



Universitat de Lleida

Document downloaded from:

<http://hdl.handle.net/10459.1/67920>

The final publication is available at:

<https://doi.org/10.1007/s10522-010-9273-8>

Copyright

(c) Springer Science+Business Media B.V., 2010

Title:

Tagging long-lived individuals through Vitamin-D receptor (*VDR*) haplotypes

Authors:

Marina Laplana, Manuel Sánchez-de-la-Torre, Alicia Aguiló, Ignasi Casado, Miquel Flores, Ramon Sánchez-Pellicer and Joan Fibla

Affiliations:

Marina Laplana, Manuel Sánchez-de-la-Torre, Joan Fibla

Departament de Ciències Mèdiques Bàsiques, Unitat de Genètica Humana, IRBLleida, Universitat de Lleida. C/ Montserrat Roig, 2. 25008 Lleida, Spain.

Alicia Aguiló, Ignasi Casado, Miquel Flores, Ramon Sánchez-Pellicer

Centre de Salut Balafia-Pardinyes-Secà. Àrea Bàsica de Lleida. Institut Català de la Salut. C/ Vidal i Codina, s/n. 25005 Lleida, Spain.

Author for correspondence:

Dr. Joan Fibla

Tel: +34 973702473

Fax. +34 973702426

e-mail address: joan.fibla@cmb.udl.cat

Abstract

Longevity is a complex phenomenon involving multiple environmental and biological factors. Genetic studies of longevity have focussed on DNA repair, oxidative damage correction and immune-related genes. Vitamin-D works by modulating mineral homeostasis and key physiological processes such as cell proliferation and immune response. Vitamin-D insufficiency has been largely considered a risk factor for life-limiting illnesses including cardiovascular and immune-related diseases and cancer. Vitamin-D acts through vitamin-D-receptor (VDR), which regulates the expression of vitamin-D-response genes. *VDR* variants have been associated with susceptibility to cancer, bacterial and viral infections, autoimmune diseases, and allergies. In the present study we evaluated *VDR* as candidate gene involved in human longevity. We selected five polymorphisms of the *VDR* gene that capture variability at the 5', coding and 3' regions. Genotype data was obtained from 104 octogenarians (>85y) and 114 controls (17-40y). Although differences in genotype and allele distribution did not reach statistical significance, haplotype distribution was distinctive, mainly for 3' region haplotypes and particularly in males. Our results suggest a role for *VDR* gene variability in aging and longevity.

Key words:

Vitamin-D, VDR, haplotypes, polymorphism, longevity, octogenarians

Introduction

Longevity is a complex phenomenon in which environmental, social, stochastic and biological factors are involved. Maximum lifespan potential of individuals has a strong biological component; population life expectancy is a multifactorial trait based on both biological and non-biological factors. In humans, as in other species, long-lived individuals emerge as a result of a successful life, lack of stochastic damaging factors and healthy aging. Recent socioeconomic improvements and advances in prevention and treatment of diseases have reduced infant mortality as well as allowing individuals to survive to extreme old age. As a result, life expectancy in humans has increased in developed countries, with no effect on maximum lifespan potential. Social and technological improvements have been crucial to increased human longevity, creating a new scenario in which longevity-related biological factors have been able to operate. The aging process has been considered the result of the accumulation of damages in cells and tissues that, left uncorrected, produces a systemic dysfunction concluding in death. Throughout the lifespan, however, cell maintenance mechanisms, such as DNA-repairing enzymes, correction of oxidative damage and immune system response against pathogens and tumour cells, attempt to correct the damage.

Among environmental factors acting on health status, vitamin-D has been largely considered protective and a promoter of good health (Holick and Chen 2008). Pleiotropic effects of vitamin-D have been largely known to affect mineral metabolism, cell proliferation and immune response modulation. Vitamin-D status has been described as involved in life-limiting diseases (Heaney 2008) such as cancer (Garland et al. 2009), cardiovascular diseases (Gouni-Berthold et al. 2009), osteoporosis (Lash et al. 2009), sarcopenia (Visser et al. 2003) and diabetes (Danescu et al. 2009). In addition, vitamin-D has an important role in the modulation of the immune response, inducing microbicide factors against bacterial infections (Wang et al. 2004) and controlling the exacerbation of the cellular immune response (Deluca and Cantorna 2001). Based on this, it has been proposed a major positive role for vitamin-D in biogerontology by reducing susceptibility in the elderly to chronic degenerative diseases (Hayes 2010).

Sources of vitamin-D come from dietary intake, mainly oily fish such as salmon, mackerel and sardines (Chen et al. 2007) and egg yolks (Lamberg-Allardt 2006), and from endogenous synthesis by photolytic reaction of vitamin-D precursors in the skin (Lips 2006). Both types of sources are strongly linked to environment, such as climate and latitude, and social behaviour, such as daily diet, sun exposure and clothing. In addition, demographic factors must be considered, such as culture habits, gender and age, which act in several ways to change social behaviour as well as race, which is related to melanin pigmentation (Chen et al. 2007). Genes coding for vitamin-D metabolic enzymes and downstream mediators of vitamin-D signalling constitute a complex gene network. Vitamin-D receptor (*VDR*) plays a pivotal role in this network, acting as a ligand-dependent transcription factor that modulates the expression of vitamin-D responsive genes (Pike 1991).

