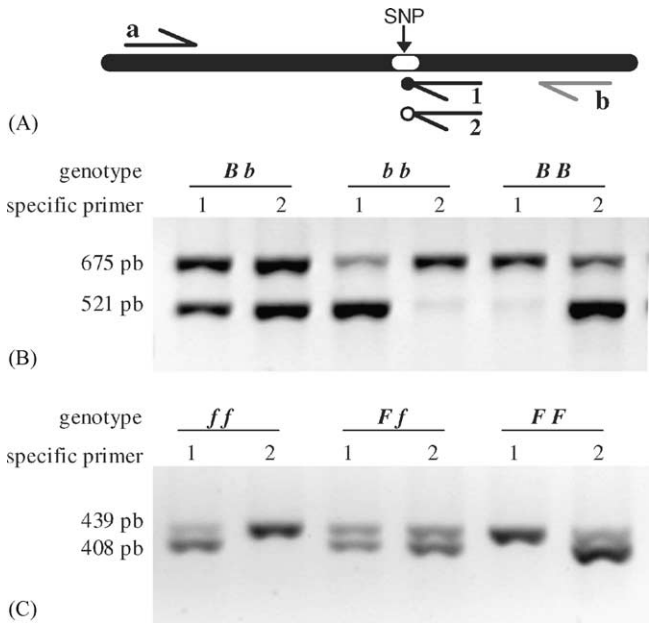

Fig. 1. Allele-specific PCR amplification. (A) Primer design. Schematic representation of the primers used in the allele-specific PCR amplifications. The primers "a" and "b" correspond to the extra common primers. The primers "1" and "2" are the allele-specific ones, being identical except at their 3′-ends. (B) AGE results. Agarose gel electrophoresis of the PCR amplifications corresponding to the *BB*, *Bb* and *bb* genotypes of the *Bsm*I polymorphism, as determined by allele-specific PCR. (C) AGE results. Allele-specific PCR of the *Fok*I polymorphism showing the *FF*, *Ff* and *ff* genotypes. See legend of part (B).

common primer) generated an amplicon that was longer than the allele-specific amplicon. The extra common primers used were 5′-AACCAGCGGGAAGAGTCAAGGG-3′ and 5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ for the *Bsm*I and *Fok*I positive controls, respectively. Fig. 1 shows a diagram of the different primers used to amplify the heterozygote and each homozygote for the *Bsm*I and *Fok*I polymorphisms.

All amplifications were carried out in a total volume of 25 μl, containing 20 ng of DNA, 1× reaction buffer, 1.5 mM of $MgCl_2$, 200 μM of each four dNTPs, 0.4 μM of each primer and 1 U of EcoTaq DNA polymerase from Ecogen (Barcelona, Spain). The samples were subjected to 34 cycles consisting of 20 s at 94 °C (denaturation); and 50 s at 70 °C (*Bsm*I) or 71 °C (*Fok*I) (annealing and extension) in a MasterCycler gradient thermocycler from Eppendorf (Hamburg, Germany).

1.6. Individual PCR genotyping and restriction analysis

The PCR-RFLP methodology was also carried out to determine the *Bsm*I and *Fok*I polymorphisms. In this case, the primers 5′-CAACCAAGACTACAAGTACCGCGTCAGTGA-3′ and 5′-AACCAGCGGGAAGAGGTCAAGGG-3′ were used for *Bsm*I [21]; and the primers 5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ and 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′ for *Fok*I [22]. The PCR amplifications were performed as previously described, but using 30 s at 63 °C for the hybridization step and 1 min at 72 °C for the primer extension. The 822 bp (*Bsm*I) and 266 bp (*Fok*I) amplicons were digested with the appropriate (*Bsm*I or *Fok*I) restrictases, respectively from MBI Fermentas (Vilnius, Lithuania). The reaction products were segregated on AGE. Digested amplicons correspond to the "*b*" or "*f*" alleles, respectively; non digested amplicons correspond to the "*B*" or "*F*" alleles, respectively.

1.7. DNA pooling methodology

The concentration of each individual DNA sample was carefully measured at 260 nm in a DU64 spectrophotometer from BeckmanCoulter (Fullerton, CA, USA). Samples were then diluted to 10 ng/μl and 30 ng of each sample were "pooled." Three different pools were constructed, according to the BMD *T*-score: (i) those showing values higher than −2 (91 individuals); (ii) those with values between −2 and −2.5 (49 individuals); and (iii) those exhibiting values less than −2.5 (85 individuals). Thus, such tree DNA pools were used to determine the frequencies of the alleles "*B*," "*b*," "*F*" and "*f*" by quantitative real-time PCR (QRT-PCR; kinetic PCR).

