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**Silvia Perea**  
Museo Nacional de Ciencias  
Naturales, CSIC. Department of  
Biodiversity and Evolutionary  
Biology, C/ José Gutiérrez  
Abascal, 2, Madrid, Spain

**Enrique Pérez-Campos**  
Asociación Nacional de Criadores  
de Ganado Ovino de Raza  
Churra. Av. Casado del Alisal 21.  
Palencia, Spain

**José Manuel Gonzalo-Orden**  
Universidad de León,  
Departamento de Sanidad  
Animal, Facultad de Veterinaria,  
León, Spain

**Markus Bastir**  
Museo Nacional de Ciencias  
Naturales, CSIC. Department of  
Paleobiology, C/ José Gutiérrez  
Abascal, 2, Madrid, Spain

**Ignacio Doadrio**  
Museo Nacional de Ciencias  
Naturales, CSIC. Department of  
Biodiversity and Evolutionary  
Biology, C/ José Gutiérrez  
Abascal, 2, Madrid, Spain

**Correspondence**  
**Ignacio Doadrio**  
Museo Nacional de Ciencias  
Naturales, CSIC. Department of  
Biodiversity and Evolutionary  
Biology, C/ José Gutiérrez  
Abascal, 2, Madrid, Spain

## Morphological and molecular assessment of hip dysplasia in two body types of Spanish Mastiff

**Silvia Perea, Enrique Pérez-Campos, José Manuel Gonzalo-Orden, Markus Bastir and Ignacio Doadrio**

### Abstract

Canine hip dysplasia is a polygenic disorder having genetic and non-genetic components. Divergent breeding lines for working dogs (livestock guardians) and non-working dogs (pets) gave rise to different selection pressure and differences in hip morphology and prevalence in this giant breed. We investigated morphological and genetic traits associated to Canine Hip Dysplasia in the Spanish mastiff. Norberg angle was used to diagnose this disorder, with agreement between evaluators assessed. Validity of a genetic test based on the *Fibrillin-2* gene in distinguishing presence of Canine Hip Dysplasia was evaluated. Relationships between Norberg angle, sex, *Fibrillin-2* genotype and morphology of working and non-working dogs were analyzed. Directional asymmetry was also found, in most cases related to animal manipulation during radiography. Standardization of radiography and Norberg angle measurement is crucial to diagnosis of Canine Hip Dysplasia and to avoid evaluator's bias.

**Keywords:** body types; canine hip dysplasia; *FBN2* gene; morphometrics; Spanish mastiff

### 1. Introduction

Canine hip dysplasia (CHD) is a condition affecting more than 20% of dog breeds that can produce laxity and eventual degenerative joint disease [1-3]. Late diagnosis and treatment may lead to substantial decrease in canine health requiring hip replacement or euthanasia. CHD is caused by both non-genetic and heritable factors, with the genetic component known to be polygenic [2, 4]. GWA studies have identified several SNPs related to CHD and osteoarthritis and demonstrated that CHD is predictable from genomics [5-6]. An insertion-deletion polymorphism of 10 bp within intron 30 of the *fibrillin-2* (*FBN2*) gene that contributes to CHD was identified [7], which so far has been reported in several breeds [8]. However, link between altered *FBN2* expression and the development of CHD remains unclear [7]. The relationship of *FBN2* with phenotype may be influenced by non-genetic factors. Moreover, some breeds frequently show asymmetric hips [9-10], but genetic basis for this is unclear. The asymmetry factor in CHD is important, due to evaluation of its grade is usually based on the most affected hip, and breeding decisions are made according to this assessment.

The Spanish mastiff is a giant breed traditionally used for safeguarding flocks against predators. Archaeological data demonstrate the presence of large dogs in central Spain associated with livestock since 2800 years BP [11]. The Spanish mastiff acquired a prominent role in safeguarding transhumance livestock in the thirteenth through eighteenth centuries. In the beginning of the 1980s more massive dogs were introduced into breeding programs. The breed became popular as a pet, and there was a trend to breeding dogs with greater body mass and exaggerated morphological traits. Nevertheless, due to an increase in predators, shepherds continued to breed dogs as livestock guardians, which maintained the smaller body size.

