



## Variability of Osteocalcin Status in Chinese Holstein Cattle: Do Phylogeny, Vitamin D or Gene Polymorphisms Matter?

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**ABSTRACT :** Osteocalcin (OC), a marker of bone turnover, displays patterns in relation to physiological and genetic factors. Here, we present an association study in a population of Chinese Holstein cattle ( $n = 24$ ) with OC serum concentration as a phenotypic trait. We hypothesised that OC status is associated with phylogeny, vitamin D serum level and single nucleotide polymorphisms (SNPs). Mitochondrial DNA (mtDNA) was used as an unlinked marker to examine phylogeny and linkage to measured phenotypic traits of vitamin D and OC status. Following an association study with OC serum variability as the trait, genotyping of SNPs ( $n = 27$ ) in OC-related genes was performed. Candidate SNPs were chosen in genes with an emphasis on the vitamin D and vitamin K pathways. Multivariate factor analysis revealed a correlation between vitamin D serum concentration and a SNP in the gene *GC* (rs43338565), which encodes a vitamin D-binding protein, as well as between a SNP in *NFATc1* (rs42038422) and OC concentration. However, univariate analysis revealed that population structure, vitamin D serum levels and SNPs were not significant determinants of OC status in the studied group. (**Key Words :** Osteocalcin, Vitamin D, Population Structure, Single Nucleotide Polymorphisms, D-loop, *Bos taurus*)

### INTRODUCTION

Epidemiological studies of bone diseases indicate that both genetic and physiological factors are likely to be involved in the variation of bone turnover markers (Sabsovich et al., 2008; Lei et al., 2009). Osteocalcin, also known as bone Gla protein, has been shown to be a sensitive biochemical marker for bone formation (Ducy et al., 1996; Szulc et al., 2000). The regulation of OC is complex, and a wide array of factors are assumed to contribute, including vitamin D (indexed by 25[OH]D) and vitamin K status as well as SNPs and phylogeny.

Vitamin D status has long been recognised as an important factor for bone health, and its association with OC has recently received attention and results have been controversial (Garnero et al., 2007; Moreno-Reyes et al., 2009). However, it has been shown that, in human osteoblastic cells, the active form of vitamin D ( $1,25(\text{OH})_2\text{D}_3$ ) stimulates OC mRNA and protein expression (Arbour et al., 1995; Martinez et al., 2001).

Crucial proteins for the activation of vitamin D are the enzyme CYP27A1, which has 25-hydroxylase activity (McCullough et al., 2009), as well as the group-specific component (GC), also known as vitamin D-binding protein, which is the major transporter of vitamin D metabolites to target tissues. Recent studies have shown that polymorphisms in *GC* are associated with vitamin D concentrations as well as affinity of the binding protein for vitamin D metabolites (Lauridsen et al., 2005; Engelman et al., 2008).

Vitamin K is known to be a determinant of OC, and SNPs in vitamin K-related genes have a reported association with bone health (Kinoshita et al., 2007). Vitamin K is a cofactor of  $\gamma$ -carboxylase, which converts three glutamic acid (Glu) residues to  $\gamma$ -carboxyglutamic acid (Gla) and, thus, is essential for  $\gamma$ -carboxylation of the bone GLA protein OC (Ferland, 1998; Stafford, 2005). The  $\gamma$ -carboxylation is mediated through the vitamin K epoxide reductase subunit one (VKORC1) (Teichert et al., 2008). An additional reported destination factor is the nuclear factor of activated T cells (NFATc1), which inhibits OC expression by decreasing OC promoter activity (Choo et al., 2009).

Given that SNPs diverge in subpopulations and

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phenotype frequency can vary across subpopulations, it can be hypothesised that phylogeny is the underlying factor for genotype-phenotype association. Therefore, population structure can lead to a spurious association (Ewens et al., 1995; Pritchard et al., 1999). If population structure is present, an unlinked marker should also show association with the phenotype (Goring et al., 2001). One phylogenetic marker unlinked to either OC or vitamin D is mitochondrial DNA (mtDNA). The non-coding D-loop region is commonly variable at the intraspecies level (Jia et al., 2007).

