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Genetic and genomic insights into morphological knee defects in Pura Raza Española horses

Nora Laseca[a,b], Antonio Molina[c], Davinia Isabel Perdomo-González[d], Chiraz Ziadi[c], Arancha Rodríguez Sainz de los Terreros[b] and Mercedes Valera[a]

[a]Departamento de Agronomía. Escuela Técnica Superior de Ingeniería Agronómica (ETSIA), Universidad de Sevilla, Sevilla, España; [b]Real Asociación Nacional de Criadores de Caballos de Pura Raza Española (ANCCE), Sevilla, España; [c]Departamento de Genética, Universidad de Córdoba, Córdoba, España; [d]Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, España

ABSTRACT

Conformation is a key selection criterion in horse breeding, influencing performance, health and appearance. Knee defects are a common morphological limb defect in horses, and their poorly understood genetics pose a major challenge to breeders. This study aimed to estimate genetic parameters and identify genomic regions associated with knee defects in Pura Raza Español (PRE) horses. Two evaluation approaches were used: continuous variables scored from −4 to 4 for side and front view knee angles (SVKA and FVKA); and multinomial variables on a 5–class scale assessing: buck and calf knee; bench and knock knee. 0 indicated no defect. Genetic analysis and genome-wide association study (GWAS) were performed using weighted single-step genomic BLUP methodology, assessing the knee angle of a total of 58,922 horses, with genotypes available for 4,057 individuals using the Axiom EQUIGENE array. The most prevalent severe defect for the population was knock knee (5.22%), while buck knee and bench knee were the least common (0.12%). Heritability estimates for continuous traits ranged from 0.19 (SVKA) to 0.20 (FVKA), and from 0.13 (bench knee) to 0.38 (knock knee) for independently evaluated traits. GWAS identified 17 genomic regions across seven chromosomes associated with knee defects. Additionally, candidate genes related to musculoskeletal diseases, bone malformations, joint disorders, and cartilage defects were identified. These findings enhance understanding of the genetic basis of knee defects and represent a first step towards genomic selection strategies in PRE horses. Further research is needed to deepen knowledge of conformation-related issues in equine breeding.

HIGHLIGHTS

- Analysis of the PRE horse genome has allowed us to locate regions associated with conformation and knee defects.
- The estimated heritability values indicated a moderate to high genetic influence on the morphological defect of the knee.
- Seventeen genomic regions were associated with knee conformational defects in horses.
- Genes related to chondrocytes, synovial joints, bone and cartilage formation, and joint disorders have been found within these regions.
- This research supports the development of genomic selection strategies to reduce knee defects in PRE horses.

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Introduction

Horse health is a fundamental requirement for good performance and durability in the equine industry. Diseases and defects of the locomotor system significantly affect the horse's functionality (Welsh et al. 2013; Jönsson et al. 2014). One example is knee defects in horses, a major concern in equine veterinary medicine because of their profound implications for both athletic performance and overall health (Axelsson et al. 2001; Anderson et al. 2004; Ripollés-Lobo et al. 2023). These conditions not only impair joint functionality but also predispose affected horses to chronic

CONTACT Nora Laseca nlaseca@us.es

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pain and reduced mobility, and lameness, underscoring their importance in equine health management (McIlwraith et al. 2011). These abnormalities can be congenital or acquired, compromising joint function, mobility and long-term health issues (Weishaupt et al. 2004). In competitive equine sports, where optimal performance is vital, knee defects often lead to decreased functionality and early retirement of affected horses (Solé et al. 2013). Additionally, the welfare of affected horses is a concern, as pain and movement problems can significantly reduce their quality of life. These defects cause significant economic losses for breeders.

The Pura Raza Española (PRE) horse is renowned for its elegance, agility and versatility, with conformation and aesthetics playing a crucial role in its overall value. Knee defects can significantly impact both the market value of the horse and its performance potential in competitions. In this breed, knee conformation is systematically assessed as part of the basic evaluation for studbook registration and genetically evaluated to guide selection decisions aimed at reducing the inheritance of such defects. The PRE horse is a native Spanish breed with an international presence, currently distributed across more than 71 countries and comprising a population of 290,407 active horses (MAPA 2024). The breed is managed by the Royal Purebred Spanish Horse Breeders' Association (ANCCE), which oversees the studbook established in 1912—as well as the implementation of the official breeding program.

The cause of knee defects is multifactorial, involving a combination of genetic predisposition, developmental influences, and environmental factors such as training intensity and nutrition (Jönsson et al. 2014). Genetic predisposition plays a crucial role, as certain breeds may inherit increased susceptibility. The study of the genetic basis of conformation and its defects has advanced in recent years, providing new insights into the heritable components of these conditions (Love et al. 2006; Kristjansson et al. 2016; Sánchez-Guerrero et al. 2016; Gmel et al. 2018). However, for knee defects, there are still few studies (Mostafa et al. 2019; Ripollés-Lobo et al. 2023). Genomic technologies, including genome-wide association studies (GWAS) and high-throughput sequencing, allow us to identify genetic variants and loci associated with conformation in horses (Frischknecht et al. 2016; Gmel et al. 2019; Rosengren et al. 2021; Gmel et al. 2023; Reich et al. 2024; Nazari-Ghadikolaei et al. 2025). However, to date, there are few genomic studies on conformational defects in limbs (Gmel et al. 2019; Reich et al. 2024) and specifically none on predisposition to knee anomalies. Therefore, our aim was to identify genomic regions associated with knee defects in Pura Raza Español horses by means of a genome-wide association study.

Materials and methods

Description of traits

Defects related to the angle of the knee in side view (SVKA) are defined as a buck knee defect when the horse's foreleg arches forward. The axis formed by the foreleg and the shank creates a broken line, with the knee positioned in front of the vertical line of the aplomb. Conversely, a calf knee defect occurs when the foreleg arches backwards, forming a broken line behind the aplomb line, between the axis of the forearm and the shank with the knee. Meanwhile, defects related to the angle of the knee in front view (FVKA) are described as bench knee defects when the knees deviate outwards from the line of aplomb and knock knee defects when the knees deviate inwards from the same reference line (Ripollés-Lobo et al. 2023).