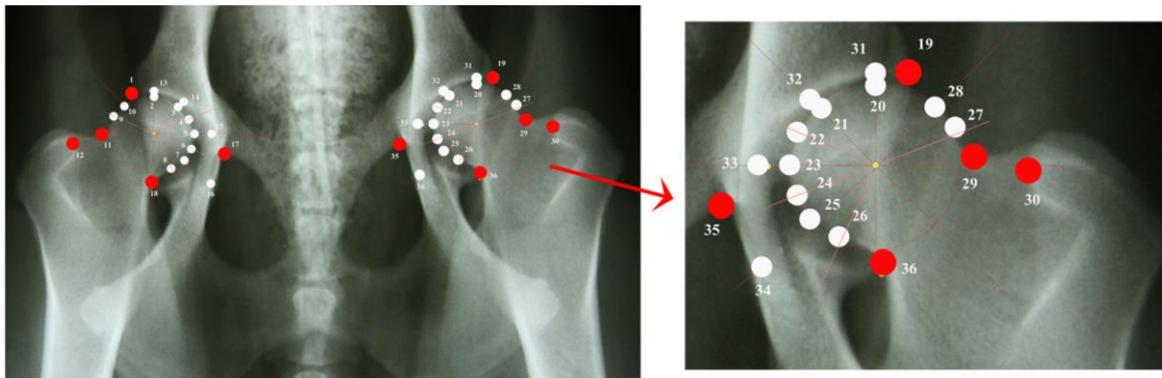
The Spanish Mastiff has been identified as a breed seriously affected by CHD [12]. This, along with the divergence of breeding lines for working (livestock guardians) and non-working dogs (show dogs or pets), mean different selection pressures and two body types, which makes the Spanish mastiff a good model for investigating CHD. We analyzed the morphological characteristics of CHD in working and non-working Spanish mastiffs using traditional and geometric morphometrics to determine CHD grade. We also perform DNA analyses to assess the contribution of the *FBN2* gene in the Spanish mastiff hip dysplasia.

Selection pressures against CHD are expected to be more intense in working dogs than in animals bred as pets and dog shows and consequently a lesser prevalence of CHD is expected in working dogs. A between *FBN2* genotype and different CHD grades in the Spanish mastiff is also expected.

## 2. Material and Methods

Ventrodorsal radiographs obtained in standardized anatomical position of 303 Spanish mastiffs 18 to 48 months old were analyzed. Age, sex, date of birth, and whether the animal was bred for working or non-working dog were recorded. Forty-six dogs (27 females; 19 males) were livestock guardians and 257 were non-working dogs (154 females; 103 males). A subsample of 187 non-working (102 females; 85 males) and 44 working (19 males; 25 females) were measured for withers height and weighted using a calibrated electronic scale. To test differences in body height and weight between working and non-working dogs, sexual dimorphism and their interactions, we carried out a two-way ANOVA in Past 3.12 [13]. Radiography was conducted of extended hind limbs of dogs following Fédération Cynologique Internationale (FCI) recommendations. Radiographs were evaluated following FCI criteria based on NA (recognized as highly accurate for discriminating between dogs with CHD-positive and CHD-negative joints [14]) and hip morphology (congruency between the femoral head and acetabulum) [15]. NA was measured in Digimizer v.4.2.2 [16]. Two evaluators independently measured NA twice, with evaluations separated by an interval of at least 48 h. A paired student's t-test was performed in MedCalc® [17] to assess the equality of means between

evaluators. Normality of data was evaluated and data were log transformed to avoid normality violation. Bland-Altman plots [18] were used to assess repeatability and reproducibility between evaluators and Spearman correlation was carried out to compare results between evaluators. Mean of the four values was used in subsequent statistical analyses. ROC curves [19] were calculated with MedCalc® [17] plotting sensitivity against 1-specificity to evaluate the validity of NA in the discrimination of dogs with and without CHD. Area under the curve (AUC) was also estimated. Construction of the curve generated a value optimal for discriminating CHD in the two tested groups associated to the Youden index [20] or highest accuracy, which was compared with the standard value for all breeds according FCI (100°). 95% confidence intervals for the optimal criterion was calculated by Bayesian bootstrapping (1000 replicates). We also estimated the positive and the negative likelihood ratios as well as positive and negative predictable values for the estimated optimal criterion. A higher positive likelihood ratio indicates a better accuracy of the test. In the same way, lower values of negative likelihood ratio mean better discriminatory accuracy. Geometric morphometric analyses were carried out to quantify the variation in size, shape and relative position of involved anatomical elements. For these analyses 2D Cartesian coordinates of 18 landmarks in each hip were recorded from digitized radiographs using TpsDigv.1.4 [21]. From all the landmarks, five in each hip were real landmarks (Type I and II) [22]. The remaining ones were semi-landmarks used to quantify the curvature of the femur head and acetabulum following Bookstein [23] (Figure 1).