Several *VDR* polymorphisms with functional effects have been described in the promoter, coding and 3' untranslated region (UTR) (Uitterlinden et al. 2004a). Two common polymorphisms, *Cdx* (rs1568820) and *A1012G* (rs4516035), have been located in the 5' regulatory region that affect binding to transcription factors. The rs1568820 polymorphism is a G to A transition between exons 1f and 1e that alters the recognition site for the intestinal-specific transcription factor "caudal-related homeodomain protein-2" (*Cdx-2*), affecting vitamin-D-

VDR-mediated intestinal calcium absorption (Arai et al. 2001). The rs4516035 polymorphism is an A to G transition located between 1e and 1a exons that modifies the GATA-3 transcription factor recognition sequence, involved in the regulation of Th2 polarization (Halsall et al. 2004). In addition, a common *FokI* (rs10735810) polymorphism has been described in the *VDR* coding region that alters the first ATG start site to an alternate ACG sequence, giving rise to a VDR protein that is three amino acids shorter (424 aas vs. 427 aas) (Gross et al. 1996). Functional differences among rs10735810 genotypes shows that VDR protein coded by the *C-VDR* allele (short 424 aas) interacted more efficiently with TFIIB and showed greater transcriptional activity than the full-length VDR protein coded by *T-VDR* allele (Jurutka et al. 2000). Finally, a common restriction fragment length polymorphism (RFLP), designated as *BsmI* (rs1544410), and a microsatellite polymorphism, designated as *PolyA* (rs17878969), has been described in the 3'-UTR region (Morrison et al. 1994; Ingles et al. 1997). Functional effects of 3'-UTR variants have been related to alterations in *VDR* mRNA stability (Fang et al. 2005).

Knowledge of the genomic architecture is important for designing association studies of complex phenotypes, as revealed by recent research on the linkage disequilibrium (LD) pattern of the human genome. LD refers to the non-random transmission of alleles of neighbouring *loci* that is high for closer *loci* and decreases with distance. In addition, recombination rate is a determinant factor for decreasing LD. The underlying structure of LD in the human genome reveals a series of discrete blocks of high LD interrupted by hotspot recombination sites. LD blocks range from 5 to 50 kb and are characterized by a reduced diversity of allelic combinations (haplotypes) (Wall and Pritchard 2003). Studies of the *VDR* genomic region in Caucasians have revealed a scattered LD pattern with rs1568820 and rs4516035 polymorphisms included in a single block of the *VDR* promoter and rs1544410 and rs17878969 polymorphisms included in a 3'UTR block. Blocks are separated by a hotspot recombination region that includes the rs10735810 polymorphism (Fang et al. 2005).

Several studies reveal that polymorphisms at *VDR locus* are associated with life-limiting diseases such as cancer, cardiovascular diseases and immune system disturbances (Uitterlinden et al. 2004b). Therefore, we propose *VDR* as a candidate gene involved in human longevity as a result of its protection from age-related diseases and modulation of the immune response. Taking advantage of the known haplotype block structure of the *VDR* genomic region, we performed an association study for longevity considering both single genotypes and haplotypes.

Material and Methods

Subjects

In total, 214 Caucasian subjects from the city of Lleida in northeast Spain were included between January and June 2004: 104 octogenarians (>85 years), 72 women (mean age 89.4±3.6; range 85-100) and 32 men (mean age 89.4±3.2; range 85-97), and 110 sex-matched healthy young individuals, 75 women (mean age 28±5.8; range 17-39) and 35 men (mean age 29±8.6; range 17-40), were selected as cases and controls, respectively. A comprehensive medical history was obtained from each octogenarian, focussing on the absence of vitamin-D related illness such as osteoporosis, autoimmune diseases, immunodeficiency and cancer. Written informed consent for enrolment in the study and for personal data management was obtained from all subjects in accordance with Spanish laws. The Ethics Committee for our institution approved the study.

DNA sources and Genotyping

Genomic DNA from controls was extracted from peripheral blood lymphocytes using a KG-Midi extraction kit (Camgen, Cambridge, UK). DNA from octogenarians was obtained from buccal swabs using the BuccalAmp DNA extraction kit (Epicentre, Madison, USA). *VDR* genotyping followed protocols described by de la Torre et al. (2008). For each subject we obtained genotype information from five polymorphic variants located in the *VDR* gene at: 5' promoter region, rs11568820 and rs4516035; coding region, rs10735810 and 3'UTR, rs1544410 and rs17878969 (Fig.1A). All markers were coded as biallelic, including the rs17878969 microsatellite polymorphism that shows a biphasic distribution of short (<18 adenosine repeats, S) and long (≥18 adenosine repeats, L) allele populations (Ingles et al. 1997).

