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# Polymorphisms in ten candidate genes are associated with conformational and locomotive traits in Spanish Purebred horses

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**Abstract** The Spanish Purebred horses, also known as Andalusian horses, compete to the highest standards in international dressage events. Gait and conformation could be used as early selection criteria to detect young horses with promising dressage ability. Although the genetic background of equine size variation has been recently uncovered, the genetic basis of horse conformational and locomotive traits is not known, hampered by the complex genetic architecture underlying quantitative traits and the lack of phenotypic data. The aim of this study was to validate the loci associated with size in 144 Spanish Purebred horses, and to seek novel associations between loci previously associated with the development of osteochondrosis (OC) lesions and 20 conformational and locomotive traits. Ten loci were associated with different conformational and locomotive traits (*LCORL/NCAPG*, *HMGA2*, *USP31*, *MECR*, *COL24A1*, *MGP*, *FAM184B*, *PTH1R*, *KLF3* and *SGK1*), and the *LCORL/NCAPG* association with size in the Spanish Purebred horse was validated. Except for *HMGA2*, all polymorphisms seem to influence both the prevalence of OC lesions and morphological characters, supporting the link between conformation and OC. Also, the implication of most genes in either immune and inflammatory responses and cellular growth, or ossification processes, reinforces the role that these mechanisms have in the aetiology of OC, as well as their reflection on the general conformation of the individual. These polymorphisms could be used in marker-assisted selection (MAS) programmes to improve desirable conformational traits, but taking into account their possible detrimental effect on OC prevalence.

**Keywords** *Equus caballus* · Morphology · Association · Single nucleotide polymorphism · SNP

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## Introduction

The Spanish Purebred horses, also known as Andalusian horses, have been bred for classical dressage since the 15th century (Lenoir 1998), and compete to the highest standards in international dressage events. Characteristics of gaits, including the walk, trot and gallop, and figures are judged in this Olympic equestrian sport and described by the Fédération Equestre Internationale (FEI 1999). Apart from competition and performance test scores, gait and conformation could be used as early selection criteria to detect young horses with promising dressage and jumping abilities, predicting locomotor performance in adult animals (Back et al. 1994; Barrey et al. 2002a, b). However, there is considerable variability in the evaluation of dressage ability, which depends on subjective assessments, although applied by experienced judging committees (Clayton and Schamhardt 2001; Hawson et al. 2010), and could result in the misjudging of the natural dressage ability of a horse that may be reflected in the low mean heritability estimates ($h^2 = 0.04–0.27$) on this discipline (Langlois et al. 1980; Bruns 1981; Huizinga and van der Meij 1989; Barrey and Biau 2002).

Conformation, as for many other traits in domestic animals, is the result of both natural and man-induced selection for

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different purposes (Saastamoinen and Barrey 2000), as reflected by the significant differences between selected and non-selected horses for several conformation variables (Holmström 2001). Conformational traits seem to be related to specific performance, e.g. race vs. jumping, or longevity, predisposition to lameness (Koenen et al. 1995; Saastamoinen and Barrey 2000; Weller et al. 2006). Intense selection for certain traits like size has led to great breed variations, ranging from one to two metres in the American Miniature and the Percherons, respectively (Brooks et al. 2010). In dressage, some relationships between certain conformational traits and body dimensions seem to be advantageous (Solé et al. 2013).