The veterinary technicians assessed for knee angle of all horses in the side and front views following the procedures described by Sánchez-Guerrero et al. (2016). The evaluation of these traits is a linear score on a nine-level scale from −4 to 4, with 0 indicating no deviation as described in Table 1 (Ripollés-Lobo et al. 2023; ANCCE 2024).

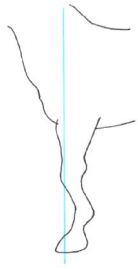
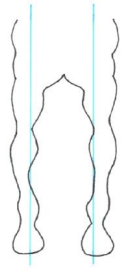
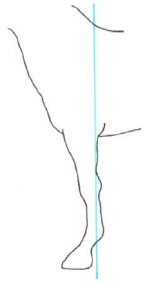
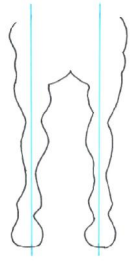
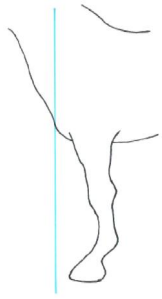
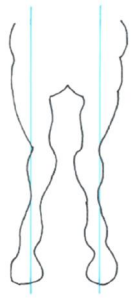
Two approaches were used to assess each knee angle trait:

1. Continuous variable, scored from −4 to 4 (9 levels) for both side view knee angle (SVKA) and front view knee angle (FVKA).
2. Two independent multinomial variables (underlying scale), scored from −4 to 0 and from 0 to 4, representing each of the knee defects separately: buck and calf knee, bench and knock knee defects. Horses with one of the defects were considered without defect for the opposite defect. That is, for example, horses with the buck defect were considered 'no defect' for the calf defect.

Animals, genotyping and quality control

In total, the dataset included records from 58,922 PRE horses (19,752 stallions and 39,170 mares), evaluated at an average age of 4.8 years during the mandatory morphological evaluation required for official studbook registration. The complete pedigree—comprising

Table 1. Description of knee defects according to the linear score of the morphological evaluation of the Pura Raza Española horse.

		Trait			
Score	Degree	Side view knee angle		Front view knee angle	
−4 −3 −2 −1	Very severe Severe Slightly	Buck knee		Bench knee	
0	Absence	On the vertical		On the vertical	
1 2 3 4	Slightly Severe Very severe	Calf knee		Knock knee	

398,866 animals across all available generations—was used to generate the relationship matrix. A total of 111,241 informative animals (i.e. those with performance control data, and a maximum of 25 generations, with an average of 5.8 complete generations) was obtained.

A total of 4,057 horses from over 700 studs were selected for genotyping. These individuals were selected to be highly representative of the PRE breed population, without direct kinship. The selection also focused on reflecting the morphological variability of the PRE, including recognised distinct morphological lines, and on individuals with assessed study traits. This sampling strategy ensures broad representativeness and minimises bias, thus supporting the validity and generalisability of our genomic analyses.

Genomic DNA was provided by the ANCCE molecular genetics laboratory. The selected horses were genotyped with Axiom™ EQUIGENE SNP array. Genotype data were analysed using the Axiom Analysis Suite 5.4 software, following the "Best Genotyping Practices Workflow" with default parameters (Dish Quality Control $>$ 0.82 and call-rate (CR) $>$ 0.95). Quality control was performed using PLINK v1.9 software (Purcell et al. 2007). SNPs with CR below 95% and minor allele frequencies (MAF) below 1% were removed. Y chromosome and mitochondrial DNA SNPs were excluded. After filtering, a total of 77,584 SNPs remained for the final analysis, distributed across all 32 Equus caballus (ECA) chromosomes (31 autosomal chromosomes and the X chromosome).

Weighted Single-step GREML (wssGREML) method

Knee defect traits were analysed using two trivariate models differentiated based on both biological (knee

angulation differentiated from lateral vs frontal anatomical planes) and statistical considerations (improve convergence and interpretability of results). The first model included SVKA, buck knee and calf knee traits, while the second model included FVKA, bench knee, and knock knee traits. The models used in matrix annotation were as follows:

$$y = Xb + Za + e$$

where **y** is the vector of phenotypic observations for the corresponding trait; **b** is the vector of fixed effects, including: sex (two levels; male and female), age assessment (two levels; young, for individuals aged between three and a half years (inclusive) and under five years old, and adult, for those aged five years or older) (Ripollés-Lobo et al. 2023), ancestral origin (two levels; Hispanic origin—phenotypically with grey coat colour, reflecting historical foundations and supported by observed phenotypic differences (Poyato-Bonilla et al. 2018; Sánchez-Guerrero et al. 2019) and genetic divergence (Poyato-Bonilla et al. 2022); Central European and Arabian breeds influence—phenotypically with non-grey coat colour), geographical area (four levels; Spain, rest of Europe, North America and Mexico, and South and Central America), stud size (three levels; less than three foals born per year, between three and nine foals born per year, and more than nine foals born per year), inbreeding (four levels; below 3.125%, between 3.125% and 6.25%, between 6.25% and 12.5%, and more than 12.5%) and proportionality index (four levels; less than 98, between 98 and 100, more than 100, and unknown), defined as the ratio between height at the withers and shoulder–ischial length (the straight-line distance from the scapulohumeral joint to the point of the buttock) (ANCCE 2025). This index was included to account for overall body conformation, as disproportionate horses (e.g. tall and short-bodied individuals) may present altered biomechanical stress and a higher likelihood of angular limb defects. Its inclusion aims to reduce potential confounding when estimating the genetic parameters of knee traits; **a** is the vector of random additive genetic effects; **e** is the vector of random residuals; and **X** and **Z** are the incidence matrices of **b** and **a**, respectively.