Statistical analysis

We compared genotype and allele distribution between controls and octogenarians by contingency table analysis performed by the OpenEpi web resource (Sullivan et al. 2009). Fisher exact test P values were considered in each comparison. Correction for multiple testing was done applying the SNP spectral decomposition approach, which takes into account the linkage disequilibrium between markers (Nyholt 2004). The significance threshold required to maintain a Type I error rate of 5% was P=0.036 for two-marker haplotypes and P=0.011 for five-marker haplotypes. Pair-wise linkage disequilibrium was measured by the normalized D' coefficient (Lewontin 1968) that was estimated using Haploview 4.2 software (Barrett et al. 2005). Absolute D' values range from 0 (no disequilibrium), indicating that alleles are randomly distributed and all expected haplotypes are observed, to 1 (complete disequilibrium), indicating a non random distribution of alleles with a less observed haplotypes than expected.

We used PHASE 2.1.1 software (Stephens et al. 2001) to perform statistical inference of haplotypes from our sample data. Only data from fully genotyped subjects, 86 octogenarians (25 males) and 101 controls (35 males), were considered. We used the "out_pair" PHASE output file to assign haplotypes for each individual, with their corresponding probability. Haplotype counts were estimated by obtaining the cumulative probability of each haplotype. In brief, for an individual bearing haplotype A with probability 0.3 and haplotype B with probability 0.7, 0.3 and 0.7 were added to the corresponding counts of haplotypes A and B, respectively. Following the same rationale, we estimated the presence of 0, 1 or 2 copies of a specific haplotype in each individual.

Comparison of global haplotype distribution between controls and octogenarians was done by the “product of approximate conditionals” (PAC)-likelihood test implemented in PHASE 2.1.1 (Li and Stevens 2003). Differences between groups for specific haplotypes were evaluated by 2x2 contingency table analysis performed by the OpenEpi web resource.

Results

The genotype and allele distribution conform to Hardy-Weinberg equilibrium for all markers in both the control and octogenarian samples. We evaluated differences in genotype and allele distribution between octogenarians and controls, considering all subjects, gender between groups and gender within each group (Table 1). No statistical differences were observed for single-marker genotype association modelled as dominant (common allele homozygotes and heterozygotes vs. rare allele homozygotes), recessive (rare allele homozygotes vs. common allele homozygotes and heterozygotes) and codominant (cross genotype comparison). Allele distribution did not differ in any comparison performed (Table 1).

Linkage disequilibrium pattern in the control sample was in accordance with available data for Caucasian populations (Fang et al. 2005) and block structure ($D' > 0.8$) was observed for markers rs11568820-rs4516035 (block-1, Fig.1B) and markers rs1544410-rs17878969 (block-2, Fig.1B). Exon-2 polymorphism (rs10735810) did not show any significant D' with the 5' or 3'UTR markers. In contrast, a modest D' value ($D' < 0.68$) was observed in the octogenarian sample (Fig.1B).

According to the observed LD pattern, haplotypes were independently inferred from 5' and 3'UTR markers (block 1 and 2, respectively). No statistical differences were observed for 5'UTR haplotype distribution (rs11568820 and rs4516035, block 1) between controls and octogenarians. However, statistically significant differences were observed for global 3'UTR haplotype distribution (rs1544410 and rs17878969, block 2) between controls and octogenarians (PHASE PAC-likelihood test $P=0.01$) (Fig.2A), mainly due to differences in male subjects (PHASE PAC-likelihood test $P=0.01$) (Fig.2B). Haplotype-specific test revealed that rs1544410-G:rs17878969-S (GS) haplotype was overrepresented in octogenarians (7.3% vs. 0.8%; odds ratio (OR): 15.3, 95% confidence interval (CI): 2.3-637; $P < 0.001$) (Fig.2A). This difference was greater in males (14.2% in octogenarians vs. 0.3% in controls, OR out of range, $P=0.006$) (Fig.2B). We also estimated the haplotype carrier status of each individual. The distribution of carriers of at least one copy of 3'UTR GS haplotype is presented in Table 2. Carriers of at least one copy of GS haplotype were common in octogenarians (13.3%) but rare in controls (1.6%) ($P=0.008$). Among males, nearly a quarter of the octogenarians carried at least one copy of GS haplotype, whereas GS carriers represent less than 1% of controls (24.4% vs. 0.7%, respectively; $P=0.013$).

Extended five-marker haplotypes were also estimated. Prevalence in controls and octogenarians is presented in Fig.3. Extended five-marker haplotype counts and frequencies were compared between groups. Global five-marker haplotype distribution showed statistically significant differences (PHASE PAC-likelihood test $P=0.020$) (Fig.3A), again mainly contributed by differences in males (PHASE PAC-likelihood test $P=0.020$) (Fig.3B).