The genetic background of equine size variation has been simultaneously uncovered by Signer-Hasler et al. (2012) and Makvandi-Nejad et al. (2012), who reported four loci (*LCORL*/*NCAPG*, *HMGA2*, *ZFAT* and *LASP1*) explaining 83% of the size variation in horses. Moreover, Frischknecht et al. (2016) have recently located selection signatures related to height in Shetland ponies. On the contrary, the genetic basis of horse conformational and locomotive traits is poorly known, apart from the work of François et al. 2016 on *MSTN* and Andersson et al. (2012) on *DMRT3*, hampered by the complex genetic architecture underlying quantitative traits and the lack of phenotypic data. However, several studies have shown heritabilities of morphological traits ranging from 0.09 to 1.00 (e.g. Koenen et al. 1995; Dolvik and Klemetsdal 1999; Love et al. 2006; Ducro et al. 2009), opening the possibility of improving these characters by selection. The aim of this study is to validate the four loci associated with size in the Spanish Purebred horse, and to seek novel associations between loci previously associated with osteochondrosis (OC) lesions (Sevane et al. 2016) and conformational and locomotive traits, based on previous studies that found associations between morphological characteristics and OC in pigs (Aasmundstad et al. 2014; de Koning et al. 2015).

## Materials and methods

### Animals and phenotypic data

Phenotypic data were collected from 144 yearling Spanish Purebred horses born between 2000 and 2012, and registered in the studbook of the breed in Spain. To reduce environmental effects, all animals belonged to one of the most important Spanish Purebred horse stud, an open stud that, every year, adds new stallions to the herd, offering a wide representation of the breed that included the offspring of 44 sires and 116 dams. The conformation of each individual was assessed in a fixed routine by a single expert observer. Twenty specific conformational traits were assessed in all animals (Table 1): six body measurements including height at withers (HW), body length (BL), knee perimeter (KP), fore cannon circumference (FCC), hind cannon circumference (HCC) and hock circumference (HC); and the grade of 'head' (HS), 'neck' (NS), 'back, withers and forelimb' (BWFS), 'chest, thorax and barrel' (CTBS), 'back and loin' (BLS), 'croup and tail' (CTS), 'fore conformation' (FCS), 'hind conformation' (HCS), 'overall conformation' (OCS), 'breed standard' (BSS), 'walk collected' (WCS), 'trot collected' (TCS) and 'canter collected' (CCS), scored within the range 0–10, where a score of 0 indicated poor conformation and a score of 10 indicated desirable conformation. Also, 'hoof colour score' (HOCS) was recorded within the range 0–4, where a score of 0 indicated clear colour and a score of 4 indicated darker colour.

**Table 1** Conformational and locomotive traits measured in 144 Spanish Purebred horses

| Trait (units) | Abbreviation | Mean ± SE |
|---|---|---|
| Height at withers (m) | HW | 1.63 ± 0.003 |
| Body length (m) | BL | 1.61 ± 0.004 |
| Knee perimeter (cm) | KP | 34.05 ± 0.15 |
| Fore cannon circumference (cm) | FCC | 21.11 ± 0.10 |
| Hind cannon circumference (cm) | HCC | 23.35 ± 0.08 |
| Hock circumference (cm) | HC | 45.29 ± 0.16 |
| Head score (0–10) | HS | 6.63 ± 0.11 |
| Neck score (0–10) | NS | 7.00 ± 0.10 |
| Back, withers and forelimb score (0–10) | BWFS | 7.02 ± 0.10 |
| Chest, thorax and barrel score (0–10) | CTBS | 6.93 ± 0.10 |
| Back and loin score (0–10) | BLS | 6.24 ± 0.09 |
| Croup and tail score (0–10) | CTS | 6.85 ± 0.12 |
| Fore conformation score (0–10) | FCS | 5.45 ± 0.07 |
| Hind conformation score (0–10) | HCS | 5.46 ± 0.06 |
| Overall conformation score (0–10) | OCS | 6.59 ± 0.09 |
| Breed standard score (0–10) | BSS | 6.60 ± 0.10 |
| Walk collected score (0–10) | WCS | 6.13 ± 0.08 |
| Trot collected score (0–10) | TCS | 6.35 ± 0.08 |
| Canter collected score (0–10) | CCS | 6.26 ± 0.07 |
| Hoof colour score (0–4) | HOCS | 3.24 ± 0.10 |