Our general assumption was that (a) the studied group differs in mtDNA sequence composition and (b) the OC serum concentration shows variability in the group. Based on these assumptions, our aim was to investigate whether the variation in OC serum concentration is associated with i) population structure, ii) vitamin D serum concentration or iii) selected SNPs in candidate genes related to the OC pathway.

## MATERIAL AND METHODS

### Sample collection

Blood samples were collected by tail vein puncture from a dairy farm in Beijing, China. To minimise the impact of environmental factors as a source of biological variability, one skeletal mature group (n = 24) from one dairy farm was studied over a period of three hours. Samples for phylogenetic analysis were stored in EDTA; those for serum level measurement were allowed to clot and were stored at 4°C until centrifugation at 12,000 rpm for 20 min at 4°C. The serum was stored at -80°C until measurement.

### Population structure inferred by mitochondrial DNA sequence analysis

Extraction of DNA was performed with the TIAamp Blood DNA Kit (Tiangen Biotech, Co., Ltd., Beijing) according to the manufacturer's protocol. The primer pair used for specific amplification (5'-CTGCAGTCTC ACCATCAACC-3' and 5'-GATTATAGAACAGGCTCC TC-3') was generated with Primer premier v.6.0 (Singh et al., 1998). The PCR was conducted in 50 µl reaction volumes with a 1 min denaturation at 95°C, followed by 35 cycles consisting of 1 min at 94°C, 1 min at 60°C, 30 s at 72°C and finally extension for 10 min at 72°C. The products were purified, and sequencing was performed on an ABI 3730 automated sequencer at Beijing Sunbiotech Inc., Beijing. The obtained chromatographic sequences were verified by eye and aligned using the program ClustalW (Larkin et al., 2007). The program DNAsp v.5 was used to analyse the alignment and compute haplotype (HT) and nucleotide diversity (Librado et al., 2009). The consensus networks were computed by TCS 1.21 (Clement et al., 2000), and phylogenies for the discrimination of

clades were assessed by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) with the maximum composite likelihood method using the Tamura 3-parameter substitution model as implemented in Mega4 (Tamura et al., 2007; Kumar et al., 2008). The tree was rooted using the outgroups *Bos grunniens* (DQ856604) and *Bubalus bubalis* (EF053552). The phylogenies obtained were verified by Bayesian statistics in MrBayes 3.1 (Ronquist et al., 2003), and conflicts of the alignment were illustrated via lentoplot in Spectronet (Huber et al., 2002).

### Serum concentration of osteocalcin and vitamin D

Measurement of serum OC and vitamin D status (n = 24) was performed with a commercially available bovine-specific OC ELISA immunoassay (Bovine Osteocalcin/Bone gla protein ELISA Kit; USCN Life Science & Technology Co., Ltd., Beijing, China) and goat anti-cattle vitamin D ELISA kit (Adlitteram Diagnostic Laboratories Co., Ltd., Beijing, China). All samples were measured in triplicate, and the mean concentration was z-transformed and visualised via boxplot to obtain categorical variables.

### SNP selection and genotyping

Data mining for SNPs in vitamin D- and vitamin K-related candidate genes was performed on the Database of Single Nucleotide Polymorphisms (Sherry et al., 2001). Genotyping of 27 SNPs located on different chromosomes was completed for each individual (Table 1). The genotype of each SNP in each DNA sample was determined using MATRIX-Assisted Laser Desorption Ionisation-Time of Flight (MALDI-ToF) mass spectrometry assessed by Benegene<sup>®</sup> Cooperation; Shanghai/China. The obtained genotypes were initially tested for Hardy-Weinberg Equilibrium (HWE) by a  $\chi^2$  test (Rodriguez et al., 2009) and minor allele frequency (MAF), whereas SNPs with values <5.0 were excluded from further analysis. To detect population stratification, we used the software Structure v.2.3 under an admixture assumption (Pritchard et al., 2000).