Five-marker haplotype-specific test revealed that rs11568820-G:rs4516035-G:rs10735810-C:rs1544410-G:rs17878969-S (GGCGS) haplotype was differentially distributed between groups (0.25% in controls vs. 4.1% in octogenarians, OR: 11.5; 95% CI: 1.2-621, $P=0.024$; Fig.3A). Again, these differences were mainly due to males (0.07% in controls vs. 9.5% in octogenarians, OR out of range; $P=0.014$; Fig.3B). The distribution of carriers of at least one copy of GGCGS haplotype (0.5% in controls and 8.3% in octogenarians, $P=0.023$) is presented in Table 2. Males carrying at least one copy of GGCGS haplotype represent just 0.1% in controls but 18.9% in octogenarians ($P=0.012$).

Discussion

Long-lived people escaped or delayed major age-related illnesses such as cardiovascular diseases and cancer, as well as life-threatening infections and immune-related diseases. It is noteworthy that vitamin-D plays a key role in these impairing diseases (Holick 2005). A general concern exists about the vitamin-D insufficiency that has been found in older persons (Holick 2006). Recent data indicates that low serum levels of 25-hydroxyvitamin-D are associated with all-cause mortality in the general population (Dobnig et al. 2008) as well as in older men and women (Pilz et al. 2009). However, the question of whether vitamin-D deficiency is a cause or a consequence of poor health status remains to be answered.

Additional evidence supports a role for vitamin-D in the aging process (Lanske and Razzaque 2007; Tuohimaa 2009). Studies in animal models have revealed a paradoxical effect of vitamin-D on aging. Genetically modified mice lacking *FGF23* and *Klotho* genes exhibit features of premature aging that have been related with an altered mineral homeostasis and hypervitaminosis-D due to increased 1- α -hydroxylase activity (Razzaque and Lanske 2006). At the same time, null mice for the vitamin-D receptor gene, in which vitamin-D action cannot occur, also show features related with premature aging (Keisala et al. 2009). According to this, it has been proposed that aging shows a hormetic U-shaped dependency on hormonal forms of vitamin-D where both hyper- and hypovitaminosis are risk factors for abnormal aging (Tuohimaa 2009). This bimodal effect of vitamin-D levels also has been described for vascular disease risk in children on dialysis (Shroff et al. 2008) and prostate cancer risk in Nordic countries (Tuohimaa et al. 2004). Therefore, it can be assumed that for good health status there is a narrow range for optimal serum concentration of vitamin-D forms.

VDR acts as a nuclear receptor for vitamin-D and is responsible for its genomic action. Several *VDR* gene polymorphic variants have been described as affecting *VDR* gene expression (rs11568820 and rs4516035), protein structure (rs10735810) and mRNA stability (rs1544410 and rs17878969) (Fang et al. 2005; Uitterlinden et al. 2004a). Variability at the *VDR* locus has been associated with cancer, osteoporosis, cardiovascular diseases and diabetes (Uitterlinden et al. 2004b) as well as mycobacterium (Bellamy and Hill 1998) and viral infections (Fibla and Caruz 2010).

While the vitamin-D effect on impairing diseases and aging has been well studied, the effect of *VDR* variability has been evaluated only in relation to diseases; no data exist about its role in aging and longevity. In the present case-control study, we compared allele, genotype and haplotype distribution of five *VDR* gene variants between healthy-living octogenarians and younger controls to assess *VDR* as a candidate gene affecting aging and longevity. Our study is the first to provide evidence of an association between *VDR* gene polymorphisms and longevity, as reflected by the differential distribution of *VDR* haplotypes that we observed. The positive significant association between longevity and the 3'UTR GS and five-marker GGCGS haplotypes was observed in octogenarian males but not in women. Epidemiological data on long-lived subjects document a clear-cut prevalence of females compared to males, suggesting that sex-specific mortality rates follow different trajectories. Demographic and behavioural risk factors have been considered in the literature, but do not account for the overall sex-related difference in mortality rates (Wingard 1984). In addition, the role of biological factors has been explored with contentious results (Austad 2006). Our finding of a sex-specific association was in accordance with previous reports that certain polymorphisms of the *interleukin (IL)-6*, *IL-10* and *interferon-gamma* genes are male-specific markers for longevity (Bonafe et al. 2001; Di Bona et al. 2009; Giacconi et al.

2004; Lio et al. 2002a; Lio et al. 2002b), supporting a finding that female longevity is less dependent on genetic background than male longevity (Franceschi et al. 2000).