It was assumed that $a \sim N(0, H\sigma_a^2)$ for all traits, $e \sim N(0, I\sigma_e^2)$ for continuous SVKA and FVKA traits, and $e \sim N(0,1)$ for threshold buck knee, calf knee, bench knee, and knock knee traits, where σ_a^2 and σ_e^2 are the additive genetic and residual variances, respectively. Matrix **H** was obtained as described by Aguilar et al. (2010) by combining the numerator relationship matrix (**A**) with the genomic relationship matrix (**G**). The inverse of **H** matrix is:

$$H^{-1} = A^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & G^{-1} - A_{22}^{-1} \end{bmatrix}$$

where A is the pedigree-based relationship matrix for all animals; A_{22} is the pedigree-based relationship matrix for genotyped animals; and **G** is the genomic relationship matrix for genotyped animals, obtained following VanRaden (2008) as:

$$G = \frac{ZZ'}{\sum_{i=1}^{N} 2\widehat{p_i}\left(1 - \widehat{p_i}\right)}$$

where Z is a matrix of SNP genotypes; N is the number of SNPs, and p_i is the minor allele frequency of *i*th SNP.

Variance components and genomic breeding values (GEBVs) of the knee defect traits were estimated using the Gibbs Sampling algorithm in the GIBBSF90+ software (Lourenco et al. 2022). Chains of 200,000 samples were used with a burn-in period of 50,000. We retained one sample every 100 iterations to avoid high correlations between consecutive samples. To calculate posterior means and high posterior density intervals, and to check convergence with the Geweke test, a post-Gibbs analysis was performed using the POSTGIBBSf90 program (Tsuruta and Misztal 2006).

In the first iteration, a single-step genomic restricted maximum likelihood (ssGREML) approach was employed using the *G* matrix. Then, the estimates of SNP effects were obtained by back-solving GEBVs from ssGREML according to Wang et al. (2012):

$$\hat{a} = DZ'(ZDZ')^{-1}\hat{u}_g$$

where $\hat{a}$ is a vector of SNP effects, *D* is a diagonal matrix of weights (*D* is equal to the identity matrix for the ssGREML in Model 1), *Z* is the centred matrix of SNP genotypes and $\hat{u}_g$ is the vector of GEBV from genotyped animals only. Estimates of SNP effects were used to estimate individual variance of each SNP effect (Zhang et al. 2010):

$$\sigma_{u,i}^2 = 2\hat{a}_i^2 p_i(1 - p_i)$$

where p_i is the allele frequency of SNP i. SNP effects and variances were calculated using the POSTGSF90 software (Aguilar et al. 2010). Then, the vector of variances of SNP effects was used as weights in matrix D to construct the weighted matrix G (G^*) as described in Wang et al. (2012):

$$G^* = \frac{ZDZ'}{\sum_{i=1}^{N} 2\widehat{p_i}\left(1 - \widehat{p_i}\right)}$$

GEBV were estimated again with Model 1 using the GIBBSF90+ program (Lourenco et al. 2022) by considering weights for each SNP *via* the G^* matrix included in the H matrix. This process was carried out iteratively with weights estimated at each iteration as described in Wang et al. (2012).

The genetic correlations between traits not included in the same trivariate model were estimated following the methodology of Calo et al. (1973), which, in summary, consists of calculating the weighted correlation between two traits based on their estimated breeding values (EBVs), adjusted according to their respective reliabilities.

Genome-wide association analysis

The percentage of genetic variance explained by the *i*th set of SNPs included in a 1 Mb window (*i*th SNP window) was calculated as described by Wang et al. (2012) as:

$$\frac{Var(a_i)}{\sigma_a^2} x\ 100\% = \frac{Var\left(\sum_{j=1}^{x} Z_j \hat{u}_j\right)}{\sigma_a^2} x\ 100\%$$

where a_i is the genetic value of the *i*th SNP window of consecutive SNPs; σ_a^2 is the total additive genetic variance; Z_j is the vector of gene content of the *j*th SNP for all individuals and $\hat{u}_j$ is the effect of the *j*th SNP within the *i*th window.

The GWAS analysis was performed with the POSTGSF90 program (Aguilar et al. 2010), with the 1 Mb overlapping windows option. SNPs with more than 1% explained additive genetic variance were selected. Manhattan plots were performed with R software.

Functional annotation

We investigated which genes are located in the significant genomic regions associated with the knee defect using the Ensembl BioMart tool (Kinsella et al. 2011), based on the EquCab 3.0 reference genome assembly (Beeson et al. 2019). The genomic windows were 1 Mb, and all genes in the region were considered, prioritising those associated with previous associations with joint traits. We report on the biological role and biological processes of annotated genes in bone and cartilage morphology and metabolism, development and incidence of bone and joint-related diseases in human and other animal models, including horses using the DAVID software (Database for Annotation Visualisation and Integrated Discovery) (Sherman et al. 2022), AmiGO 2 software (Carbon et al. 2009) and the literature available in public databases.

Results and discussion

Morphological defects in the limbs—particularly in the knee—pose a significant challenge for breeders and owners, as they adversely affect the horse's welfare, functionality and performance, ultimately reducing its economic value (Anderson et al. 2004; Mostafa and Elemmawy 2020). One of the main selection criteria in the Pura Raza Española breeding program is the improvement of morphology and conformation, alongside traits related to sporting performance (Sánchez-Guerrero et al. 2016). Incorporating genomic tools into breeding programs offers a promising avenue for reducing the incidence of knee and other conformational defects, thereby enhancing the overall quality and athletic potential of horses. Using a weighted single-step genomic BLUP (wssGBLUP) approach, which integrates phenotypic and genomic data, we aimed to identify genomic regions associated with knee defects in PRE horses.

