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Article

Association of VDR Polymorphisms with Muscle Mass Development in Elite Young Soccer Players: A Pilot Study

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Abstract: Vitamin D receptor (*VDR*) is an important candidate gene in musculoskeletal phenotypes. Polymorphisms in *VDR* have been previously associated with several pathologies and muscular strength in athletes and elderly people, however literature reported contradictory results. The object of this research is to verify the association between the most studied *VDR* variants (rs2228570, rs7975232, and rs1544410) and the increase of muscle mass in elite young soccer players. A sample of 55 soccer players (15-18 years old) from a professional team was selected for this study. DNA was extracted by salting out method and polymorphisms were genotyped by PCR-RFLP, followed by 2% agarose gel electrophoresis. To test the effect of the three SNPs, logistic regression analysis was applied. Body composition was carried out through skinfold thickness method, and muscular area of arm and lower limb were calculated using Frisancho formulas. All three polymorphisms met Hardy-Weinberg equilibrium ($p > 0.05$) and their frequencies fell within the worldwide variability. A significant correlation between rs1544410 and increase of calf muscle mass was observed. Individuals carrying A allele showed higher calf muscular mass than those carrying the G allele ($p = 0.034$). Moreover, haplotype analysis applied to the two SNPs in linkage disequilibrium (rs7975232 and rs1544410), showed that AG haplotype resulted negatively correlated to calf muscle area. In conclusion, we confirm an association between *VDR* polymorphisms and muscular mass that could encourage the genetic screening of *VDR* gene to identify a potential risk of injury and for individual nutritional interventions.

Keywords: vitamin D receptor; SNPs; genotype; muscle mass

1. Introduction

Vitamin D, also called calciferol, is a fat-soluble molecule belonging to the family of steroid hormones and plays an important role in the regulation of calcium metabolism, bone [1], and muscle development. Vitamin D is involved in the mechanism of muscle contraction [2] and a strong relation between Vitamin D level and sarcopenia has been demonstrated [3]. Moreover, it has been shown that sufficient levels of vitamin D enable the maintenance and development of athletic performance; consequently, reduced levels of the vitamin inhibit muscle relaxation required after training and increase muscle pain. This leads to a major risk of injury and stress fractures, causing loss of muscle power and reducing bone mineral density [4].

Vitamin D can either be assumed with the diet or synthesized by the skin: UVB rays, by penetrating the epidermis, convert 7-dehydrocholesterol (7DHC) into vitamin D₃ (cholecalciferol).

Two subsequent hydroxylation steps occurring, respectively, in the liver and in the kidney convert vitamin D₃ into 1 α -25-dihydroxyvitamin D (1,25(OH)₂D or calcitriol), which is the biologically active molecule. Calcitriol enters the cell where it binds to the cytoplasmic vitamin D receptor (VDR), a protein constituted by 427 amino acids in humans, and then the hormone-receptor complex migrates to the nucleus, where it binds to the retinoid X receptor α (RXR α); finally, this trimeric complex binds to DNA, affecting the expression of several vitamin D target genes, orchestrating various biological processes [5].

VDR gene is located on human chromosome 12q13.11 and comprises 11 exons, encompassing about 75 kb. Exons 2 and 3 are involved in DNA binding, whereas exons 4 and 9 are involved in ligand binding [6].

It has been suggested that different polymorphisms in VDR gene can determine different response to strength training [7] and muscle injury [8], but results are still controversial [9]. In addition, VDR overexpression has been linked to the hypertrophic response to resistance exercise in humans [10].

Among the more than one hundred SNPs (Single Nucleotide Polymorphisms) present in the VDR gene, the most common and studied are rs228570 (FokI) located in exon 2, rs1544410 (BsmI) and rs7975232 (ApaI) both located in intron 8.

The FokI polymorphism is located at the 5' end of exon 2, corresponding to the start codon. This mutation involves the replacement of a guanine with an adenine, guanine being the ancestral allele. The A allele was found to be correlated to the maintenance of bone homeostasis [11–13]. The same allele has been significantly associated with total Fat Free Mass (FFM), appendicular FFM, and relative appendicular FFM, demonstrating a correlation with sarcopenia [14]; accordingly, males carrying GG genotype had significantly less FFM, appendicular fat-free mass and skeletal muscle index (SMI) compared to A allele carriers, and two-fold higher risk of being sarcopenic when compared to carriers of the A allele.

Other studies demonstrated an association between FokI polymorphism and muscle phenotypes with male GG homozygotes showing significantly lower values of knee extensor (KE) [15] and handgrip (HG) strength [16] than A allele carriers. On the contrary, a study conducted by [17] found AA individuals displaying significantly greater HG strength than G carriers.