### SNP selection and genotyping

Forty-eight single nucleotide polymorphisms (SNPs), 24 previously associated with different manifestations of OC in several horse breeds and the other 24 located in candidate genes from equine, porcine and human studies, were genotyped in the full set of samples (see Sevane et al. 2016 for more detail on the SNPs and the genotyping conditions). Four SNPs previously associated with size variation in horses were also included in this study (Makvandi-Nejad et al. 2012) (Table 2). These four SNPs were included in a multiplex capillary primer extension assay following the procedure described by Sevane

**Table 2** Mutations explaining 83% of the size variation in horses (Makvandi-Nejad et al. 2012) and allele frequencies in 144 Spanish Purebred horses

| Locus | SNP location | Effect on size from literature | | Frequency of the small allele |
|---|---|---|---|---|
| | | Small | Big | |
| *HMGA2* | Ch6 81, 481, 064 | C | T | 0.05 |
| *LASP1* | Ch11 23, 259, 732 | G | A | 0.78 |
| *LCORL* | Ch3 105, 547, 002 | T | C | 0.53 |
| *ZFAT* | Ch9 75, 550, 059 | G | A | 0.91 |

et al. (2010). Table S1 details the multiplex and primer extension primers and polymerase chain reaction (PCR) conditions. Replication of SNP genotyping was performed in 5% of the samples for repeatability purposes and Mendelian inheritance was checked in four trios for reliability.

### Statistical analysis

SNPs with minor allele frequency (MAF) less than 0.05 were excluded from the association analysis to avoid bias of the data. Linear regression analysis was then applied to test associations between genotypes and phenotypes using R programming (http://www.r-project.org) and the *lme4* statistical package, which fits linear models and generalised linear mixed models (GLMMs) to data (Bates and Maechler 2008). The main assumption was that the SNP effect on any of the traits is additive.

The effect of the SNP on each of the traits was estimated by including them as a covariate into a linear model. The model used in this study was:

$$Y_{ijk} = S_k + BY_i + \alpha G_{ijk} + e_{ijk}$$

where:

- $Y_{ijk}$ Is the trait registered in the *j*th individual for the *k*th sex and *i*th birth year
- $S_k$ Is the fixed effect of the *k*th sex
- $BY_i$ Is the fixed effect of the *i*th birth year
- $\alpha$ Is the regression coefficient for the relation between Y and G
- $G_{ijk}$ Are the ordered genotype constants with values 1, 2 or 3
- $e_{ijk}$ Are independent $N(0, \sigma^2)$
- j 1,..., 144

In order to correct for multiple testing in each group, a permutation analysis was carried out to calculate the experiment-wise significance threshold within each trait (Churchill and Doerge 1994). For each permutation, SNP genotypes were randomised across all animals. The effect of each SNP was then estimated and the maximum F-statistic across all SNPs was used to calculate the distribution of the null hypothesis. A total of 10,000 permutations was used to calculate the null distribution, from which the 5% experiment-wise significance thresholds were inferred.

Spearman correlations were determined between the different conformational traits using the CORR procedure of SAS implemented in the SAS statistical package v.9.1.3 and considering the whole set of data on all animals.

## Results

Twenty conformational and locomotive traits assessed in 144 Spanish Purebred horses were included in this study to seek novel associations between loci implicated in the development of OC lesions and body size and morphological characters in the Spanish Purebred horse breed. The full set of samples included 76 males (52.8%) and 68 females (47.2%). Means and standard errors (SEs) for the different measured traits are shown in Table 1.

Correlations were significant and positive between many morphological characters, and especially high between the breed standard and head scores ($r = 0.96$, $p < 0.0001$), knee perimeter and body length ($r = 0.90$, $p < 0.0001$), hind cannon circumference and body length ($r = 0.82$, $p < 0.0001$), and fore and hind conformation scores ($r = 0.96$, $p < 0.0001$) (Fig. 1). The traits walk, trot and canter collected, as well as hoof colour, showed the lowest correlations with the rest of characters.