With respect to the biogerontological significance of the observed association, we can only suggest a reasonable potential explanation. From functional studies, the common 3'UTR haplotypes GL and AS have been linked to low and high mRNA stability, respectively (Fang et al. 2005). However, no causative effect has been assigned to rs1544410 or rs17878969 polymorphisms. The main resulting hypothesis is to consider those polymorphisms as markers for an unknown causative element located in the 3'UTR region. In octogenarians, we found an increase of the GS haplotype prevalence. GS haplotype combines elements of the two common GL and AS haplotypes, from which we can expect an intermediate phenotype. Assuming the vitamin-D effect on the aging process follows a hormetic U-shaped model (Tuohimaa 2009), haplotypes conferring low (GL) and high (AS) *VDR* mRNA stability contributes to an adverse Vitamin-D response caused by deficiency and toxicity, respectively. However, haplotypes conferring a middle *VDR* mRNA stability (i.e. GS) can be considered as protective, as Vitamin-D response can remain in the homeostatic region, producing neither deficiency nor toxicity.

No previous study has been specifically designed to evaluate differences in *VDR* variants between young and elderly subjects. The literature contains indirect data on genotype frequencies of *VDR* markers, but comparison with our data is inappropriate due to differences in the origin of each population and in selection criteria. We acknowledge that our study has a number of potential limitations. Although octogenarian Caucasian cohorts may have common characteristics, such as the limited medical care available until the 1950s, we cannot exclude potential confounding factors. In particular, two historical events must be taken into account: the Spanish flu epidemic at the beginning of the 20th century, which depleted the childhood population, and the Civil War (1936-1939) that had an especially important impact on male mortality (González et al. 1996). The first event could have produced biological consequences, while the second had a stochastic effect. Another concern refers to the comparison of different birth cohorts, within which we assumed that initial frequencies are similar (Capri et al 2008). We cannot exclude this confounding factor in our study. However, our cohorts fit Hardy Weinberg equilibrium in all *loci* analyzed, and neither disturbances on random mating nor selective factors attributable to the *VDR locus* can be expected to act before reproductive age. Therefore, we could hypothesize that genotype and allelic frequencies are maintained over generations.

In conclusion, the well-documented impact of vitamin-D levels on the aging process and on age-related diseases is bolstered by our results. In addition, the study results propose a new scenario in which the variability of the *VDR* gene is also relevant and emphasize the role of *VDR* genetic background in determining optimal vitamin-D levels for healthy aging.

Acknowledgements

ML was recipient of a fellowship from the University of Lleida. This work was supported by grants to the University of Lleida (JF, principal investigator) from *Fondo de Investigación Sanitaria* (FIS, PI051778) and *Ministerio de Ciencia e Innovación* (CGL 2007-62875). We thank Elaine Lilly, Ph.D. (Writer's First Aid), for English language revision of the manuscript. We would like to thank the elderly participants and their families, who made this study possible.

References

- Arai H, Miyamoto KI, Yoshida M et al (2001) The polymorphism in the caudal-related homeodomain protein Cdx-2 binding element in the human vitamin D receptor gene. *J Bone Miner Res* 16: 1256-1264.
- Austad SN (2006) Why women live longer than men: sex differences in longevity. *Gend Med* 3: 79-92.
- Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263-265.
- Bellamy RJ, Hill AV (1998) Host genetic susceptibility to human tuberculosis. *Novartis Found Symp* 217: 3-13.
- Bonafe M, Olivieri F, Cavallone L et al (2001) A gender--dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity. *Eur J Immunol* 31: 2357-2361.
- Capri M, Salvioli S, Monti D et al (2008) Human longevity within an evolutionary perspective: the peculiar paradigm of a post-reproductive genetics. *Exp Gerontol* 43: 53-60.
- Chen TC, Chimeh F, Zhiren L et al (2007) Factors that influence the cutaneous synthesis and dietary sources of vitamin D. *Arch Biochem Biophys* 460: 213-217.
- Danescu LG, Levy S, Levy J (2009) Vitamin D and diabetes mellitus. *Endocrine* 35: 11-17.
- de la Torre MS, Torres C, Nieto G et al (2008) Vitamin D receptor gene haplotypes and susceptibility to HIV-1 infection in injection drug users. *J Infect Dis* 197: 405-410.
- Deluca HF, Cantorna MT (2001) Vitamin D: its role and uses in immunology. *FASEB J* 15: 2579-2585.
- Di Bona D, Vasto S, Capurso C et al (2009) Effect of interleukin-6 polymorphisms on human longevity: a systematic review and meta-analysis. *Ageing Res Rev* 8: 36-42.
- Dobnig H, Pilz S, Scharnagl H et al (2008) Independent association of low serum 25-hydroxyvitamin d and 1,25-dihydroxyvitamin d levels with all-cause and cardiovascular mortality. *Archiv Intern Med* 168: 1340-1349.
- Fang Y, van Meurs JB, d'Alesio A et al (2005) Promoter and 3'-untranslated-region haplotypes in the vitamin d receptor gene predispose to osteoporotic fracture: the rotterdam study. *Am J Hum Genet* 77: 807-823.
- Fibla J, Caruz A (2010) Vitamin D and HIV Infection. In: Alfano M, (ed) Soluble factors mediating innate immune responses to HIV infection. Bentham eBooks, pp. 180-229.
- Franceschi C, Motta L, Valensin S et al (2000) Do men and women follow different trajectories to reach extreme longevity? Italian Multicenter Study on Centenarians (IMUSCE). *Aging (Milan, Italy)* 12: 77-84.
- Garland CF, Gorham ED, Mohr SB, Garland FC (2009) Vitamin D for cancer prevention: global perspective. *Ann Epidemiol* 19: 468-483.
- Giacconi R, Cipriano C, Albanese F et al (2004) The -174G/C polymorphism of IL-6 is useful to screen old subjects at risk for atherosclerosis or to reach successful ageing. *Exp Gerontol* 39: 621-628.
- González ER, Benavides FG, Mestre J (1996) La Transición Sanitaria en España desde 1900 a 1990. *Rev Esp Salud Pública* 70: 221-233.
- Gouni-Berthold I, Krone W, Berthold HK (2009) Vitamin D and cardiovascular disease. *Curr Vasc Pharmacol* 7: 414-422.
- Gross C, Eccleshall TR, Malloy PJ et al. (1996) 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: 1850-1855.
- Halsall JA, Osborne J E, Potter L, Pringle J H, Hutchinson P E (2004) A novel polymorphism in the 1A