Landmarks: 1 and 19: cranial effective acetabular rim; 11 and 29: femoral neck notch; 12 and 30: greater trochanter edge; 17 and 35: obturator foramen; 18 and 36: caudal acetabular rim

**Fig 1:** Landmarks (red dots) and semi-landmarks (white dots) used for geometric morphometric analyses

Shape analyses were carried out following the generalized Procrustes analyses method [23-25]. Bilateral symmetry was assessed in MorphoJ [26]. Asymmetry of hips was analyzed following Klingenberg and Nihout [27]. Shape data were obtained by Procrustes superimposition and thin plate splines transformation grids (TPS-grids) were used for visualization [22, 28-29]. TPS-grid transformations between two landmark configurations provide intuitive visual diagrams of those shape features that differ between these configurations [24]. Principal component analysis (PCA) of Procrustes shape coordinates in shape space considering the five FCI dysplasia grades was used to explore overall variation in the sample. Since variation showed by PCA analysis was continuous for the five dysplasia grades, we performed a multivariate regression analyses of shape of CHD grades A and E in order to cover the whole variation range and to facilitate the visualization of the changes of the shape [24]. A canonical analysis of variance (CVA) was also applied to identify shape

differences in the hip relative to the three different *FBN2* genotypes.

To analyze genotype, DNA from blood and saliva samples was collected from 232 and 71 individuals, respectively. The 303 samples corresponded to the radiographs. Blood samples were preserved in EDTA until DNA isolation using the QiAquick DNA isolation kit (QIAGEN). DNA from saliva samples, preserved in Oragene-Animal transport and purification tubes (DNAgenotek), was isolated using the Oragene-Animal DNA collection kit (DNAgenotek). A fragment of intron 30 of the *FBN2* gene was amplified by PCR [7] in order to check the presence or not of the 10bp *indel* considered to contribute to CHD in dogs homozygous for deletion. Positive PCR amplifications were digested by the restriction enzyme ApaI (BioLabs) following the manufacturer's protocol. Digested fragments were separated by electrophoresis on 2% agarose gel and visualized with SYBR® Safe (Life Technologies). To avoid the use of closely

related animal (full or half siblings), the pedigree of each Spanish mastiff was asked to the owners. Additionally, we performed genetic complementary analyses to ensure the use of unrelated individuals. Therefore, pairwise coefficients of relatedness ( $r$ ) between dysplastic and non-dysplastic dogs were inferred using allele frequencies at microsatellite loci based on seven relatedness estimators using COANCESTRY<sup>[30]</sup>. For these analyses, we randomly chose a subsample of 169 individuals and analyzed 18 microsatellite markers recommended by the International Society of Animal Genetics (ISAG), which are included in a commercial kit (Canine Genotype TM Panel 1.1; Finnzymes, Vantaa, Finland). Microsatellite alleles were processed using GENEMAPPER v 4.0 (ABI, Foster City, CA, USA) and manually checked. Significant differences among body type (working/non-working dogs), *FBN2* genotype, and sex relative to NA were evaluated with one-way ANOVA, followed by Tukey's HSD *post hoc* comparisons in Past 3.12<sup>[13]</sup>. Data were log transformed when the assumption of normality was violated. A Chi-square test was used to assess association between genotypes and body type. In order to have a statistically representative sample for analyzing the relationship between body type and *FBN2* genotype we added 183 not radiographed but genotyped working dogs to the study.

### 3. Results

#### 3.1 Working/non-working dogs

The two-way ANOVA analysis showed significant differences in body weight between males and females

( $F=127.7$ ,  $p<0.001$ ) and between working and non-working dogs ( $F=52.92$ ,  $p<0.001$ ), but the interaction between sex-related dimorphism and working/non-working dogs was not significant ( $F=4.68$ ,  $p=0.0316$ ). Males were heavier than females (Males  $\bar{X}=83.1$ ; Median=84; females  $\bar{X}=72.6$ ; Median=72.8) and the non-working group was heavier than the working group (Males  $\bar{X}=73.6$ ; Median=74; females  $\bar{X}=67.1$ ; Median=67). There was not significant correlation between weight and grades of dysplasia ( $r=-0.0771$ ,  $p=0.245$ ).

#### 3.2 Norberg angle measurements

No significant differences were observed in NA with repeated measures by an individual evaluator. However, significant between-evaluator differences were found (Table 1), especially in cases of severe osteoarthritis. Bland-Altman plots established limits of agreement between evaluators at  $95\% \pm 1.96$  times the standard deviation, these limits were from -1.8 to 1.2 for both hips (mean differences=0.3). Correlations between the individual evaluators and the consensus mean of measurements of both evaluators were significant at  $p<0.0001$ , with  $r$  higher than 0.8. Nevertheless, FCI grade assigned to each given radiograph was consistent between evaluators, possibly as consequence of their previous joint training. Distribution (%), mean, standard deviation, and range of FCI grades using the smaller NA of the two hips based on the mean value of the four measurements are presented in Table 2.