1.8. QRT-PCR with DNA pools

The allelic frequencies of the DNA pools were determined by QRT-PCR using the LightCycler from Roche (Basel, Switzerland). The PCR amplifications were performed as previously described for the individual allele-specific genotyping, with the following modifications: no extra common primer was added, the $MgCl_2$ concentration was increased to 2.5 mM and SYBR green from Molecular Probes (Eugene, OR, USA) was added at dilutions of $1 : 15 \times 10^4$.

The relative quantitation of each allele was determined after Germer et al. [14]. To that goal, the Cycle threshold (Ct) of each amplification reaction was obtained. The Ct corresponds to the PCR cycle at which the fluorescence intensity of the reaction mixture is higher than a predetermined threshold. The Ct value can be fractional, being the difference between the calculated Ct values of alleles a function of the relative abundance of each allele in the DNA pool. Thus, a Ct difference of one cycle means that the allele with lower Ct is twice as abundant and a Ct identical for both alleles indicates a 50% percentage for each.

A total of four QRT-PCR reactions were carried out from each DNA pool and for each of the two alleles of a particular polymorphism, calculating the average mean of the different Ct values (Ct of allele 1 versus Ct of allele 2). Since the extension efficiency of each allele-specific primer is not usually the same, five replicas were obtained for each heterozygote allele, thus calculating the corresponding Ct averages (Ct′ of allele 1 versus Ct′ of allele 2). These means

were used to normalize the Ct values obtained on the DNA pools. The frequency of each allele in the pool was calculated with the formula $1/(2^{-Ct} + 1)$, where $\Delta Ct = (Ct \text{ of allele } 1 - Ct \text{ of allele } 2) - (Ct' \text{ of allele } 1 - Ct' \text{ of allele } 2)$ [14,15]. It should be indicated that the figure "2" in the formula denominator is actually "1 plus the extension efficiency." Yet, the PCR efficiency is near 100% at the initial exponential slope, thus the value "2" is a good fit under such experimental conditions.

1.9. Statistical analyses

Data was stored and managed using in the program dBASE III from Vestal (NY, USA) and analyses were performed with the "Statistical Package for Social Sciences" SPSS 9.0 (Chicago, IL, USA). The disparity among the groups was evaluated by analysis of variance (ANOVA) and Student's *t*-test as appropriate. The relationship between numerical variables was assessed by Pearson's correlation test. The distribution frequencies of the different genotypes were compared by using the χ^2 test. Evaluation of the influence of independent variables on BMD was tested by multivariate logistic regression. All tests were two-tailed and differences with $P < 0.05$ were considered significant.

2. Results

2.1. Allele-specific PCR amplification

Fig. 1 illustrates the allele-specific PCR amplification of the *Bsm*I and *Fok*I genes. The procedure amplifies differentially the two alleles when using the primers "1" or "2," respectively. It should be indicated that when the corresponding allele is not present at the SNP site, a faint amplification band was observed. We have also applied the RFLP-PCR methodology to 42 subjects, showing a full concordance with the results obtained with the allele-specific approach (data not shown).

The prevalence of the three *Bsm*I VDR genotypes in the cohort was 19.1, 44.9 and 36.0% for *BB*, *Bb* and *bb*, respectively. The allele frequencies were 41.6 and 58.4% for the *B* and *b* alleles, respectively. In the case of the *Fok*I locus, the prevalence of the genotypes was 40.4, 48.0 and 11.6% for *FF*, *Ff* and *ff*, respectively; the allele frequencies were 64.4 and 35.6% for the *F* and *f* alleles, respectively.

No statistical differences were found for age, weight or BMI between the *Bsm*I and *Fok*I polymorphisms. Moreover, no significant differences were found in lumbar spine and hip *T*-score of DEXA and quantitative ultrasound calcaneous densitometry between the Vitamin D receptor gene variants studied. Likewise, no statistical differences were found in the genotype distribution of the osteoporotic subjects using WHO criteria (*T*-score < -2.5) or using NOF criteria (*T*-score < -2).