A total of 52 SNPs, including loci previously associated with different manifestations of OC (Sevane et al. 2016) and four loci explaining 83% of the size variation in horse (Makvandi-Nejad et al. 2012), were genotyped in the full set of samples. Allele frequencies of the analysed SNPs can be found in either Sevane et al. (2016) for the OC-associated loci or in Table 2 for the mutations associated with body size variation. After eliminating two SNPs with MAF under 0.05 (Chr4-851132 and AOAH), 50 polymorphisms were analysed through linear regression analysis. Ten SNPs located in or near ten different genes were found to be associated with different conformational and locomotive traits, with effects ranging from 0.25 to 1.09 standard deviation units (Table 3). Significant as well as suggestive (F Reg > 8) associations are shown.

Four loci, *LCORL*/*NCAPG*, *HMGA2*, *USP31* and *MECR*, were found to be significantly associated with morphometric characters. The CC genotype of the intergenic SNP at position

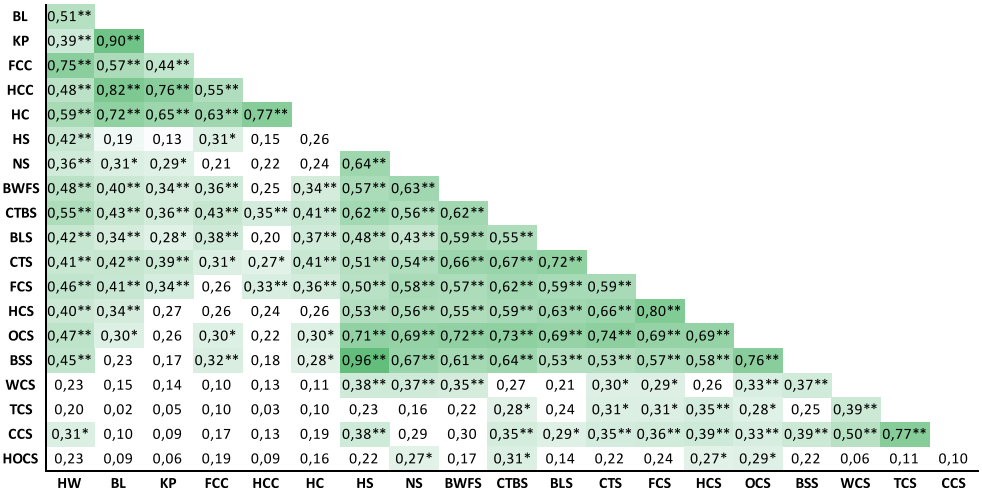


**Fig. 1** Correlations between conformational and locomotive traits in the Spanish Purebred horse. Level of significance: $^{**}p < 0.0001$, $^{*}p < 0.001$. The darker colours indicate higher correlations

Ch3 105,574,002 (near the *LCORL*/*NCAPG* genes) was associated with an increase in height at withers and body length of 3.6 and 4.5%, respectively, when compared with the TT genotype. The increase in size also leads to bigger hock (6.2%),

**Table 3** Significant (when F Reg > F Th) and suggestive (F Reg > 8) associations between single nucleotide polymorphisms (SNPs) and different conformational traits in the Spanish Purebred horse breed