**Table 1:** Norberg angle (NA) measurements made by two independent evaluators (N=303) and paired *t*-student test for assessing mean differences (MD) within and between evaluators. RH = Right hip; LH = Left hip; 1 = evaluator 1; 2 = evaluator 2.

Comparison	NA Mean (range)	MD	SD MD	<i>t</i> statistic	p-level
LH 1	99.4 (66-118.7)	0.31	0.02	1.779	0.08
LH 2	99.2 (65-118.4)	0.32	0.02	2.413	0.02
RH 1	101.1 (55.4-122.5)	0.37	0.02	1.074	0.28
RH 2	100.8 (55.1-122.4)	0.39	0.02	0.295	0.77
LH 1 and 2	99.2 (65.6-118.4)	0.33	0.76	7.536	<0.0001
RH 1 and 2	100.9 (55.2-122.4)	0.34	0.76	7.632	<0.0001

**Table 2:** Distribution (%) of FCI grades, mean value of NA for each grade, standard deviation and the range of the smaller NA per FCI grade.

FCI category	Individuals (%)	Mean	Standard deviation	Range of the smaller Norberg angle
A	77 (25.41%)	106.9	3.0	102.5-117.0
B	59 (19.47%)	101.5	1.3	99.0-104.5
C	60 (19.80%)	97.6	1.9	90.2-100.3
D	46 (15.18%)	92.6	1.6	89.9-95.3
E	61 (20.13%)	81.6	8.4	55.1-90.0

The receiver operating characteristic (ROC) curve (Figure 2) confirmed high validity of NA in discriminating between CHD-positive and CHD-negative individuals. The angle estimated as the optimal discriminant of CHD was  $99.4^\circ$  (Youden index  $J=0.93$ ; CI 95% based on Bayesian bootstrapping:  $98.7^\circ$ - $100.1^\circ$ ), but the confidence interval at 95% included the FCI standard value ( $100^\circ$ ). Table 3 shows

the percentage of individuals of each body type showing each FCI CHD grade, based on NA values established by the ROC curve. We estimated a CHD prevalence of 55.8% in the Spanish mastiff in all the individuals analyzed. When we considered separately working and non-working dogs, CHD prevalence was higher in non-working (56 %) in relation to working (43.5 %) dogs.

**Table 3:** Distribution after NA correction ( $99.4^\circ$ ) based on ROC curve to discriminate CHD-positive and CHD-negative. NWD = Non-working dogs; WD = working dogs.

Grades CHD FCI	Males (n=124)	Females (n=179)	NWD (n=257)	WD (n=46)	All Dogs (n=303)
A	27 (21.77%)	41 (22.91%)	54 (21.01%)	14 (30.43%)	68 (22.44%)
B	30 (34.19%)	41 (22.91%)	59 (22.96%)	12 (26.08%)	71 (23.43%)
C	41 (33.06%)	53 (29.61%)	80 (31.13%)	14 (30.4%)	94 (31.02%)
D	19 (15.32%)	29 (16.2%)	42 (16.34%)	6 (13.04%)	48 (15.84%)
E	7 (5.65%)	15 (8.38%)	22 (8.56%)	0	22 (7.26%)

CHD-negative	55 (44.4%)	78 (43.6%)	107 (41.6%)	26 (56.5%)	134 (44.2%)
CHD-positive	69 (55.6%)	101 (56.4%)	150 (58.4%)	20 (43.5%)	169 (55.8%)

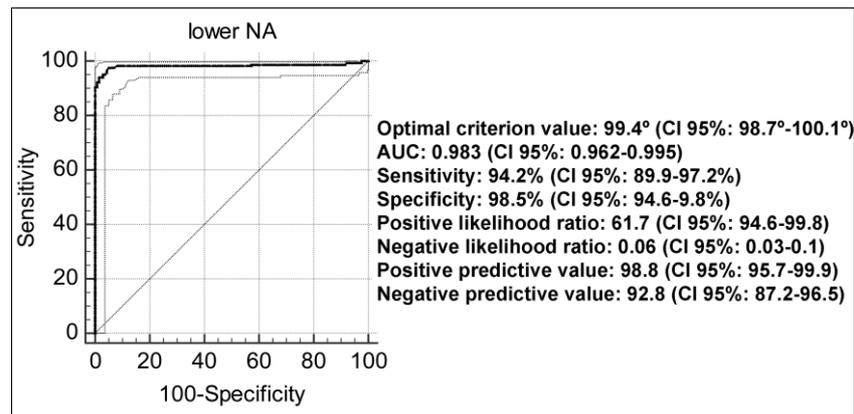


Fig 2: ROC curve

### 3.3 Geometric morphometrics

PCA analysis revealed FCI grades of CHD-positive and CHD-negative individuals (Figure 3), and, along with a multivariate regression of the shape of grades A and E (23%,  $P < 0.0001$ ), clearly separated non-dysplasia (A) from severe dysplasia (E). TPS grids (Figure 3) showed a separation of the femur from the acetabular cavity in dysplastic dogs. In these dogs, landmarks and semi-landmarks located in the acetabulum (15-17 in the right hip and 33-35 in the left hip) were oriented laterally towards the caudal region; while those