Finally, a study conducted on soccer players [18] showed that analysis of the FokI polymorphism could help select young athletes who possess the most favorable genetic potential to succeed in soccer. Indeed, it was observed that the homozygous AA genotype of the FokI polymorphism was significantly more represented in young soccer players than in the control group.

BsmI, located at the 3' end of the VDR gene, shows a change from A to G, with the G allele representing the ancestral variant. Functional implications of this SNP include the possibility of altering splice sites involved in mRNA transcription or affecting intronic regulatory elements of the VDR gene.

Its relationship with risk of osteoporosis has been widely debated with conflicting results: a metaanalysis conducted on 26 studies [19] reported the possible association between BsmI polymorphism and the risk of bone fracture, suggesting a protective role for GG for osteoporosis risk. A different metaanalysis conducted on 41 studies [20] did not confirm such an association.

Studies on the association between BsmI polymorphism and muscle mass and strength also highlighted contrasting results. While several research denied any association between rs1544410 polymorphism and muscle mass phenotype [17], a work displayed higher quadriceps and grip strength for elderly with G allele [16]. In contrast, an association between AA genotype and higher hamstring strength was demonstrated in a sample of elderly women [21,22], suggesting a higher knee extensor strength for AA genotypes when compared with GG and AG genotypes.

ApaI, also located at the 3' end, shows a change from A to C, with the C allele representing the ancestral variant. The VDR ApaI is associated with increased bone mass concomitant with higher calcium intake [23], and it seems to influence the severity of musculoskeletal injuries in professional football players [8]. A recent meta-analysis [24] highlighted an increased risk for osteoporotic fracture

for AA genotype in the European population but not in the overall sample that included individual from the Americas (northern, central and southern).

Several studies are based on the analysis of the association between *VDR* polymorphisms and bone mineral density (BMD). Limited research focused on the association with muscular mass and strength and has led to contrasting results. A work by Iki [25] negated any difference in developing muscular strength for the different genotypes. On the contrary, an association between genotypes and hand grip strength has been suggested in research on elderly from Taiwan [26]. Moreover, a study by Wang [27] found lower knee and elbow concentric or eccentric peak torque among individuals carrying the *ApaI* A allele.

The aim of the present research is to verify the association between the most studied *VDR* variants (rs2228570, rs7975232, and rs1544410) and the increase of lean and muscle mass in young soccer players.

2. Materials and Methods

2.1. Sampling

55 male elite soccer players, 15 to 18 years old (mean: 16.65 ± 1.55 years), were selected for this study. All players included in the study were from the same professional team, participated in the Official National Football Championship (Primavera, Allievi, and Giovanissimi), and self-reported to be Italians for at least 3 generations. All study participants read and signed an informed consent, which was signed by the parents of underage athletes. This study was approved by the Ethics Committee of Azienda Ospedaliera Universitaria (AOU) of Cagliari University (Italy).

2.2. DNA Analyses

A buccal swab was taken by using a sterile cytobrush from each participant and DNA was extracted using the salting-out method.

Concentration and quality of the extracted DNA were measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific).

DNA amplification was performed by classical PCR method using the following primers for the three polymorphisms:

ApaI forward: 5'-CAGAGCATGGACAGGGAGCAAG-3'; reverse: 5'-CAACTCCTCATGGCTGAGGTCT-3';

BsmI forward 5'-CAACAAGACTACAAGTACCGCGTCAGTGA-3'; reverse 5'-AACCAGCGGAAGAGGTCAAGGG-3';

FokI forward 5'-GATGCCAGCTGGCCCTGGCACTG-3'; reverse 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'.

Each reaction mix (total 25 μ l) for all three polymorphisms included 2 μ l of DNA, 0.2 μ l of the forward and reverse primers, 10.6 μ l of water, and 12 μ l of NZYSupreme qPCR Green Master Mix 2x (Nzytech, Portugal).

Enzymatic digestion was performed with restriction enzymes *ApaI*, *BsmI*, and *FokI* for allele recognition.

Genotyping was performed by electrophoresis technique, with a 2% agarose gel for *ApaI* and *BsmI* and a 10% polyacrylamide gel for *FokI*, observing the following DNA fragments: for *ApaI* (A:740 bp, C:515+225 bp); for *BsmI* (A: 825 bp, G:650+175 bp); for *FokI* (G:272 bp, A:198+74 bp).

2.3. Body Composition Determination

For each subject height and weight were measured according to standard procedure [28].