### Association study

The associations between mtDNA clades and OC as well as vitamin D z-transformed serum levels were demonstrated via scatterplot. Single SNP association analyses with OC and vitamin z-scores were performed by linear regression and bivariate analyses of variance (ANOVA) (Balding, 2006). The subpopulation structures for mtDNA and genotyped SNPs were compared by structure analyses in Structure v.2.3 using an estimation of three populations in an admixture model. Multivariate statistics with the purpose of structure detection were applied by factor analysis. Consequently, the underlying relationships between the variables (OC, vitamin D, SNPs and mtDNA) were visualised in a biplot. All statistics were

**Table 1.** Summary of results from 27 genotyped SNPs (Shown are all 12 polymorphic SNPs in the population, which are linked to seven haplotypes (HTa- g). Single point analyses revealed no significant correlations between SNPs and phenotypic traits.)

Gene	HT	Chr	Index SNP	X <sup>2</sup>	MAF	Association	
						VitD	OC
CYP27A1	HTa	2	rs43315171	0.42	39.5	r = -0.145; p = 0.5	r = -0.280; p = 0.186
		2	rs43315173	0.42	39.5	r = -0.145; p = 0.5	r = -0.280; p = 0.186
		2	rs43315176	0.42	39.5	r = -0.145; p = 0.5	r = -0.280; p = 0.186
	HTb	2	rs43315174	2.12	22.9	r = 0.095; p = 0.659	r = 0.287; p = 0.174
		2	rs43315175	2.12	22.9	r = 0.095; p = 0.659	r = 0.287; p = 0.174
OC (BGLAP)	HTc	3	rs41257354	0.96	16.7	r = -0.266; p = 0.208	r = -0.301; p = 0.154
GC	HTd	5	rs43338560	2.67	22.9	r = 0.173; p = 0.419	r = 0.068; p = 0.751
	HTe	5	rs43338565	1.66	20.8	r = 0.150; p = 0.484	r = -0.067; p = 0.755
NFATC1	HTf	24	rs42038422	0.62	27.1	r = -0.16; p = 0.941	r = 0.081; p = 0.706
VKORC1		25	rs42065182	0.96	16.7	r = -0.037; p = 0.863	r = -0.110; p = 0.608
	HTg	25	rs42065186	0.96	16.7	r = -0.037; p = 0.863	r = -0.110; p = 0.608
		25	rs42065187	0.96	16.7	r = -0.037; p = 0.863	r = -0.110; p = 0.608

carried out with SPSS v.16 (SPSS Inc., Chicago IL).