located in the femoral head (3-8 in the right hip and 21-26 in the left hip) were oriented laterally perpendicularly to the mesial axis. Separation of the femur from the acetabulum is associated with greater laxity in dysplastic hips. A further characteristic of dysplastic individuals is a relatively wider femoral neck, causing extension of the landmarks and semi-landmarks 9, 10, and 11 in the right hip and 27, 28, and 29 in the left hip. The etiology and the mechanism by which the thickening of the femoral neck is associated with hip laxity are known [31].

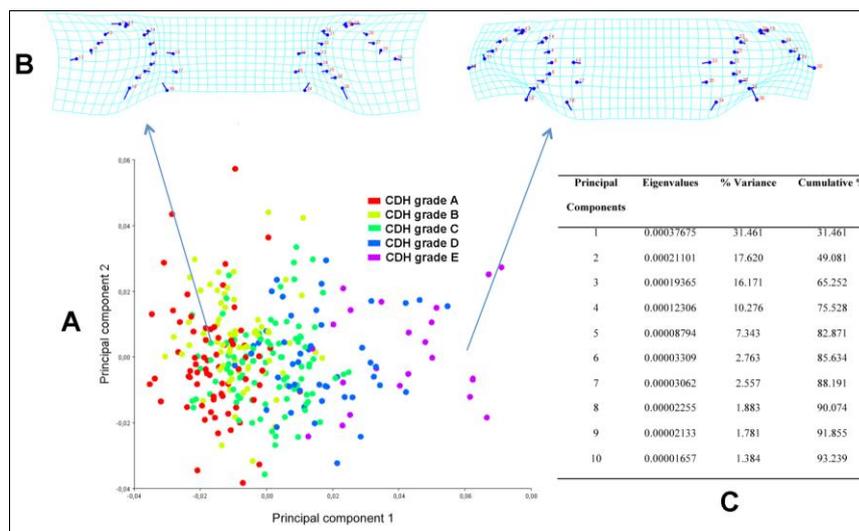
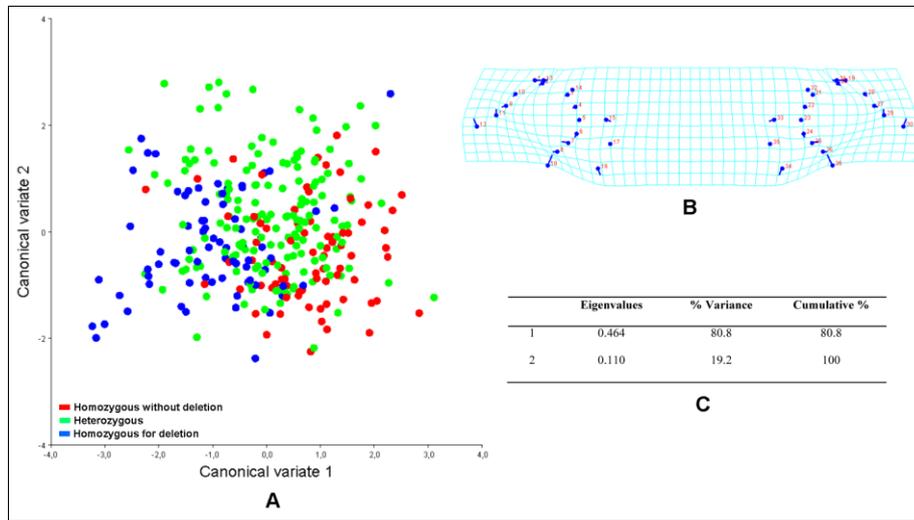


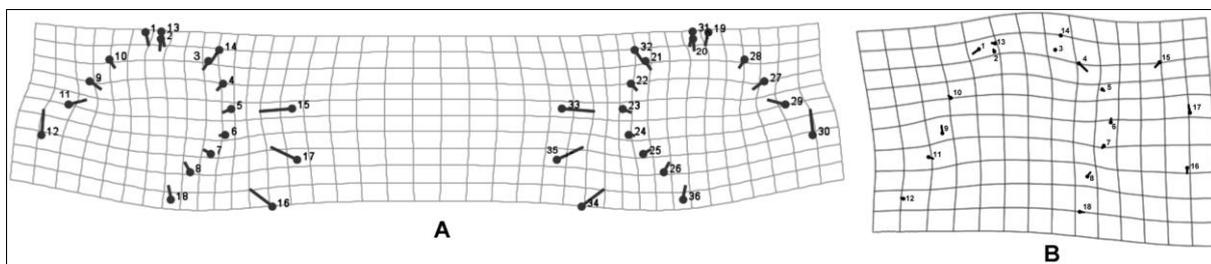
Fig 3: A) PCA analysis of the five FCI grades of CHD. B) thin-plate spline grid of the two extreme grades of CHD (A and E). C) Eigenvalues and % of cumulative variance.