Body composition parameters were calculated using the skinfold equations. Two different formulas were used to calculate the percentage of Fat Mass (FM) and Fat Free Mass (FFM): Slaughter's formula [29], designed specifically for boys (up to age 18) using measurements of the tricipital skinfold and subscapular skinfold; and Reilly's formula [30], specifically validated for young soccer

players (age 16.67 ± 0.5 years) by [31], that uses measurements of the thigh, abdominal, triceps, and calf skinfold.

The muscle area of the arm (AMA), calf (CMA), and thigh (TMA) were calculated according to Frisancho formula [32]. For AMA correspondence to proper percentile for each participant was also verified.

2.4. Statistical Analyses

Allele and genotype frequencies, as well as Hardy-Weinberg were calculated with Genepop software (ver.4.4.3).

One-way ANOVA was used to evaluate the hypothesis of equality of means among genotypes in relation to all body composition measures. Analyses were performed with the R 4.2.3 program.

Genotypic association analysis with fat-free mass and muscle mass was performed with software SNPstats (<https://snpstats.net/>)

Given the variability of the three polymorphisms across 1000 genomes, the population branch statistic (PBS), a summary statistic that exploits pairwise F_{ST} values among three populations, was calculated to check for evidence of natural selection within the *VDR* gene [33]. A population’s PBS value represents the amount of allele frequency change at a given locus in the history of this population.

The VCF (Variant Call Format) file of chromosome 12 was downloaded by the 1,000 Genomes Database Phase 3. From the VCF file of the whole gene, only SNPs with Minimum Allele Frequency (MAF) ≥ 0.05 were selected, using Plink 1.9 [34]; next, F_{ST} index was calculated among three potential population pairs: YRI, CEU, and CHB, using the program VCFtools [35]; finally, PBS was calculated and the two-dimensional graph created using R Studio [36]. In order to obtain further information regarding the possible natural selection of the *VDR* gene, Tajima’s D was calculated using VCFtools.

3. Results

Allele and genotype frequencies are shown in Table 1. Hardy-Weinberg test revealed that all samples were in equilibrium for all loci ($p > 0.05$). The three SNPs showed a high worldwide genetic variability (1,000 Genomes dataset), and allele frequencies of our sample fell within the worldwide range; ApaI and BsmI frequencies were also included within the European range (Table 1).

Table 1. Allele and genotype frequencies. Values are indicated in percentages, while absolute values are shown in parentheses.

VDR ApaI rs7975232 (N = 55)				
A	C	AA	AC	CC
58.18	41.82	30.91 (17)	54.54 (30)	14.55 (8)
VDR BsmI rs1544410 (N = 54)				
G	A	GG	AG	AA
55.56	44.44	35.18 (19)	40.74 (22)	24.08 (13)
VDR FokI rs2228570 (N = 54)				
A	G	AA	AG	GG
25.93	74.07	3.71 (2)	44.44 (24)	51.85 (28)

Considering the high variability of allele frequency and the correlation of the gene with several diseases, we examined, through PBS, possible traces of natural selection using data from 1000 genomes. Some SNPs had PBS values near or equal to the limits of the distribution, but not the specific polymorphisms we focus on. They fell on both lines and had consistently low PBS values, suggesting the absence of selection (Figure 1).