## RESULTS

### Population structure inferred by mitochondrial DNA sequence analysis

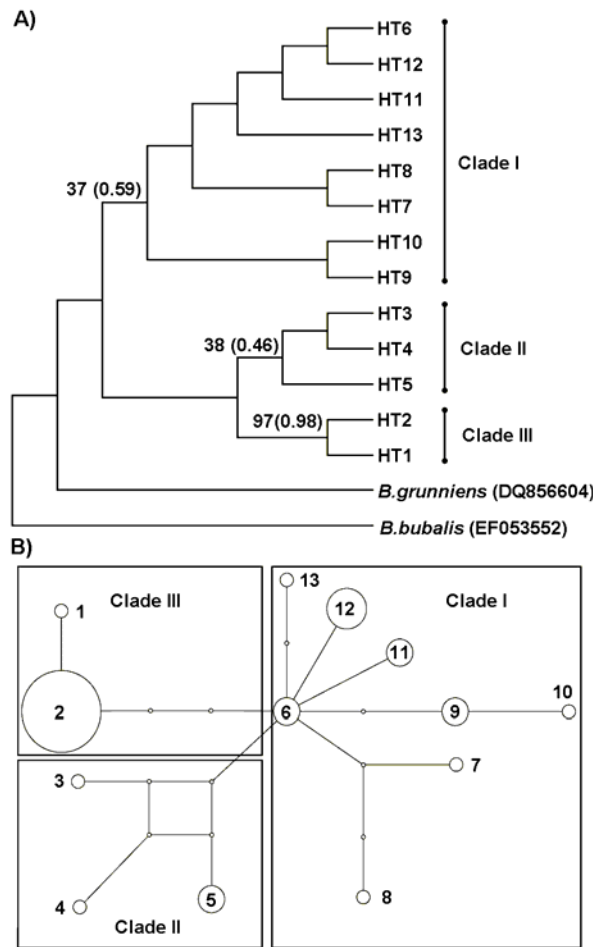
Basic indices of the D-loop alignment (n = 24) revealed 13 haplotypes (HT) and nucleotide frequencies of T = 28.8, C = 23.3, A = 32.6 and G = 15.3. The obtained sequences were deposited at GenBank under the accession numbers HM637893 - HM637905. Phylogenetic relationships of the HTs were analysed by constructing an UPGMA tree based on pairwise distances that revealed three clades (Figure 1A). Bootstrap values and Bayesian analyses were applied to support the obtained phylogeny, and both nodal values demonstrated relatively low support for population stratification. Visualisation of the relationships between HTs via a network indicated a possible alternative inner topology, shown as a loop (Figure 1B). Conflicting parsimonious alignment sites (n = 9) were examined by splitting the sequence and visualising it with a Lento plot (Figure 2). The haplotypes contained conflicting or supporting information in a series of splits, where haplotypes of clade III were well supported, and clade I was also supported by

several splits. In contrast, splits of haplotypes (HT 3-5) that clustered in clade II showed strong conflicts. Therefore, the inner topology of clade II could be only poorly resolved.

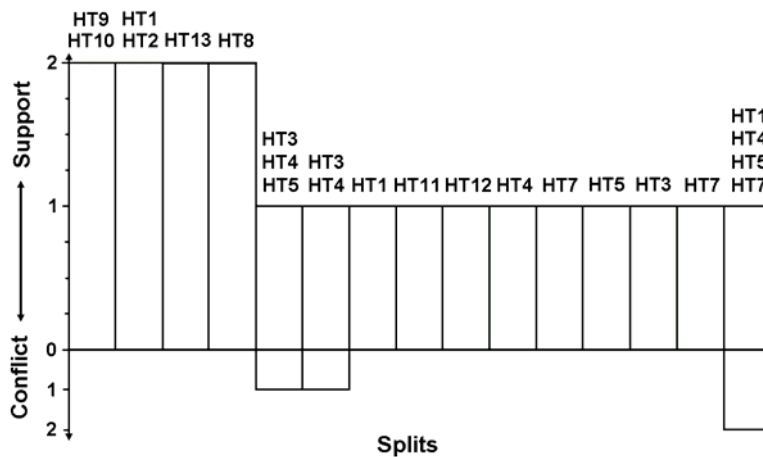
### Association analysis

The studied population displayed a biological variability in OC and vitamin D serum concentration (Figure 3A). A scatterplot revealed that OC and vitamin D concentration were independent of each other (r = -0.188; p = 0.380; Figure 3B). Furthermore, individual levels of OC and vitamin D concentration did not form distinctly different groups which depended on the mtDNA-based clades (Figure 3C, 3D). Twenty-eight SNPs were genotyped and found to contain 12 polymorphisms in the studied population. For all SNPs, a test of Hardy-Weinberg Equilibrium and a chi-square test were conducted; SNPs with a low allele frequency (>5.0) or departure from HWE were removed from further analysis.