Prevalence and genetic parameters of knee angle conformational defects in PRE horses

No deviation was observed in 56.37% of horses (33,215) in the PRE population studied. The prevalence of the different levels of knee deviation in the evaluated PRE population is shown in Table 2. Most horses exhibited proper knee alignment, with no deviation recorded from either the side (72.44%) or front (71.76%) views. The most frequent deviations were knock knee (25.28%) and calf knee (22.19%), whereas bench knee was the least common (2.96%). Slight deviations were relatively common, particularly for calf knee and knock knee, accounting for 18.01% and 20.06% of the population, respectively. In contrast, deviations with a high or very high degree of severity were rare, ranging from 0.12% for bench knee and buck knee to 5.22% for knock knee. Recently, Ripollés-Lobo et al. (2023) reported that the most prevalent knee defect in PRE horses was knock knee, which is consistent with the findings in our study. In comparison, 31% of Thoroughbred jumping horses (Mostafa et al. 2019) and 36.8% of Norwegian cold-blooded trotters horses (Dolvik and Klemetsdal 1999) presented with calf knee, a greater prevalence than observed in the PRE breed (22.19%). The incidence of this abnormal conformation in elite Swedish Warmblood jumping horses was 18.7% (Holmström et al. 1990), which is similar to that of the PRE.

Differences in the prevalence of abnormal knee conformation across breeds are likely due to the specific usage and athletic demands placed on each

Table 2. Prevalence of the different levels of knee defect in the Pura Raza Española population evaluated.

Score	−4	−3	−2	−1	0	1	2	3	4
Degree	Very Severe	Severe	Slightly		Absence	Slightly	Severe		Very Severe
Side View (%)	25 (0.04)	48 (0.08)	573 (0.97)	2,517 (4.27)	42,684 (72.44)	10,613 (18.01)	2,174 (3.69)	231 (0.39)	57 (0.10)
		Buck knee (5.36%)			On the vertical		Calf knee (22.19%)		
Front View (%)	35 (0.06)	38 (0.06)	295 (0.50)	1,377 (2.34)	42,284 (71.76)	11,819 (20.06)	2,922 (4.96)	145 (0.25)	7 (0.01)
		Bench knee (2.96%)			On the vertical		Knock knee (25.28%)		

Table 3. Genetic parameters and heritability for knee defect traits in the Pura Raza Española population evaluated.

	Trait	σ^2_a (s.d.)	σ^2_e (s.d.)	σ^2_p (s.d.)	h^2 (s.d.)
Side view	*Buck knee*	0.42 (0.043)	1.21 (0.066)	1.62 (0.072)	0.26* (0.023)
	Calf knee	0.29 (0.015)	0.57 (0.036)	0.85 (0.04)	0.33* (0.02)
	SVKA	0.08 (0.004)	0.35 (0.003)	0.44 (0.003)	0.19 (0.009)
Front view	*Bench knee*	0.30 (0.058)	1.91 (0.127)	2.21 (0.129)	0.13* (0.025)
	Knock knee	0.29 (0.013)	0.48 (0.024)	0.76 (0.026)	0.38* (0.016)
	FVKA	0.08 (0.004)	0.33 (0.003)	0.42 (0.003)	0.20 (0.009)

SVKA- side view knee angle; FVKA- front view knee angle; σ^2_a- additive genetic variance; σ^2_e- residual variance; σ^2_p – phenotypic variance; h^2- heritability; s.d.- standard deviation; * Underlying scale.

breed. For instance, breeds specialised for high-impact disciplines like jumping or eventing disciplines where horses frequently perform powerful take-offs and landings, may experience increased stress on their knee joints. Similarly, dressage horses, which execute highly collected movements involving deep flexion and repetitive loading of the limbs, could develop unique patterns of joint wear. These intense and specialised movements place distinct biomechanical stresses on the knee joint, contributing to specific types of abnormalities over time. In addition, certain bone and joint conditions may have a genetic basis, influenced by artificial selection in some breeds (Anderson et al. 2004; Love et al. 2006; Jönsson et al. 2014). This genetic predisposition may be increased or selected for in breeding programs that prioritise performance traits, potentially at the expense of joint robustness. Other contributing factors may include differences in management practices, nutrition or environmental conditions, all of which can impact conformation and, consequently, the prevalence of such defects.

The heritability (h^2) of conformation traits depends on several factors, including the method of data collection (e.g. subjective assessment, different scoring scales, linear profiling or independent trait evaluation), the methodology applied (with or without genomic information), the sample size used for the estimation, the breed and the underlying genetic architecture (Sánchez et al. 2013; Gmel et al. 2022). The genetic parameters and heritability estimates of the knee defects are presented in Table 3. The h^2 values for knee defects assessed as continuous traits were moderate: 0.19 (±0.009) for SVKA and 0.20 (±0.009) for FVKA. The h^2 estimates of independently assessed traits ranged from 0.13 (±0.025) for the bench knee to 0.38 (±0.016) for the knock knee. As previously demonstrated in a study by Ripollés-Lobo et al. (2023), the h^2 values of independently evaluated traits -often measured on an underlying categorical scale tend to be higher than those of continuously evaluated traits. This may be due to threshold models overestimating variance on underlying liability scales. Recently, Ripollés-Lobo et al. (2023) reported h^2 estimates for knee defects in the PRE, which were very similar to those observed in our study, both for traits assessed on a linear scale and those evaluated independently. The most notable differences were found in the traits calf knee (0.33 vs. 0.26) and bench knee (0.25 vs. 0.13), likely reflecting the impact of incorporating genomic information into our model.

In Swiss Franches-Montagnes horses, the carpal joint angle was measured using two different landmark placements—one located in front of the limb and the other within the limb—resulting in heritability estimates of 0.13 and 0.27 (Gmel et al. 2022). Another study by Gmel et al. (2019) on the same breed and on Lipizzan horse estimated a genome-wide heritability of 0.35. These findings support the notion that heritability estimates can vary depending on the measurement method and the statistical approach used. Nevertheless, all reported values fall within the range of our estimated heritabilities. In German Warmblood horses, Reich et al. (2024) reported a h^2 of 0.05 for carpus position (over at knee—back at knee) and 0.01 for frontal carpal angle (wide at knees (bow-legged)—narrow at knees (knock-kneed)). These estimates were substantially lower than ours (0.19 for SVKA and 0.20 for FVKA), possibly due to differences in estimation

Table 4. Genetic and phenotypic correlations and their standard deviations for knee defects in Pura Raza Española population evaluated.