A CVA analysis did not show a clear association of *FBN2* genotype with CHD, although there was a tendency to group those dogs homozygous without deletion on one side and homozygous with deletion on the opposite side of the axes (Figure 4). Some homozygous with deletion individuals were grouped with homozygous without deletion and heterozygous individuals overlapped with both homozygous groups. Although mean shapes differed, Procrustes distances were significantly different only between the two homozygous types ( $d=0.014$ ). Mahalanobis distances were also significantly different between the two homozygous types ( $d=1.912$ ) as well as between heterozygous and homozygous for deletion ( $d=0.009$ ). The thin-plate spline grid showed the same pattern of transformation of homozygous for deletion into homozygous without deletion (Figure 4).

Mean shape comparisons showed sexual dimorphism. Both Procrustes ( $d=0.013$ ) and Mahalanobis ( $d=1.512$ ) distances were significantly different in sexes. Transformation from female to male (Figure 5) showed the relative position of the birth canal to be the primary source of dimorphism. We found directional asymmetry, with left hip malformation more frequent than right in the analyzed dogs. ANOVA of shape based on Procrustes analysis revealed differences between both hip sides ( $F=3.07$ ;  $P < 0.0001$ ). However, these apparent differences between hips (Figure 5) could be caused by manipulation during radiography and could mask real morphological significance. The Procrustes ( $d=0.017$ ) and Mahalanobis ( $d=2.4846$ ) distances were significantly different between left and right hips.



**Fig 4:** A) CVA of hip conformation relative to *FBN2* genotype B) thin-plate spline grid for the transformation from hip of dogs homozygous for deletion to homozygous without deletion C) Eigenvalues and variance of CVA.



**Fig 5:** A) Deformation grid showing the transformation of the means of a female hip to a male hip. B) Deformation grid of left hip to right hip.

### 3.4 Genetic analyses

Pairwise relatedness (*r*) between dysplastic and non-dysplastic individuals using the software COANCESTRY was always  $r < 0.25$  for all the seven relatedness estimators (Table

4), indicating low level of familial relationship among analyzed dogs. Table 5 represents distribution and frequency of *FBN2* genotypes among the different FCI dysplasia grades.

**Table 4:** Mean and variance (Var) of pairwise coefficient relatedness (*r*) between dysplastic and non-dysplastic dogs based on seven relatedness estimators.

	TrioML	Wang	LynchLi	LynchRd	Ritland	QuellerGt	DyadML
<b>Dysplastic dogs (FCI grades: C, D and E)</b>							
Mean	0.074	-0.038	-0.034	-0.013	-0.014	-0.014	0.089
Var.	0.010	0.032	0.033	0.014	0.012	0.026	0.013
<b>Non-dysplastic dogs (FCI grades: A and B)</b>							
Mean	0.077	-0.028	-0.024	-0.012	-0.013	-0.010	0.091
Var.	0.011	0.030	0.032	0.014	0.009	0.023	0.013

**Table 5:** Distribution of *FBN2* genotypes among FCI classifications.

FCI Class / Genotype	Homozygous without deletion (n=79)	Heterozygous (n=153)	Homozygous with deletion (n=72)
A	32 (40.5%)	42 (27.4%)	3 (4.2%)
B	18 (22.8%)	33 (21.6%)	6 (8.3%)
C	19 (24.0%)	26 (17.0%)	19 (26.4%)
D	7 (8.9%)	15 (9.8%)	19 (26.4%)
E	3 (3.8%)	36 (23.5%)	25 (34.7%)

### 3.5 Statistical analyses

A one-way ANOVA yielded significant *p*-values for several independent factors, showing significant differences in NA between body types and *FBN2* genotypes (Figure 6). Differences in NA between sexes were not significant (Figure 6; Tukey's HSD test: male=96.7, female=96.1). Tukey's *post hoc* comparisons showed higher NA in genotypes homozygous without *FBN2* deletion (homozygous without deletion = 100.4, homozygous with deletion = 91.4,

heterozygous = 96.6) as well as in working dogs (working = 100.6, non-working = 95.6). However, a  $\chi^2$  test did not show an association between *FBN2* genotype and body type ( $\chi^2=3.957$ ,  $df=2$ ,  $P=0.1382$ ) when we considered only the original 46 radiographed working dogs included in the study. When the working dog sample was increased to 183, the association between genotype and body type became significant:  $\chi^2=10.953$ ,  $df=2$ ,  $P=0.004$ ; 44/229 homozygous for deletion in working dogs vs. 84/257 in non-working dogs.