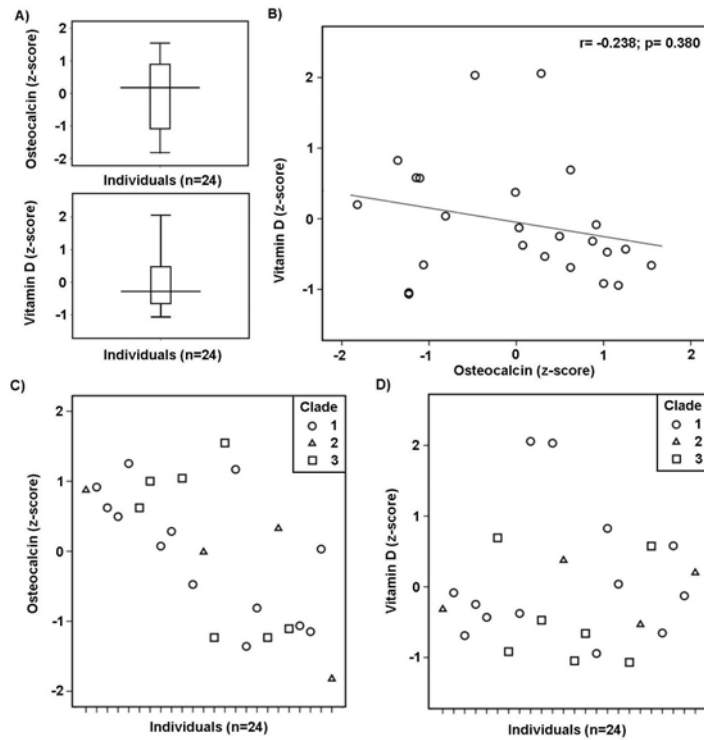
Structure analysis of 12 polymorphic SNPs displayed a different phylogenetic structure than inferred by mtDNA (Figure 4A, 4B). The population structure inferred from SNPs, compared with the structure of categorical serum vitamin D concentration (Figure 4C) and OC categorical concentration (Figure 4D), revealed unequal structure.



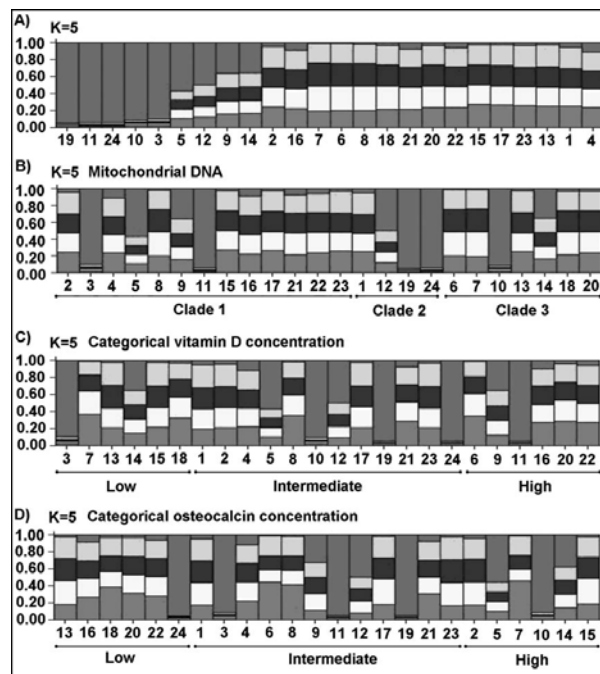
**Figure 1.** Phylogenetic relationship of 24 Chinese Holstein cattle inferred by mtDNA sequence analysis of 711 bp of the D-Loop region. (A) Clustering of haplotypes into three clades based on the UPGMA method. The distances were computed with the maximum composite likelihood method using the Tamura 3-parameter substitution model ( $GC \neq AT$ ), and a consensus tree is shown. The bootstrap test values (1,200 replicates) and Bayesian values (in parenthesis) are relatively low and are shown next to the branches. (B) Minimum spanning network of thirteen haplotypes (HT). Nodes in the branch represent nucleotide differences. Circle sizes correspond to the number of individuals. The inner topologies of clade II (HT 3-6) form a loop, which indicates possible alternative resolutions.



**Figure 2.** A Lento plot displaying, in ranked order, the data support and conflict for each split (a partition of the alignment). Taxa are separated into conflicting or supporting groups. Conflicting sites with the highest values are in clade II, the topology of which can therefore be alternative, whereas the topology between clades shows an absence of conflict.