Trait	SVKA	Buck knee	Calf knee	FVKA	Bench knee	Knock knee
SVKA		−0.43 (0.815)	0.93 (0.135)	0.35 (0.878)	−0.22 (0.952)	0.36 (0.870)
Buck knee	−0.55 (0.698)		−0.37 (0.863)	0.16 (0.974)	0.10 (0.990)	0.11 (0.988)
Calf knee	0.89 (0.208)	−0.11 (0.988)		0.38 (0.856)	−0.06 (0.996)	0.56 (0.686)
FVKA	0.16 (0.974)	0.002 (0.999)	0.19 (0.964)		−0.54 (0.708)	0.95 (0.098)
Bench knee	−0.01 (0.999)	0.05 (0.998)	0.01 (0.999)	−0.45 (0.798)		−0.39 (0.848)
Knock knee	0.17 (0.971)	0.02 (0.999)	0.22 (0.952)	0.93 (0.135)	−0.08 (0.994)	

The phenotypic correlations (r_p) are below the diagonal, and the genetic correlations (r_g) estimated following the methodology of Calo et al. (1973), are above the diagonal and their corresponding standard deviations (s.d.); SVKA- side view knee angle; FVKA- front view knee angle.

methodology, sample size and the quality of phenotype recording. In our study, we included all evaluated animals and used the wssGBLUP method, which integrates both genotyped and non-genotyped individuals, while Reich et al. (2024) based their estimates solely on genotyped animals and SNP-based heritability.

The estimates of the phenotypic and genetic correlations for knee defects are presented in Table 4. To ensure interpretability and comparability, we opted to present only genetic correlations derived from EBV (Calo et al. 1973), instead of mixing correlations estimated within the trivariates (analysed on the underlying scale from threshold models with those from linear traits on the actual scale) with those estimated following Calo et al. (1973) between traits included in different trivariate models. This choice allows all correlations to be expressed in a common, comparable scale, avoiding the difficulty of interpreting results across different model assumptions.

The obtained correlations showed a highly consistent pattern with the nature and prevalence of knee defects in the PRE population, showing the same pattern in the case of genetics and phenotypic factors, although, as expected, with a lower magnitude in the case of the latter.

Negative correlations were observed between opposite defects in the same angle view (buck knee vs. calf knee (–0.37); bench knee vs. knock knee (–0.39)). These values reflect that as the frequency of one defect increases, its opposite defect tends to disappear from the population, confirming their mutually exclusive nature. Furthermore, high positive correlations were found between continuously assessed defects and the most prevalent individually assessed defects in the same view (FVKA vs. knock knee, 0.95; SVKA vs calf knee, 0.93). Conversely, correlations were negative when comparing continuously assessed defects with the less prevalent individual defects (FVKA vs. bench knee, −0.54; SVKA vs buck knee −0.43). These results suggest that prevalence dictates both the magnitude and direction of the correlations, with prevalent defects explaining a significant portion of the angular variability, and rare defects exhibiting an inverse relationship. Furthermore, continuously evaluated defects showed moderate positive correlations with the most prevalent defects of the opposite view angle. Specifically, FVKA correlated positively with calf knee (0.38) and SVKA with knock knee (0.36). These findings suggest that continuously evaluated defects in one view also capture a portion of the transverse variability in knee alignment, which reinforces the concept of a structural continuum connecting lateral and frontal deviations.

On the other hand, correlations between defects that were not directly opposed were generally low, with values close to zero in most cases (or magnitude statistically not differentiated from zero). This indicates independence between traits from different views or without a direct anatomical relationship. Only one association was of moderate magnitude, knock knee vs. calf-knee (0.56), which suggests a tendency for the co-occurrence of lateral deviations and "calf" angulations, linked to the biomechanics of the hind limbs.

Comparing our study with that of Ripolles-Lobo et al. (2023), it is observed that of the four correlations reported by those authors, the calf knee vs. knock knee correlation was the highest in both studies (0.70 in Ripollés-Lobo et al. (2023) vs 0.56 in our case), and buck knee vs. knock knee correlations are similar in both (0.13 vs. 0.11). However, bench knee vs. buck knee and bench knee vs. calf knee were lower in our study. These differences should not be interpreted as inconsistencies but rather as a consequence of the low prevalence of the bench knee defect, very sensitive to the different methodology used. Specifically, in the study of Ripollés-Lobo et al. (2023), all variables were treated on linear appreciation scales with a smaller number of categories, whereas in this study, we distinguished between continuously evaluated defects (in nine classes) and individual opposing discrete defects (modeled as threshold traits).

We contributed to the understanding of knee defects by presenting new correlations that reveal a structural continuum within and between the frontal and lateral views. These previously unreported

Table 5. Significant regions identified in the genome-wide association study associated with knee angle defect in Pura Raza Española horses and protein coding candidate genes annotated in the regions.