2.2. Allele frequencies of DNA pools by kinetic PCR

We have established a functional osteoporosis classification based on the *T*-score values, taking into account both the WHO and NOF rankings. The Table 1 shows the allele frequencies and *T*-score data obtained using the DNA pooling approach, as compared to the previously known frequencies for the individuals used to generate the DNA pools. No differences were found when such methodologies were compared, as indicated in Table 1. The observed differences were just 0.06 or even less.

3. Discussion

In this work, we have use an allele-specific methodology to genotype both the *Bsm*I and *Fok*I loci of the *vdr* gene in postmenopausal women. This approach has several advantages when compared to the previous RFLP-PCR procedure

Table 1
Comparison of allelic frequencies obtained by individual genotyping and "DNA pooling" approaches

Group ($n = 225$)	Polymorphism	Allele	Frequency (known)[a]	Frequency (DNA pooling)	Frequency[b]
T-score > -2 ($n = 91$)	*Bsm*I	*B*	0.43	0.40	0.086
		b	0.57	0.60	
	*Fok*I	*F*	0.64	0.66	0.046
		f	0.36	0.34	
$-2 >$ *T*-score > -2.5 ($n = 49$)	*Bsm*I	*B*	0.36	0.30	0.055
		b	0.64	0.70	
	*Fok*I	*F*	0.69	0.72	0.030
		f	0.31	0.28	
T-score < -2.5 ($n = 85$)	*Bsm*I	*B*	0.43	0.37	0.060
		b	0.57	0.63	
	*Fok*I	*F*	0.62	0.64	0.058
		f	0.38	0.36	

[a] Frequencies obtained after individual genotyping.
[b] Calculated after Chen et al. [15].

[21,22]. Thus, no restrictase digestion is required, it is quicker and cheaper. Besides, the RFLP approach is prone to generate false positives due to partial or lack of DNA digestion; in fact this may be a common problem depending on DNA purity and the restrictase used. We have used an extra primer in the allele-specific amplification to avoid false positives. We have further optimized the methodology including an extra mismatch at the second 3′-end base, thus each PCR primer pair amplifies efficiently (i.e., exponentially) the target allele (just one mismatch) but not the alternative allele (two mismatches). It should be indicated that when no target allele is present in the reaction vial, a faint amplification band was observed (lines 2 and 1 of the genotypes *bb* and *BB*, respectively, on Fig. 1 panel B). The rationale of this result is that in the absence of a perfect target match, the DNA polymerase can sometimes extend the mismatched primer, albeit with very low efficiency [23].

Our results demonstrate that the new allele-specific approach can be used as a more convenient alternative to the RFLP-based procedure. In fact, the results obtained with both methodologies are identical. Moreover, the genotype and allele frequency of the population studied are similar to those obtained in the same ethnicity and geographical area (either Spain or Europe) both at the *Bsm*I [3,24,25] and *Fok*I [3]. There were no differences in age, weight or height, BMI and other parameters among the different genotypes in postmenopausal women.

No positive correlation was found between the BMD and the studied polymorphisms. This is in agreement with some published reports [26–28], yet not with others [22,29,30]. Since BMD is a subrogate of osteoporosis, our methodology could be applied to study patients with radiological criteria of osteoporotic fractures, because there have been relatively few studies of VDR gene variants and the risk of osteoporotic fractures [3,25,31,32].

In conclusion, we have applied for the first time the DNA pooling methodology to evaluate the allelic frequencies of the *vdr* polymorphisms in postmenopausal women. We have validated this procedure, obtaining similar results as those generated with other established approaches like individual RFLP-PCR or allele-specific amplification. In our hands, this is a very useful approach to study quantitative (thus polygenic) traits like the osteoporosis susceptibility, since very large number of individuals can be scored quickly. Other authors have already used the DNA pooling approach for large-scale association studies [13]. This is a high throughput methodology, being also fast, accurate and amenable to automation. Additionally, it requires very low DNA amounts, thus preserving scarce sample stocks.

Our data support the advantages of the DNA pooling approach to analyze other putative associations between the *vdr* polymorphism and some diseases like colon, breast and prostate cancer, osteoarthritis, atherosclerotic coronary artery disease, diabetes, primary hyperparathyroidism, infection, and psoriasis [5–11], among others.

Acknowledgements

This work was supported by “SANYRES XXI” (Grupo PRASA), “Fondo de Investigación de la Junta de Andalucía” 96/02 and “Grupo PAI” CTS413.