**Figure 3.** (A) Variation of z-transformed OC and vitamin D concentrations as visualised by boxplots. The upper and lower quartiles represent the 25% of cases in the low- and high-concentration categories, respectively. The central box represents 50% of cases that can be categorised as having intermediate concentration levels. (B) Plot to visualise the relationship between OC and vitamin D concentration showed a lack of correlation (C) The scatterplot for all individuals, marked by clades, against z-transformed OC concentrations shows a lack of relationship. (D) The scatterplot for all individuals, marked by clades, against z-transformed vitamin D does not cluster according to the mtDNA clades.



**Figure 4.** (A) The population structure of 24 individuals assessed by 12 polymorphic SNPs and assuming five ancestral population clusters in comparison with defined populations. (B) SNP structure in contrast with the assessed three-clade structure inferred from 711 bp mtDNA. (C) The SNP structure obtained differs with categorical levels of OC concentration and (D) dependent on categorical levels of vitamin D structure. It can be shown that mtDNA clades and the population structure assessed by SNPs differ.

In univariate single marker analyses, no evidence for an association between any of the variables was detected (Table 1). Multivariate factor analysis revealed three principal components with eigenvalues greater than one that explained a cumulative 63.68% of the variance in the data set. Plotting of the loadings from the first two principal components explained 47.73% of the variance and demonstrated patterns of variables (Figure 5). A biplot displayed that the mtDNA, OC and vitamin D variables had different structures, indicated by a wide angle. Furthermore, the plot showed that vitamin D status and rs43338560\_GC clustered together and OC status clustered with rs42038422\_NFATc1.

## DISCUSSION

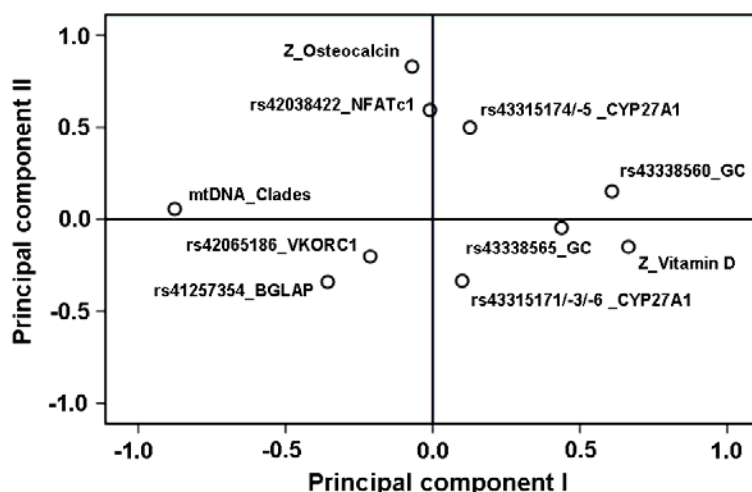
The investigated group showed population structure and clustered into three distinct clades. Nevertheless, the topology of the D-loop based mtDNA network remains unclear, as the bootstrap and Bayesian analyses indicated. Relatively low values can be explained by the occurrence of conflicting sites in the alignment. These conflicts can be displayed by a Lento plot. Visualisation here was in ranked order, by the data support and conflict for each split (a partition of the alignment). The most valuable conflicting sites were in clade II, the topology of which could therefore be alternative, whereas the topology between clades showed less conflict. Conflicting topology could be explained by the strong phylogeographic dynamic in the genetic history of cattle breeds in China (Lai et al., 2006; Lei et al., 2006). Holstein cattle are an introduced breed in China and could be crossbred with indigenous Chinese cattle breeds. It is challenging to infer the population structure of recently

admixed populations (Sankararaman et al., 2008). In these populations, two or more ancestral populations have been mixed for a relatively small number of generations. Therefore, the maternally inherited mtDNA could be of several origins in the studied population, and it contains conflicting phylogenetic information.