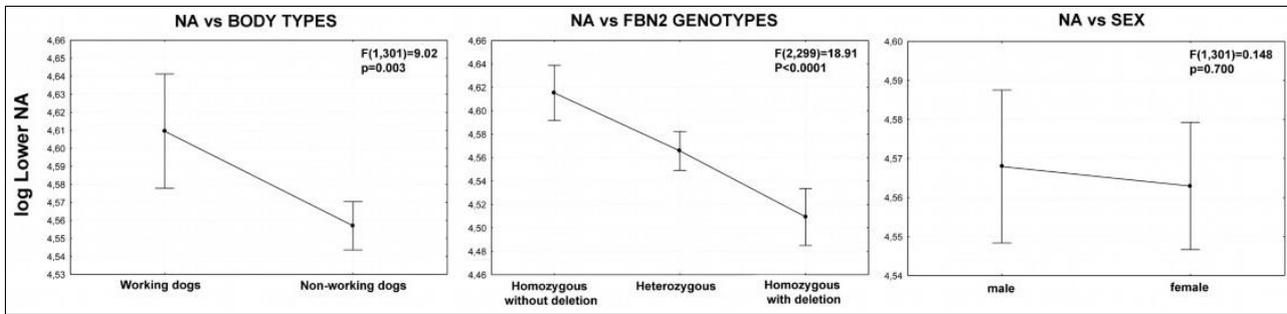


Fig 6: ANOVA analyses. Vertical bars denote confidence intervals at 95%

#### 4. Discussion

Although hip dysplasia and osteoarthritis have different genetic bases [5], hip laxity can induce osteoarthritis through improper mechanical function. Assessments of hip laxity such as Penn HIP™ or S-measurement approaches have been developed [32-33], but the most commonly used, and that adopted by the European FCI organization, is the NA. Estimation of NA is usually based on ventrodorsal radiographs [34], and may be affected by radiograph quality, particularly with respect to the position and orientation of the hip [35]. Our analyses of asymmetry demonstrated that it is crucial to control the manipulation of radiographed specimens to avoid bias in CHD diagnosis. Our outcomes reveal a rotation when one hip side is superimposed onto the other. This displacement of the landmarks when both sides are superimposed could be caused by differing rotation on hips when the radiograph is made, and could cause a bias in the NA value. Hence, it is important to take data accurately, especially since the evaluation of CHD is based on the most severely affected hip (smaller NA).

Significant differences were found in between-evaluator measurements, but due to the small range of error, the evaluators consistently agreed on FCI grade. These data are similar to previously published studies showing satisfactory reproducibility of NA measurements [14, 16], but point out possible evaluator bias and the need for comprehensive training in radiography evaluation. In general, dogs with a NA higher than  $105^\circ$  are designated non-dysplastic (A), and values of NA between  $100^\circ$  and  $105^\circ$  are considered borderline (B), depending on congruency between the femoral head and acetabulum [13]. Nonetheless, some studies suggest that the threshold angles discriminating dysplasia from non-dysplasia vary according to breed [16]. For the Spanish mastiff, the determined NA dysplasia cut-off ( $99.4^\circ$ ) slightly differed from the commonly used standard value ( $100^\circ$ ) between non-dysplastic (A and B) and dysplastic (C, D and E) grades. Nevertheless, this difference is not significant because the value of  $100^\circ$  is included within the 95% confidence interval estimated for the threshold value of  $99.4^\circ$ . However, according to results found by other studies [16] we emphasize the importance of calculating the threshold NA for diagnosis of CHD for each breed.

The prevalence of CHD varies among breeds [8]. The prevalence is high in the Spanish mastiff, approximately 56% in our study. The rate in Spanish mastiffs bred as pet for show may be underestimated, because dogs affected by the most severe CHD are not usually seen for evaluation. A high prevalence of CHD (65.8%) has also been found in the Estrela Mountain breed [3]. In our study, the Spanish mastiffs affected by CHD did not show obvious clinical symptoms, and this was also true of the Estrela Mountain dog [3]. However, the dogs in the present study were younger than 48 months, and osteoarthritis is more symptomatic at advanced age, and pet

owners do not routinely subject dogs to clinical evaluation. There were no significant differences between males and females in the CHD prevalence. Males were not more affected by CHD due to larger size, as is common in other breeds [36]. However, when size was eliminated, sexual dimorphism in hip morphology was revealed, with hips of females being wider and more separated relative to males, due to the position of the birth canal. As a consequence of the increased width of the female pelvis, the angle formed by the femoral neck with the body is more nearly a right angle than it is in males. However, these differences in hip morphology do not seem to influence other anatomical features of the hip or the NA.