Trait	ECA	Genomic Region (bp)	v.e. (%)	Candidate genes
Bench knee	3	34,564,428-35,562,054	1.08	*SLC7A5; ZNF469*
Buck knee	4	36,357,670-37,282,457	1.06	*CDK6; SAMD9L; CALCR*
Bench knee	4	62,791,008–63,734,743	1.25	*PDE1C*
Bench knee	7	51,586,388-52,487,426	1.22	*DNMT1; COL5A3; PIN1*
Bench knee; Buck knee	7	52,647,348-53,634,285	1.72; 1.14	
Bench knee	7	63,443,669-64,406,448	1.19	*DLG2; PRCP*
Buck knee	10	23,490,372-24,452,786	1.01	*VSTM1; OSCAR; RPS9; TNNT1*
FVKA	10	23,993,540-24,965,648	1.20	*TNNT1*
FVKA	10	27,356,213-28,346,515	1.65	*ZNF606*
FVKA; Knock knee	10	40,539,712-41,516,383	2.62; 1.44	*HTR1E*
Bench knee	11	40,332,518-41,329,098	1.28	*PSMD11; RHOT1; ADAP2; SUZ12; RAB11FIP4; NF1*
Bench knee	11	42,243,180-43,220,579	1.07	*NOS2; POLDIP2; SARM1; TRAF4*
Bench knee	11	44,163,659-45,130,619	1.13	
FVKA; Bench knee	11	45,864,496-46,845,582	1.04; 1.85	*OVCA2; HIC1; SMG6*
Bench knee	11	49,348,422-50,327,155	1.34	*NLRP1; MINK1; VMO1; CXCL16; ALOX15*
Buck knee	19	57,663,111-58,661,402	1.08	*COL8A1*
FVKA; Knock knee	23	24,843,814-25,843,782	1.31; 1.36	*GLIS3; SLC1A1*

FVKA: front view knee angle; ECA: equine chromosome; bp: base pair; v.e.: variance explained.

connections strengthen the concept of a holistic biomechanical structure. Supported by a large sample size, genotyped data, and a robust methodology based on phenotypic correlations from genetic values (Calo et al. 1973), our results offer a more complete and reliable basis for interpreting the genetics of these defects and developing more effective breeding strategies for PRE horses.

Genome-wide association study of knee defects and candidate genes

The GWAS for knee angle conformation traits revealed 17 genomic regions across seven chromosomes (Table 5 and Figure 1). These regions explained more than 1% of the genetic variance, with four regions accounting for over 1.5%. Ten of the significant regions were associated with bench knee, two with knock knee, four with buck knee and five with the continuously evaluated trait front view knee angle (FVKA). Interestingly, we observed shared genomic regions influencing multiple knee angle traits. For instance, one region on ECA7 was associated with both the bench and buck knee. In addition, three of the regions identified for FVKA were also associated with specific angular knee deformities, either bench knee or knock knee (Table 5), suggesting common genetic underpinnings for both independent variation in knee angle and categorical defects. These findings suggest that certain genomic regions may serve as "hotspots" for broader limb conformation traits. Such regions represent key targets for breeding strategies aimed at improving overall limb soundness and minimising the risk of multiple joint-related issues. The observed overlap with regions previously associated with different limb conformational defects supports the hypothesis of pleiotropic effects, where a single genomic region influences multiple phenotypes. This is biologically plausible given the conserved developmental pathways and integrated biomechanics of the equine limb, whereby genes involved in cartilage, bone, or extracellular matrix homeostasis may exert effects across multiple synovial joints. Alternatively, or in addition to pleiotropy, these overlaps may reflect linkage disequilibrium, whereby distinct causal variants for different joint traits reside in close physical proximity and are co-inherited. Further fine-mapping and functional validation studies will be crucial to disentangle pleiotropic effects from linkage and to better elucidate the genetic architecture underlying limb morphology in horses. Notably, one of the identified regions has been previously reported as significant in other GWAS. The region on ECA4 (36,357,670-37,282,457) was associated with carpal joint angle in both Franches-Montagnes and Lipizzan horses (Gmel et al. 2019).

Within fifteen of the significant genomic regions, we identified a total of 38 genes related to chondrocytes and chondrocyte-associated processes, synovial joints, bone and cartilage development, and joint disorders (Table 5). Full details are provided in Supplementary Table S1, but the most prominent genes are discussed below.

When joint trauma or inflammation occurs, the synovial membrane plays a key role in the progression of joint damage. One of the genes expressed in the equine synovial membrane is *SAMD9L* (Sterile alpha motif domain containing 9–Like) gene, located on ECA4, proposed as a potential marker for fibroblast-like synoviocytes (Thomsen et al. 2017). Another gene expressed in fibroblast-like synoviocytes was the *CXCL16* (C-X-C motif chemokine ligand 16) gene on ECA11 (Li et al. 2016; Chwastek et al. 2022). Interestingly, the *CALCR* (calcitonin