- promoter region of the vitamin D receptor is associated with altered susceptibility and prognosis in malignant melanoma. *Br J Cancer* 91: 765-770.
- Hayes DP (2010) Vitamin D and ageing. *Biogerontology* 11: 1-16.
- Heaney RP (2008) Vitamin D in health and disease. *Clin J Am Soc Nephro* 3: 1535-1541.
- Holick MF (2005) Vitamin D: important for prevention of osteoporosis, cardiovascular heart disease, type 1 diabetes, autoimmune diseases, and some cancers. *South Med J* 98: 1024-1027.
- Holick MF (2006) High prevalence of vitamin D inadequacy and implications for health. *Mayo Clin Proc* 81: 353-373.
- Holick MF Chen TC (2008) Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr* 87: 1080S-1086S.
- Ingles SA, Ross RK, Yu MC et al (1997) Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. *J Natl Cancer Inst* 89: 166-70.
- Jurutka PW, Remus LS, Whitfield GK et al (2000) The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB. *Mol Endocrinol* 14: 401-420.
- Keisala T, Minasyan A, Lou Y-R et al (2009) Premature aging in vitamin D receptor mutant mice. *J Steroid Biochem Mol Biol* 115: 91-97.
- Lamberg-Allardt C (2006) Vitamin D in foods and as supplements. *Prog Biophys Mol Biol* 92: 33-38.
- Lanske B, Razzaque MS (2007) Vitamin D and aging: old concepts and new insights. *J Nutr Biochem* 18: 771-777.
- Lash RW, Nicholson JM, Velez L, Van Harrison R McCort J (2009) Diagnosis and management of osteoporosis. *Primary care* 36: 181-198, x.
- Lewontin R (1988). On measures of gametic disequilibrium. *Genetics* 120: 849-852.
- Li N, Stephens M (2003) Modeling linkage disequilibrium and identifying recombination hotspots using single-nucleotide polymorphism data. *Genetics* 165: 2213-2233.
- Lio D, Scola L, Crivello A et al (2002) Allele frequencies of +874T-->A single nucleotide polymorphism at the first intron of interferon-gamma gene in a group of Italian centenarians. *Exp Gerontol* 37: 315-319.
- Lio D, Scola L, Crivello A et al (2002) Gender-specific association between -1082 IL-10 promoter polymorphism and longevity. *Genes Immun* 3: 30-33.
- Lips P (2006) Vitamin D physiology. *Prog Biophys Mol Biol* 92: 4-8.
- Morrison NA, Qi JC, Tokita A et al (1994) Prediction of bone density from vitamin D receptor alleles. *Nature* 367: 284-287.
- Nyholt DR (2004) A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am J Hum Genet* 74: 765-769.
- Pike JW (1991) Vitamin D3 receptors: structure and function in transcription. *Annu Rev Nutr* 11: 189-216.
- Pilz S, Dobnig H, Nijpels G et al (2009) Vitamin D and mortality in older men and women. *Clin Endocrinol (Oxf)* 71: 666-672.
- Razzaque MS, Lanske B (2006) Hypervitaminosis D and premature aging: lessons learned from Fgf23 and Klotho mutant mice. *Trends Mol Med* 12: 298-305.
- Roth SM, Zmuda JM, Cauley JA, Shea PR Ferrell RE (2004) Vitamin D receptor genotype is associated with