References

[1] R. Bouillon, W.H. Okamura, A.W. Norman, Structure–function relationships in the Vitamin D endocrine system, Endocr. Rev. 16 (1995) 200–257.
[2] T.V. Nguyen, P.J. Kelly, N.A. Morrison, P.N. Sambrook, J.A. Eisman, Vitamin D receptor genotypes in osteoporosis, Lancet 344 (1994) 1580–1581.
[3] J.M. Zmuda, J.A. Cauley, R.E. Ferrell, Vitamin D receptor gene variants and osteoporosis, Epidemiol. Rev. 22 (2000) 203–217.
[4] C.S. Glinda, Genetic studies of osteoporosis: what have we learned? J. Bone Miner. Res. 14 (1999) 1646–1648.
[5] T. Carling, P. Ridefelt, P. Hellman, J. Rastad, G. Akerstrom, Vitamin D receptor polymorphisms correlate to parathyroid cell function in primary hyperparathyroidism, J. Clin. Endocrinol. Metab. 82 (1997) 1772.
[6] A.G. Uitterlinden, H. Burger, Q. Huang, E. Odding, C.M. Duijn, A. Hofman, J.C. Birkenhager, J.P. Van Leeuwen, H.A. Pols, Vitamin D receptor genotype is associated with radiographic osteoarthritis at the knee, J. Clin. Invest. 100 (1997) 259–263.
[7] F.J. Van Schooten, A. Hirvonen, L.M. Maas, B.A. De Mol, J.C. Kleinjans, D.A. Bell, J.D. Durrer, Putative susceptibility markers of coronary artery disease: association between VDR genotype, smoking, and aromatic DNA adduct levels in human right atrial tissue, FASEB J. 12 (1998) 1409–1417.
[8] A.C. Lundin, P. Soderkvist, B. Eriksson, M. Bergman-Jungestrom, S. Wingren, Association of breast cancer progression with a Vitamin D receptor gene polymorphism, South–East Sweden Breast Cancer Group, Cancer Res. 59 (1999) 2332–2334.
[9] L. Correa-Cerro, P. Berthon, J. Haussler, S. Bochum, E. Drelon, P. Mangin, G. Fournier, T. Paiss, O. Cussenot, W. Vogel, Vitamin D receptor polymorphisms as markers in prostate cancer, Hum. Genet. 105 (1999) 281–287.
[10] W.J. Fassbender, B. Goertz, K. Weismüller, B. Steinhauer, H. Stracke, D. Auch, T. Linn, R.G. Bretzel, VDR gene polymorphisms are overrepresented in German patients with type 1 diabetes compared to healthy controls without effect on biochemical parameters of bone metabolism, Horm. Metab. Res. 34 (2002) 330–337.
[11] B.-Y. Park, J.-S. Park, D.-Y. Lee, J.I. Youn, I.G. Kim, Vitamin D receptor polymorphism is associated with psoriasis, J. Investig. Dermatol. 112 (1999) 113–116.
[12] T. Sasaki, T. Tahira, A. Suzuki, K. Higasa, Y. Kukita, S. Baba, K. Hayashi, Precise estimation of allele frequencies of single-nucleotide polymorphisms by a quantitative SSCP analysis of pooled DNA, Am. J. Hum. Genet. 68 (2001) 214–218.
[13] P. Sham, J.S. Bader, I. Craig, M. O’Donovan, M. Owen, DNA pooling: a tool for large-scale association studies, Nat. Rev. Genet. 3 (2002) 862–871.
[14] S. Germer, M.J. Holland, R. Higuchi, High-throughput SNP allele-frequency determination in pooled DNA samples in kinetic PCR, Genome Res. 10 (2000) 258–266.
[15] J. Chen, S. Germer, R. Higuchi, G. Berkowitz, J. Golbold, J.G. Wetmur, Kinetic polymerase chain reaction on pooled DNA: a high-throughput, high-efficiency alternative in genetic epidemiological studies, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 131–136.
[16] K. Xu, R.H. Lipsky, W. Mangal, E. Ferro, D. Goldman, Single nucleotide polymorphism allele frequencies determined by quantitative kinetic assay of pooled DNA, Clin. Chem. 48 (2002) 1605–1608.