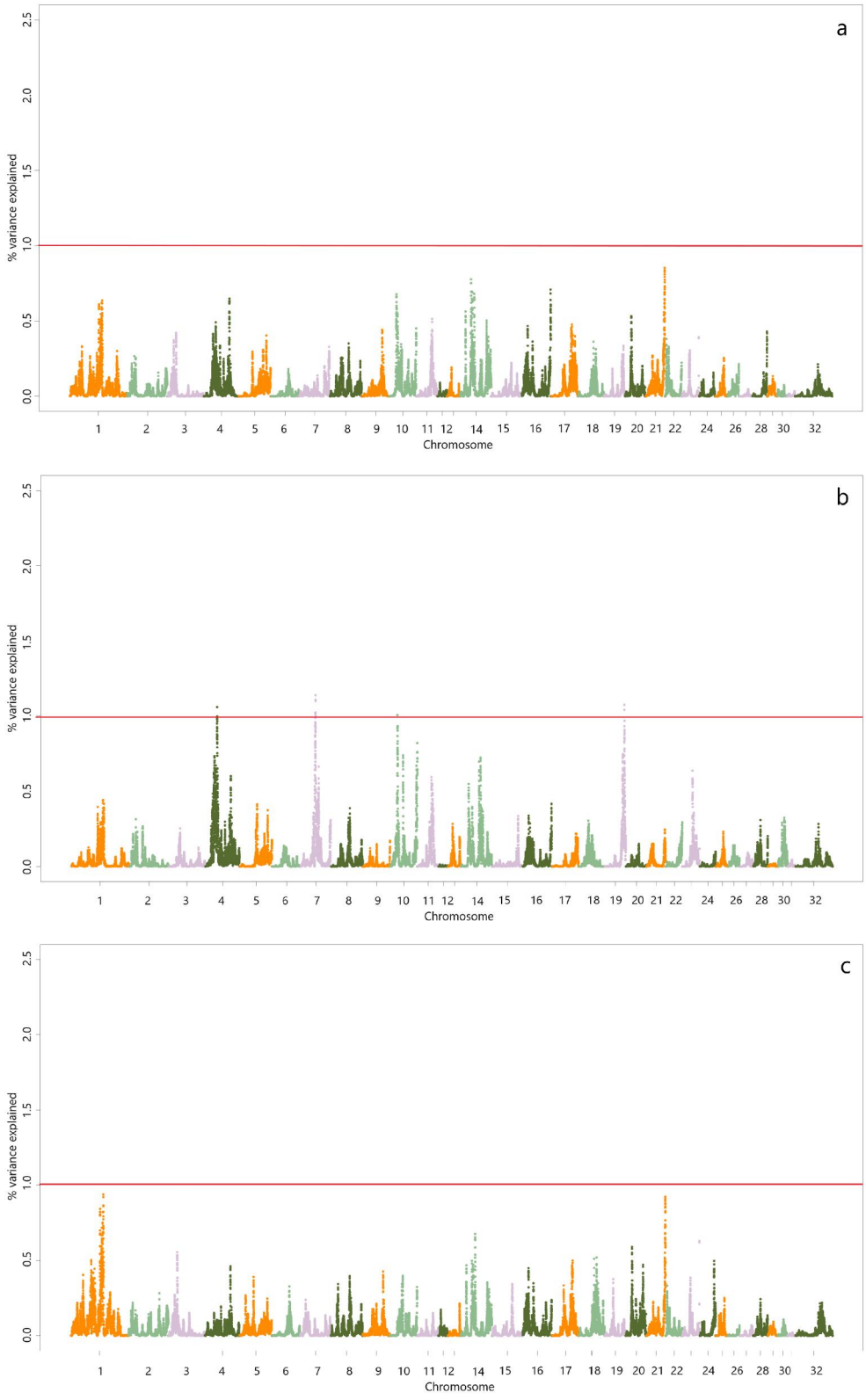


Figure 1. Manhattan plot of the variance explained (y axis) of each SNPs per 1 Mb window of adjacent SNPs for the trait. (a) Side view knee angle, (b) buck knee, (c) calf knee, (d) front view knee angle, (e) bench knee, and (f) knock knee. The red lines indicate the genome-wide significance threshold with explained variance equal to 1%.