- fat-free mass and sarcopenia in elderly men. *J Gerontol A Biol Sci* 59: 10-15.
- Shroff R, Egerton M, Bridel M et al (2008) A bimodal association of vitamin D levels and vascular disease in children on dialysis. *J Am Soc Nephro* 19: 1239-1246.
- Stephens M, Smith NJ Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 68: 978-989.
- Sullivan KM, Dean A Soe MM (2009) OpenEpi: a web-based epidemiologic and statistical calculator for public health. *Public Health Rep* 124: 471-474.
- Tuohimaa P (2009) Vitamin D and aging. *J Steroid Biochem Mol Biol* 114: 78-84.
- Tuohimaa P, Tenkanen L, Ahonen M et al (2004) Both high and low levels of blood vitamin D are associated with a higher prostate cancer risk: a longitudinal, nested case-control study in the Nordic countries. *Int J Cancer* 108: 104-108.
- Uitterlinden AG, Fang Y, van Meurs JB, Pols HA van Leeuwen JP (2004a) Genetics and biology of vitamin D receptor polymorphisms. *Gene* 338: 143-156.
- Uitterlinden AG, Fang Y, van Meurs JB, van Leeuwen H Pols HA (2004b) Vitamin D receptor gene polymorphisms in relation to Vitamin D related disease states. *J Steroid Biochem Mol Biol* 89-90: 187-193.
- Visser M, Deeg DJ, Lips P Longitudinal Aging Study A (2003) Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia): the Longitudinal Aging Study Amsterdam. *J Clin Endocr Metab* 88: 5766-5772.
- Wall JD Pritchard JK (2003) Haplotype blocks and linkage disequilibrium in the human genome. *Nat Rev Genet* 4: 587-97.
- Wang TT, Nestel FP, Bourdeau V et al (2004) Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol* 173: 2909-2912.
- Wingard DL (1984) The sex differential in morbidity, mortality, and lifestyle. *Annu Rev Public Health* 5: 433-58.

Figure legends

Figure 1.- Gene structure of the *VDR* locus and linkage disequilibrium (LD) pattern of populations analysed. (A) Graphical representation of the *VDR* gene with the location of polymorphisms selected. Polymorphisms are named according to *rs* code and alias. Small open arrows indicate alternative *VDR* promoters that incorporate alternative exon 1 (1a-1f). Grey boxes correspond to untranslated exons, empty boxes to coding exons. UTR, untranslated region. All markers were coded as biallelic including the rs17878969 microsatellite polymorphism that shows a biphasic distribution of short (<18 adenosine repeats, S) and long (\geq 18 adenosine repeats, L) allele populations (Ingles et al. 1997). (B) Graphical representation of the LD pattern in Controls and Octogenarians. D' values were calculated and plotted by Haploview 4.2 software (<http://www.broad.mit.edu/mpg/haploview>). Numbers inside boxes correspond to $D' \times 100$. Block structure was considered for marker pairs showing $D' > 0.8$, following the “solid-spine” block definition implemented in Haploview. In controls, two blocks were identified, block-1 at the 5' regulatory region including markers rs4516035-rs11568820 and block-2 at the 3'UTR region including markers rs17878969-rs1544410. No block pattern was observed in octogenarians.

Figure 2.- 3'UTR haplotype prevalence in controls (CTR) and octogenarians (OCT). Histograms plot of the 3'UTR haplotype prevalence in all subjects (A) and in males (B). Bars indicate 95% confidence interval of the proportion. Global distribution of haplotypes was compared by the PAC-likelihood test implemented in PHASE 2.1.1 (Li and Stevens 2003). Haplotype-specific test was performed by 2x2 contingency table analysis. Experiment-wide significance threshold required to keep Type I error rate at 5% was $P=0.036$.

Figure 3.- Five-marker haplotype prevalence in controls (CTR) and octogenarians (OCT). Histogram plot of five-marker haplotype prevalence in all subjects (A) and in males (B). Bars indicate 95% confidence interval of the proportion. Global distribution of haplotypes was compared by the PAC-likelihood test implemented in PHASE 2.1.1 (Li and Stevens 2003). Haplotype-specific test was performed by 2x2 contingency table analysis. Experiment-wide significance threshold required to keep Type I error rate at 5% was $P=0.011$.