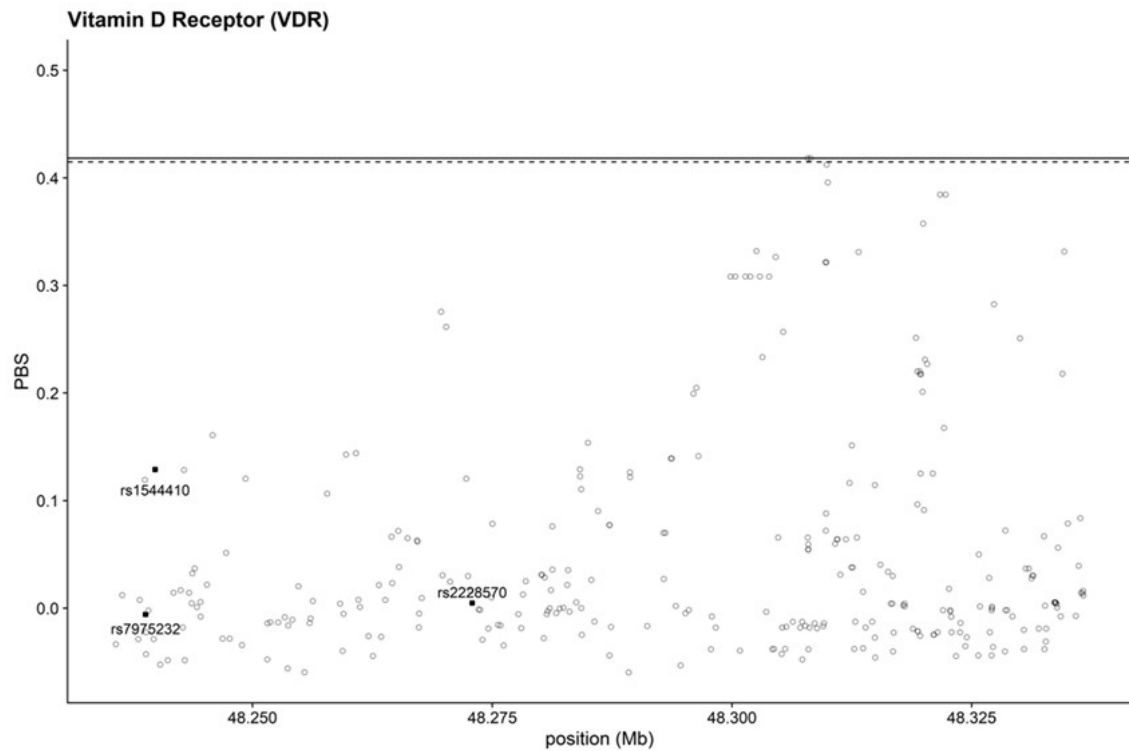


Figure 1. PBS representation of *VDR* gene. Dashed and horizontal lines represent the 99.5th and 99.9th percentiles of the empirical distribution, respectively. SNPs under scrutiny are indicated with a square.

The absence of selection was confirmed by *D'* Tajima test: positive values were obtained for the three SNPs (2.486 for *ApaI*, 1.957 for *BsmI*, and 2.113 for *FokI*), indicating low levels of low- and high-frequency polymorphisms.

One-way ANOVA test was used to assess any difference in fat-free mass between the different genotypes. The data showed no significant difference between genotypes for any considered variables ($p > 0.05$, Table 2). It is noteworthy a weak trend for increasing CMA values from CC to AA for *Apa I* (88.09, 94.27, 95.72 for CC, AC, and AA, respectively).

Table 2. p values calculated for ANOVA test.

	ApaI		BsmI		FokI	
	F value	p	F value	p	F value	p
FFM Slaughter	0.997484	0.375752	0.105224	0.900317	1.016038	0.369237
FFM Reilly	0.538281	0.586968	0.046829	0.954292	0.290343	0.749235
AMA	1.022569	0.366788	0.08582	0.917892	1.449376	0.244221
CMA	1.132188	0.330143	2.263845	0.1143	0.100998133	0.90411506
TMA	0.536284	0.588118	1.382144	0.260282	0.536284	0.588118

F value: the test statistic from the F test. p: the p-value of the F statistic.

The logistic regression analyses revealed a significant association between *BsmI* polymorphism and CMA under the dominant model: athletes carrying the A allele developed higher calf muscle mass than GG homozygous (Table 3). Moreover, although not statistically significant, we found higher values of CMA and AMA in individuals with *FokI* AA genotype under the recessive model (97.32 vs 93.49 and 54.22 vs 49.71 respectively).

Table 3. BsmI association with CMA.

Model	Genotype	n	Response means (s.e.)	Difference (95% CI)	p-value	AIC	BIC
Codominant	GG	19	89.04 (2.58)	0.00	0.11	420	430
	AG	22	95.37 (2.93)	6.93 (0.03–13.82)			
	AA	13	97.18 (2.34)	6.79 (–1.18–14.77)			
Dominant	GG	19	89.04 (2.58)	0.00	0.034	418	426
	AG – AA	35	96.05 (2.02)	6.88 (0.68–13.08)			
Recessive	GG – AG	41	92.44 (2.02)	0.00	0.4	422	430
	AA	13	97.18 (2.34)	3.16 (–4.15–10.46)			
Overdominant	GG -AA	32	92.34 (1.92)	0.00	0.19	421	429
	AG	22	95.37 (2.93)	4.26 (–1.99–10.52)			
Log-additive				3.71 (–0.25–7.67)	0.072	419	427

AIC: Akaike Information Criterion. BIC: Bayesian Information Criterion.

To test the combined effects of rs7975232 and rs1544410 SNPs, we apply logistic regression analysis to the haplotypes. The linkage disequilibrium of these variants for European population was previously verified through the LDlink program [37], that attested a strong linkage between ApaI and BsmI. This result was confirmed in our sample applying D statistics through SNPstats program (<https://www.snpsstats.net/start.htm>).