We found incongruence between the mtDNA (haploid) and SNP (diploid) approaches for assessment of population structure. Gene trees can differ substantially for each sampled locus. The different data sets include different genes that could evolve in such radically different ways that their phylogenetic signals could be very divergent (Huelsenbeck et al., 1996). A recent admixture hypothesis for the breed would also explain why the SNPs and mtDNA data indicate different population structures. Due to recombination, the genome of each individual is fragmented into shorter regions of different ancestry and, therefore, gives diverse phylogenetic signals. Consequently, the phylogeny inferred by mtDNA and SNPs resolves into different structures.

Nevertheless, neither marker approach correlated with the phenotype variability as indicated in the scatterplot visualisation and structure and factor analysis (Figures 3C, 3D, 4 and 5). Thus, the observed variability of OC and Vitamin D cannot be explained by phylogeny. In comparison, it was reported previously that horses show a breed-associated pattern of OC serum concentration variability (Lepage et al., 1998). In contrast, breeds of dogs show less support for breed-dependent OC concentration patterns (Breur et al., 2004).

There was no association between serum OC concentration and serum Vitamin D levels. This is in accordance with a recent study in humans where no



**Figure 5.** Principal components with the highest eigenvalues, which account for a total of 47.73% variation, are plotted. The plotted variables include mtDNA clades, OC and vitamin D concentration as well as all genotyped SNP haplotypes. The osteocalcin, vitamin D and mtDNA clades show contrary patterns, as indicated by their wide angle. Vitamin D shows the pattern most similar to that of GC\_rs43338565. Osteocalcin shows the pattern most similar to that of NFATc1\_rs42038422.

association between OC and Vitamin D was found (Garnero et al., 2007). In contrast, low Vitamin D serum concentrations in humans were associated with higher mean serum OC concentrations (Moreno-Reyes et al., 2009). Our finding suggests that OC and vitamin D have independent roles in bone metabolism.

Factor analysis revealed a linkage of vitamin D and SNPs in the *GC* gene, as indicated by a small angle. In general, it was shown that the different forms of *GC* differ in their affinity for vitamin D (Pani et al., 2002). Recent studies have shown that allelic variation in *GC*, the vitamin D-binding protein, is associated with changes in serum 25(OH)D concentrations as well as higher affinity of the binding proteins to vitamin D metabolites (Lauridsen et al., 2005; Engelman et al., 2008). It was shown that polymorphism in the *GC* promoter region was linked to bone mineral density in humans (Ezura et al., 2003). Nevertheless, no association between vitamin D status and *GC*\_rs43338565 could be supported by significant values in this study.

A relationship between rs42038422\_NFATc1 and OC serum concentration was illustrated by factor analysis, as indicated by a small angle and similar loading biplot (Figure 5). It is known that NFATc1 regulates bone mass by functioning in osteoblasts. Recent studies showed that a mouse variant expressing a constitutively nuclear NFATc1 in osteoblasts developed high bone mass and expressed a higher serum OC than the wild type (Winslow et al., 2006). This finding contrasts with the findings of an *in vitro* study, where NFATc1 was found to decrease OC promoter activity (Choo et al., 2009). Nevertheless, an impact of NFATc1 on OC status is described. The association can be explained by the OC promoter activity, as the coding gene for NFATc1 is distal to the OC gene in the promoter region (Desbois et al., 1994; Zhang et al., 1997).

Therefore, the source of OC variability should be other factors. Environmental factors are a well-described source for biological variability that may influence clinical interpretation. Bone turnover markers are a subject of biological variability, which includes the effects of circadian rhythms, diet, ageing and sex (Srivastava et al., 2001; Gundberg et al., 2002). We minimised the impact of such factors by sampling at the same time point, in the same geographical location and from a cohort with the same living environment, including fodder, water and microenvironment. We conclude that further OC association studies should focus on SNPs like NFATc1 in the OC promoter region, and studies of vitamin D serum concentration should focus on SNPs in *GC*. In general, the number of discovered SNPs in candidate genes with a possible impact on bone health in *Bos taurus* is limited and should be further extended.

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