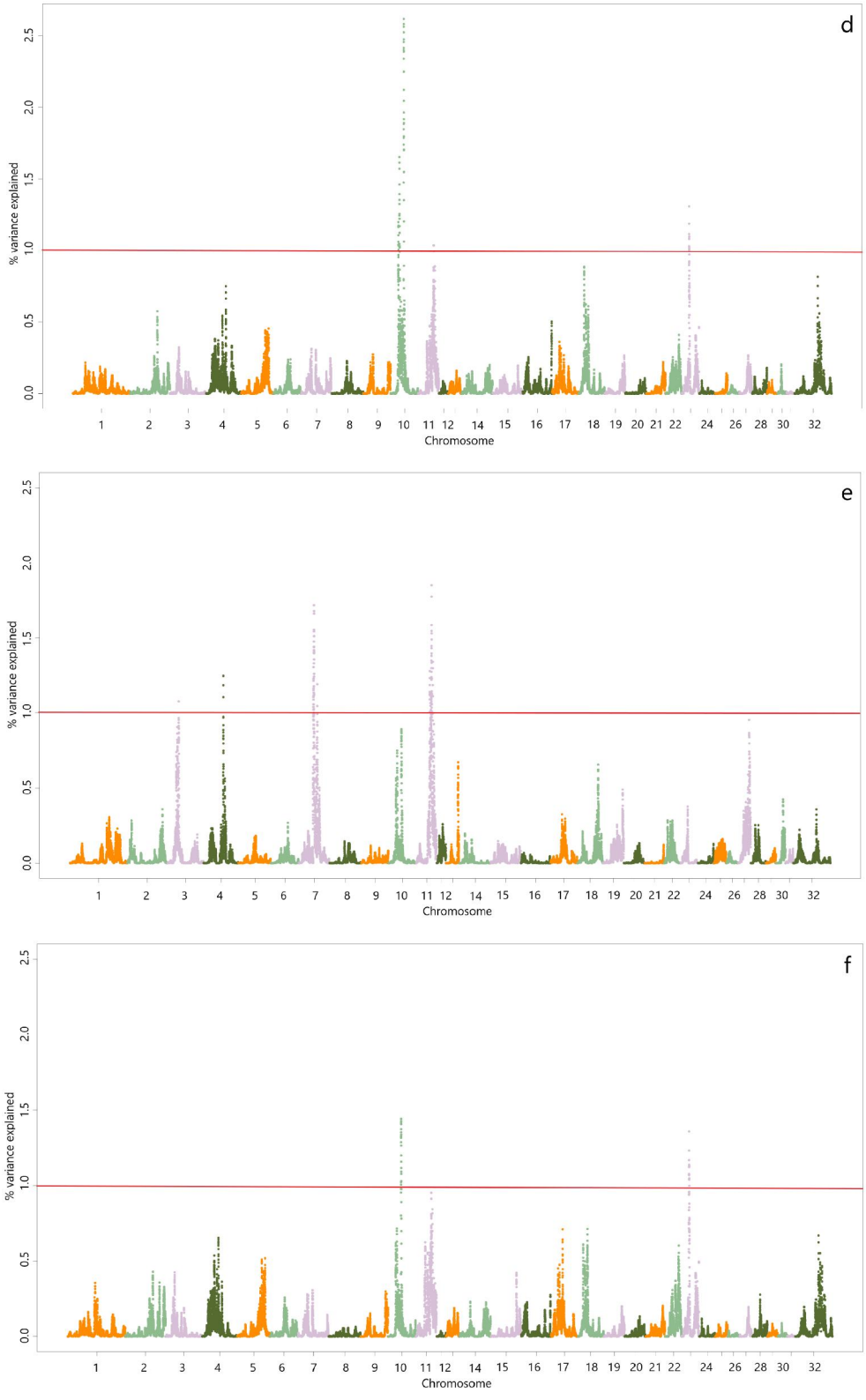


Figure 1. Continued

receptor) gene, also located on ECA4, was previously identified in a GWAS on carpal joint angle and linked to weakened carpal bones in horses with reduced carpal angles, increasing the risk of fractures (Gmel et al. 2019). On ECA7, we found genes related to degenerative suspensory ligament desmitis -a progressive idiopathic disease causing scarring and rupture of suspensory ligaments in multiple limbs in horses- such as the *COL5A3* (collagen type V alpha 3 chain) gene involved in collagen synthesis and the *PIN1* (peptidylprolyl cis/trans isomerase, NIMA-interacting 1) gene associated with tendon ageing (Momen et al. 2022). Finally, the *VSTM1* (V-set and transmembrane domain containing 1) gene was identified on ECA10 and previously associated with a QTL linked to navicular bone disease in Hanoverian Warmbloods (Lopes et al. 2010).

In addition, we identified several genes associated with chondrocytes and their formation and differentiation, including the gene *CDK6* (cyclin dependent kinase 6) on ECA4, the *ZNF606* (zinc finger protein 606) gene on ECA10 and the *RHOT1* (ras homolog family member T1) gene, the *SUZ12* (SUZ12 polycomb repressive complex 2 subunit) gene and the *RAB11FIP4* (RAB11 family interacting protein 4) gene on ECA11. Regarding the *CDK6* gene, Ito et al. (2014) reported that its overexpression in chondrocytes inhibited their maturation. The *ZNF606* gene functions as an inhibitor of chondrocyte differentiation (Zhou et al. 2016). The *RHOT1* gene contributes to chondrocyte proliferation and plays a role in repressing autophagy and extracellular matrix production (Man et al. 2022). The *SUZ12* gene is implicated in the regulation of chondrocyte maturation (Baronas et al. 2023). More recently, Lindberg et al. (2024) identified *RAB11FIP4* as a gene involved in the dedifferentiation process of bovine chondrocytes.

Finally, we identified several genes associated with osteoarthritis (OA), especially in humans, a prevalent joint disorder characterised by articular cartilage degradation and subchondral bone remodelling (Sharma 2021). These associations include: On ECA3, we found the *SLC7A5* (solute carrier family 7 member 5) gene, previously proposed as a biomarker for knee OA (Zhao et al. 2022). On ECA4, the *PDE1C* (phosphodiesterase 1 C) gene was identified, notable for its overexpression in OA cartilage, where its inhibition has been shown to reduce extracellular matrix degradation and chondrocyte apoptosis (Zhang et al. 2021). The *DNMT1* (DNA methyltransferase 1) gene on ECA7 showed decreased expression in OA cartilage (Liu et al. 2024), with its polymorphisms also linked to OA susceptibility (Miranda-Duarte et al. 2020). On ECA10, the *OSCAR* (osteoclast-associated Ig-like receptor) gene was found, playing a role in chondrocyte apoptosis (Park et al. 2020) and previously involved in navicular bone disease in Hanoverian Warmblood horses (Lopes et al. 2010). Furthermore, several genes located on ECA11 were associated with OA. These include *NF1* (neurofibromin 1) gene, involved in bone regeneration (Tan et al. 2020), and associated with knee OA in GWAS as well as *SMG6* (SMG6 nonsense mediated mRNA decay factor) gene (Primorac et al. 2020); *NOS2* (nitric oxide synthase 2) gene, whose increased expression in OA cartilage indicates inflammatory activity (Dranitsina et al. 2019); *NLRP1* (NLR family pyrin domain containing 1) gene, where inflammasomes were upregulated in OA cartilage (Zhao et al. 2018); and *MINK1* (misshapen like kinase 1) gene, whose deficiency protects against cartilage degeneration in aged joints (Yu et al. 2020). Finally, the *COL8A1* gene (collagen type VIII alpha 1 chain) on ECA19 was identified as a biomarker for knee OA (Fang et al. 2019; Wen et al. 2024), and the *GLIS3* gene (GLIS family zinc finger 3) on ECA23 featured functional variants significantly associated with a reduced risk of OA (Zhang et al. 2021; Kenny et al. 2023).

Conclusion

The estimated heritability values indicated a moderate-to-high genetic influence on knee morphology, underscoring the feasibility of genomic selection to reduce the prevalence of these defects. The genome-wide association study identified 17 genomic regions and several candidate genes associated with knee defects in Pura Raza Española horses. These findings support the genetic basis of knee conformational traits and highlight the potential of integrating genomic information into breeding programs. Furthermore, our results were consistent with previous studies in different horse breeds, supporting the relevance of these genomic regions in joint conformation and function. Overall, the integration of genetic markers and associated genomic regions into selection strategies could improve the morphology and athletic performance of PRE horses, ultimately benefiting both their welfare and economic value.

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Disclosure statement

The authors report there are no competing interests to declare.

Ethical approval

The approval of the work by an ethics committee was not required.

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ORCID

Nora Laseca http://orcid.org/0000-0003-3753-6725
Antonio Molina http://orcid.org/0000-0002-9566-6600
Davinia Isabel Perdomo-González http://orcid.org/0000-0003-2618-105X
Chiraz Ziadi http://orcid.org/0000-0002-1866-7028
Mercedes Valera http://orcid.org/0000-0003-1742-550X

Data availability statement

The dataset supporting the results of this study was supplied by the Real Asociacion Nacional de Criadores de Caballos de Pura Raza Española (ANCCE). The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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