A significant association has been found between CMA and haplotype AG (Table 4): the negative value of difference (95% CI) suggests significant low muscle mass for individuals carrying AG haplotype.

Table 4. Haplotype association with response with CMA.

ApaI	BsmI	Frequency	Difference (95% CI)	p-value
A	A	0.4138	0.00	---
C	G	0.3807	–3.68 (–8.74–1.39)	0.16
A	G	0.168	–6.49 (–12.59–0.4)	0.04
C	A	0.0375	–11.09 (–27.62–5.45)	0.19

In addition, despite not reaching the level of significance, haplotype AG showed lower muscle mass also for arm and thigh area (data not shown).

4. Discussion

Vitamin D has been proven to influence muscle activity and performance [38]. Literature shows some evidence on the association between Vitamin D receptor polymorphisms and muscle strength, but the issue is still under debate [5]. Most of the literature deals with the relationship between VDR polymorphisms and decrease muscle mass with aging or the analysis of muscular trait in young athletes [17].

In this study, we analyzed the association between VDR polymorphisms and muscle mass, in a sample of elite young soccer players from a professional Italian team, to verify if the increase of muscular mass can be influenced by VDR variants.

PBS analysis showed no evidence of selection for the three polymorphisms: their high level of variability is likely due to random evolutive factors, such as genetic drift.

Previous research has focused on the correlation between VDR polymorphisms, muscle mass and sarcopenia in elderly [14,39] and found that individuals with FokI GG genotype were more likely to have lower muscle mass and to develop sarcopenia than AA and AG genotypes. Our sampling includes only young athletes and therefore we could not detect correlation with sarcopenia; however, our data are in good agreement with previous reports, suggesting that FokI rs 2228570 can lead to different response to training by increasing muscle size and strength [20] even in young soccer players. Indeed, logistic regression highlighted higher values of CMA in individuals with AA genotype, suggesting that the AA genotype is a favorable factor to develop muscle mass and protect

from sarcopenia [14,18], confirming the role of FokI rs2228570 in developing muscular mass. The molecular and physiological basis for this association is still uncertain, but previous studies suggested that the G allele produces an isoform with a decreased ability to induce transcription compared to the isoform coded by the A allele variant [40], and, consequently, a reduced muscle mass.

Moreover, our study suggested a significant correlation between BsmI polymorphism rs1544410 and the development of calf muscle mass. The dominant model shows a statistically significant increase of CMA among individuals carrying A allele. This result partially confirms previous studies, that demonstrated higher values of hamstring strength in individuals with AA genotype, although the authors did not find any correlation with muscle mass [21,22].

Finally, ApaI rs7975232 did not show any statistical association with muscle mass, even though a weak trend versus a higher increase of CMA was evident in CC carriers respect to AA carriers. To the best of our knowledge only one study found similar results: a research carried out on elderly from Taiwan showed an association between hand grip strength and homozygous CC females [26].

The role of rs7975232 ApaI and rs1544410 BsmI SNPs in VDR activity is not fully understood, but it has been hypothesized that, despite being located in a not translated region of the gene, these two SNPs could have a role in mRNA stability, due to their proximity to the poly-A tail [41].

To verify the combined effect of the two SNPs in linkage disequilibrium on muscle mass, we analyzed the correlation between haplotype and muscle mass, and, to our knowledge, this is the first time that this approach was used. Haplotype AG appeared negatively correlated with muscular mass of arm, calf, and thigh, reporting a significant value in the correlation with CMA. This datum is particularly useful for its possible application in the field of sports science, since individuals carrying the AG haplotype, given the same amount of training, seem to develop less muscle mass. It would be important to be able to confirm whether this datum also determines earlier sarcopenia in the elderly age.

5. Conclusions

This study supports the importance of studying the simultaneous effect of different allelic variants to better define the relationship between genotype and phenotype.

Our data confirm the role of FokI rs2228570 in developing muscular mass: AA homozygotes are more likely to develop muscle mass and therefore to be protected from sarcopenia. In addition, the association found between BsmI rs1544410 and AG haplotype (ApaI rs7975232 and BsmI rs1544410) with increase of calf muscle mass suggests the potential use of genetic screening for the VDR gene in athletes or in elderly individuals, to identify a potential risk of injury and/or for individual nutritional interventions, such as vitamin D supplementation, as suggested by previous papers [38,42].

Although the sample size is not large, we believe that the highly homogeneous population tested may be considered adequate for a pilot study. However, analysis of a much larger sample size is required to validate our conclusions.

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Conflicts of Interest: The authors declare no conflicts of interest.

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