[17] M. Diaz Curiel, J.L. Carrasco de la Peña, J. Honorato Perez, R. Perez Cano, A. Rapado, I. Ruiz Martinez, Study of BMD in lumbar spine and femoral neck in a Spanish population, multicentre research project on osteoporosis, Osteoporos. Int. 7 (1997) 59–64.
[18] J.A. Kanis, L.J. Melton, C. Christiansen, C.C. Johnston, N. Khaltaev, The diagnosis of osteoporosis, J. Bone Miner. Res. 9 (1994) 1137–1141.
[19] National Osteoporosis Foundation, The Physician's Guide to Prevention and Treatment of Osteoporosis, National Osteoporosis Foundation, Washington, DC, 1998.
[20] M. Sosa, P. Saavedra P, M. Muñoz-Torres, J. Alegre, C. Gomez, J. Gonzalez-Macias, N. Guanabens, F. Hawkins, C. Lozano, M. Martinez, J. Mosquera, R. Perez-Cano, M. Quesada, E. Salas, GIUMO Study Group, quantitative ultrasound calcaneous measurements: normative data and precision in the Spanish population, Osteoporos. Int. 13 (2002) 487–492.
[21] N.A. Morrison, J.C. Qi, A. Tokita, P.J. Kelly, L. Crofts, T.V. Nguyen, P.N. Sambrook, J.A. Eisman, Prediction of bone density from Vitamin D receptor alleles, Nature 367 (1994) 284–287.
[22] C. Gross, T.R. Eccleshall, P.J. Malloy, M.L. Villa, R. Marcus, D. Feldman, The presence of a polymorphism at the translation initiation site of the Vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women, J. Bone Miner. Res. 11 (1996) 1850–1855.
[23] S. Kwok, D.E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, J.J. Sninsky, Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies, Nucleic Acids Res. 18 (1990) 999–1005.
[24] J. Guardiola, X. Xiol, R. Sallie, J.M. Nolla, D. Roig-Escofet, E. Jaurrieta, L. Casais, Influence of the Vitamin D receptor gene polymorphism on bone loss in men after liver transplantation, Ann. Intern. Med. 131 (1999) 752–755.
[25] C. Gomez, M.L. Naves, Y. Barrios, J.B. Diaz, J.L. Fernandez, E. Salido, A. Torres, J.B. Cannata, Vitamin D receptor gene polymorphisms, bone mass, bone loss and prevalence of vertebral fracture: differences in postmenopausal women and men, Osteoporos. Int. 10 (1999) 175–182.
[26] P. Garnero, O. Borel, E. Sornay-Rendu, P.D. Delmas, Vitamin D receptor gene polymorphisms do not predict bone turnover and bone mass in healthy premenopausal women, J. Bone Miner. Res. 10 (1995) 1283–1288.
[27] J.E. Looney, H.K. Yoon, M. Fischer, S.M. Farley, J.R. Farley, J.E. Wergedal, D.J. Baylink, Lack of a high prevalence of the BB Vitamin D receptor genotype in severely osteoporotic women, J. Clin. Endocrinol. Metab. 80 (1995) 2158–2162.
[28] T.R. Eccleshall, P. Garnero, C. Gross, P.D. Delmas, D. Feldman, Lack of correlation between start codon polymorphism of the Vitamin D receptor gene and bone mineral density in premenopausal French women: the OFELY study, J. Bone Miner. Res. 13 (1998) 31–35.
[29] G. Gong, H.S. Stern, S.C. Cheng, N. Fong, J. Mordeson, H.W. Deng, R.R. Recker, The association of bone mineral density with Vitamin D receptor gene polymorphisms, Osteoporos. Int. 9 (1999) 55–64.
[30] S.S. Harris, T.R. Eccleshall, C. Gross, B. Dawson-Hughes, D. Feldman, The Vitamin D receptor start codon polymorphism (*Fok*I) and bone mineral density in premenopausal American black and white women, J. Bone Miner. Res. 12 (1997) 1043–1048.
[31] D. Feskanich, D.J. Hunter, W.C. Willett, S.E. Hankinson, B.W. Hollis, H.L. Hough, K.T. Kelsey, G.A. Colditz, Vitamin D receptor genotype and the risk of bone fractures in women, Epidemiology 9 (1998) 535–539.
[32] L. Gennari, L. Becherini, R. Mansani, L. Masi, A. Falchetti, A. Morelli, E. Colli, S. Gonnelli, C. Cepollaro, M.L. Brandi, *Fok*I polymorphism at translation initiation site of the Vitamin D receptor gene predicts bone mineral density and vertebral fractures in postmenopausal women, J. Bone Miner. Res. 14 (1999) 1379–1386.