Table 1.- Genotype and allele distribution

Markers (<i>rs code</i> & <i>alias</i>)	Genotypes. alleles	Controls			Octogenarians		
		All subjects n (%)	Males n (%)	Females n (%)	All subjects n (%)	Males n (%)	Females n (%)
rs11568820 <i>Cdx</i>	GG	61 (59.2)	21 (60)	40 (58.8)	63 (69.2)	18 (69.2)	45 (69.2)
	GA	38 (36.9)	12 (34.3)	26 (38.2)	25 (27.5)	6 (23.1)	19 (29.3)
	AA	4 (3.9)	2 (5.7)	2 (3)	3 (3.3)	2 (7.7)	1 (1.5)
	G	160 (77.7)	54 (77.1)	54 (77.1)	151 (83)	42 (80.8)	109 (83.8)
	A	46 (22.3)	16 (22.9)	16 (22.9)	31 (17.0)	10 (19.2)	21 (16.2)
	Genotype failure ^a	7 (6.4)	0 (0)	7(9.3)	13 (12.5)	6 (18.8)	7 (9.7)
rs4516035 <i>A1012G</i>	GG	41 (37.6)	14 (40)	27 (36.5)	29 (29.3)	9 (30)	20 (29)
	GA	56 (51.4)	18 (51.4)	38 (51.4)	55 (55.5)	15 (50)	40 (58)
	AA	12 (11)	3 (8.6)	9 (12.1)	15 (15.2)	6 (20)	9 (13)
	A	138 (63.3)	46 (65.7)	46 (65.7)	113 (57.1)	33 (55)	80 (58)
	G	80 (36.7)	24 (34.3)	24 (34.3)	85 (42.9)	27 (45)	58 (42)
	Genotype failure	1 (0.9)	0 (0)	1 (1.3)	5 (4.8)	3 (9.4)	2 (2.8)
rs2228570 <i>FokI</i>	CC	43 (39.1)	13 (37.1)	30 (40)	46 (46.5)	15 (46.9)	31 (46.3)
	CT	58 (52.7)	18 (51.4)	40 (53.3)	39 (39.4)	10 (31.2)	29 (43.3)
	TT	9 (8.2)	4 (11.5)	5 (6.7)	14 (14.1)	7 (21.9)	7 (10.4)
	C	144 (65.5)	44 (62.9)	44 (62.9)	131 (66.2)	40 (62.5)	91 (67.9)
	T	76 (34.5)	26 (37.1)	26 (37.1)	67 (33.8)	24 (37.5)	43 (32.1)
	Genotype failure	0 (0)	0(0)	0 (0)	5 (4.8)	0 (0)	5 (6.9)
rs1544410 <i>BsmI</i>	GG	37 (33.6)	10 (28.6)	27 (36)	30 (29.2)	10 (31.2)	20 (28.2)
	GA	50 (45.5)	17 (48.5)	33 (44)	56 (54.3)	20 (62.5)	36 (50.7)
	AA	23 (20.9)	8 (22.9)	15 (20)	17 (16.5)	2 (6.3)	15 (21.1)
	G	124 (56.4)	37 (52.9)	37 (52.9)	116 (56.3)	40 (62.5)	76 (53.5)
	A	96 (43.6)	33 (47.1)	33 (47.1)	90 (43.7)	24 (37.5)	66 (46.5)
	Genotype failure	0(0)	0(0)	0(0)	1(1.0)	0(0)	1(1.4)
rs17878969 <i>PolyA</i>	LL	43 (39.8)	10 (28.6)	33 (45.2)	36 (35.3)	11 (34.4)	25 (35.7)
	LS	48 (44.5)	17 (48.6)	31 (42.5)	44 (43.1)	12 (37.5)	32 (45.7)
	SS	17 (15.7)	8 (22.8)	9 (12.3)	22 (21.6)	9 (28.1)	13 (18.6)
	L	134 (62)	37 (52.9)	37 (52.9)	116 (56.9)	34 (53.1)	82 (58.6)
	S	82 (38)	33 (47.1)	33 (47.1)	88 (43.1)	30 (46.9)	58 (41.4)
	Genotype failure	2 (1.8)	0 (0)	2 (2.7)	2 (1.9)	0 (0)	2 (2.8)
Global Genotype failure		10 (1.8)	0 (0)	10 (2.6)	26 (5.0)	9 (5.6)	17 (4.7)

a) Number of subjects with missing genotypes

Table 2.- Distribution of carriers of at least one copy of selected haplotypes in controls and octogenarians

Carriers ^a for:	Controls counts ^b (%)			Octogenarians counts ^b (%)		
	All subjects (n=101)	Males (n=35)	Females (n=66)	All subjects (n=86)	Males (n=25)	Females (n=61)
3'UTR markers haplotype GS ^c	1.6 (1.6)	0.2 (0.7)	1.4 (2.1)	11.4 (13.3)	6.1 (24.4)	5.3 (8.7)
Five-marker haplotype GGCGS ^d	0.5 (0.5)	0.05 (0.1)	0.45 (0.7)	7.1 (8.3)	4.7 (18.9)	2.4 (3.9)

a) Carriers were those bearing one or two copies of the corresponding haplotype

b) Counts refer to the number of haplotype carriers that were statistically inferred from PHASE 2.1.1 "out_pairs" output file

c) Haplotype rs1544410-G:rs17878969-S. Fisher exact test, octogenarians vs. controls: all subjects, odds ratio (OR): 7.1; 95% confidence interval (CI): 1.5-66.3, P=0.008; males, OR out of range, P=0.013. Experiment-wide significance threshold required to keep Type I error rate at 5% was P=0.036.

d) Haplotype rs11568820-G:rs4516035-G:rs10735810-C:rs1544410-G:rs17878969-S. Fisher exact test, octogenarians vs. controls: all subjects, OR: 11.9; 95%CI: 1.3-650, P=0.023; males, OR out of range, P=0.012. Experiment-wide significance threshold required to keep Type I error rate at 5% was P=0.011.

Note: As the number of each haplotype carrier was statistically inferred values obtained for counts were in fractional numbers.