Our results showed a significantly lower prevalence of CHD in working dogs. A factor suggested to improve CHD is a consistent level of moderate exercise and lower protein diet to maintain lower body weight [37]. Dog owners included in this study move livestock between regions biannually over distances  $>100$  km. In the Estrela Mountain dog, an association of feeding regimen, exercise, and body condition with CHD was not found [38]. This lack of association has been explained by the capacity of some dog breeds to self-monitor nutrition, which is common in the Spanish mastiff [38]. However, although Spanish mastiff breeders use similar feeds, surveys performed to breeders showed that non-working dogs are often overfed.

Geometric morphometric analyses showed that joint laxity and a thickening of the femoral neck characterize CHD. We found evidence of directional asymmetry [39] of CHD, with the left hip being most frequently affected. These results agree with those found in Portuguese water dogs, where a significant asymmetry was observed with greater laxity in the left hip relative to the right [9]. Directional asymmetry is considered to have a genetic basis [40]. However, studies of associations of QTLs with directional asymmetry have not found a clear heritable basis. In dogs, the majority of asymmetric traits appear to be environmentally based [9]. An exhaustive study of features in addition to laxity produced similar results for the German shepherd [10]. In our study, when we superimposed one hip onto the other, we observed rotation due to manipulation of the hip to be the main component of asymmetry. This emphasizes the difficulty in obtaining accurate ventrodorsal radiographs and their importance for accurate evaluation of CHD.

In the present study, the working dogs exhibited better hip structure than that of non-working. A serious problem for selection by breeders of working dogs is that difficulties in mobility appear late in age. Shepherds do not radiograph dogs for evaluation for CHD, and hip deformities are often not revealed until after dogs have reproduced. For this reason, genetic studies to evaluate CHD are necessary whenever economically feasible. Currently, no program for controlling CHD in the Spanish mastiff exists. The only genetic trait

evaluated in the present study was *FBN2* gene deletion. Dogs homozygous for this deletion showed a significantly smaller NA and poorer hip configuration, a tendency observed in the CVA, in which Mahalanobis and Procrustes distances were significant between the two homozygous *FBN2* groups. This trend to greater hip malformation in dogs with *FBN2* deletion was also found by Friedenber *et al.* [7]. However, homozygosity for *FBN2* deletion does not guarantee development of CHD or vice-versa. Formation of abnormal elastin in the connective tissues induced by the deletion may cause problems in the development of, or to repairing damage in, ligaments and joint capsules. Nevertheless, the association of altered *FBN2* expression with the development of CHD remains unclear [41]. We found a higher incidence of *FBN2* deletion in dogs bred as non-working, although this was not significant, possibly due to the lower sample of working dogs analyzed. When we increased the working dog sample to 229, we found a significantly lower proportion homozygous for deletion compared to non-working. Thus, it seems that there is probably indirect selection for complete *FBN2* in working dogs, although CHD is a polygenic inherited disorder involving several QTLs that possibly control its expression [5, 42]. Breeding programs could include selection for the *FBN2* gene without deletion but the mechanisms of expression of *FBN2* gene must be investigated. Currently, in the absence of better knowledge about the molecular basis of CHD inheritance, quantitative genetics (for instance, calculating Estimated Breeding Values) may help reduce the incidence of this disease in the Spanish mastiff.

## 5. Conclusion

FCI criteria for CHD are based on measurements of hip laxity and the NA. Our study reports bias due to the manipulation of the animal during radiography, with apparent asymmetry. Consequently, current evaluation of FCI based on the conformation of the most affected hip may be biased, and it seems more appropriate to take into account the scores for both hips. Inter-evaluator discrepancies in NA measures also pointed to the need for training programs for CHD evaluation. A threshold NA value of 99.4° allows discrimination of dysplastic from non-dysplastic Spanish mastiffs. Males and females differed in hip morphology, with hips being wider in females, but this difference did not influence traits analyzed for CHD. Working dogs had better hip conformation and were less affected by CHD. Although CHD expression has a polygenic source, homozygous *FBN2* deletion showed a significant correlation with CHD. The relationship of *FBN2* with CHD remains unclear, but the presence of complete gene variants, more frequent in working dogs, seems to be associated with normal hip configuration. This supports the idea of greater selection pressure for normal hip configuration in dogs used by nomadic shepherds. In Spain, the Spanish mastiff is the most popular livestock guardian breed used to protect against predators. Implementing programs to facilitate shepherd access to radiological and genetic tests is a priority.

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