| Locus | GenBank/dbSNP[a] | Trait | Mean | SD | F Th[b] | Allele[c] | F Reg[d] | SE | *p*-Value | Effect | Effect/SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *LCORL* | Ch3 105,547,002 | HC | 45.290 | 1.875 | 9.670 | C | 57.138* | 0.186 | $8.4{\cdot}10^{-12}$ | 1.403 | 0.75 |
| | | KP | 34.048 | 1.715 | 9.683 | | 43.130* | 0.143 | $1.3{\cdot}10^{-9}$ | 0.941 | 0.55 |
| | | HW | 1.633 | 0.040 | 9.744 | | 42.247* | 0.004 | $1.8{\cdot}10^{-9}$ | 0.029 | 0.73 |
| | | BL | 1.607 | 0.049 | 9.630 | | 41.930* | 0.006 | $2.1{\cdot}10^{-9}$ | 0.036 | 0.74 |
| | | HCC | 23.349 | 0.967 | 9.615 | | 36.668* | 0.106 | $1.6{\cdot}10^{-8}$ | 0.642 | 0.66 |
| | | FCC | 21.114 | 1.112 | 9.672 | | 13.550* | 0.103 | 0.0003 | 0.380 | 0.34 |
| | | CTBS | 6.926 | 1.209 | 9.552 | | 10.175* | 0.143 | 0.002 | 0.457 | 0.38 |
| *HMGA2* | Ch6 81,481,064 | HCS | 5.463 | 0.739 | 9.747 | C | 19.966* | 0.179 | $1.8{\cdot}10^{-5}$ | 0.802 | 1.09 |
| | | FCS | 5.449 | 0.778 | 9.760 | | 8.680 | 0.203 | 0.004 | 0.598 | 0.77 |
| | | FCC | 21.114 | 1.112 | 9.672 | T | 11.400* | 0.215 | 0.001 | 0.726 | 0.65 |
| *USP31* | rs68599858 | KP | 34.048 | 1.715 | 9.683 | T | 11.942* | 0.147 | 0.001 | 0.507 | 0.30 |
| | | HW | 1.633 | 0.040 | 9.744 | | 9.054 | 0.005 | 0.003 | 0.014 | 0.35 |
| | | HC | 45.290 | 1.875 | 9.670 | | 8.446 | 0.201 | 0.004 | 0.584 | 0.31 |
| *MECR* | g.30801 G>T AAWR02028280 | BWFS | 7.022 | 1.195 | 9.529 | C | 10.187* | 0.199 | 0.002 | 0.634 | 0.53 |
| *COL24A1* | rs69511701 | WCS | 6.125 | 0.938 | 9.540 | A | 9.441 | 0.114 | 0.003 | 0.349 | 0.37 |
| *MGP* | rs68649284 | FCC | 21.114 | 1.112 | 9.672 | G | 8.462 | 0.095 | 0.004 | 0.278 | 0.25 |
| *FAM184B* | rs68534880 | HC | 45.290 | 1.875 | 9.670 | G | 8.118 | 0.403 | 0.005 | 1.149 | 0.61 |
| | | BL | 1.607 | 0.049 | 9.630 | | 8.029 | 0.011 | 0.005 | 0.032 | 0.65 |
| *PTH1R* | rs69065715 | WCS | 6.125 | 0.938 | 9.540 | C | 8.363 | 0.130 | 0.005 | 0.375 | 0.40 |
| *KLF3* | rs68512502 | HCC | 23.349 | 0.967 | 9.615 | A | 8.183 | 0.103 | 0.005 | 0.294 | 0.30 |
| | | BL | 1.607 | 0.049 | 9.630 | | 8.153 | 0.005 | 0.005 | 0.016 | 0.33 |
| *SGK1* | rs68863106 | CCS | 6.257 | 0.869 | 9.591 | C | 8.059 | 0.218 | 0.005 | 0.620 | 0.71 |

*HC* hock circumference, *KP* knee perimeter, *HW* height at withers, *BL* body length, *HCC* hind cannon circumference, *FCC* fore cannon circumference, *CTBS* chest, thorax and barrel score, *HCS* hind conformation score, *FCS* fore conformation score, *BWFS* back, withers and forelimb score, *WCS* walk collected score, *CCS* canter collected score

[a] GenBank accession numbers for *Equus caballus* sequences including the interrogated SNPs or dbSNP accession numbers

[b] Trait significant thresholds

[c] Allele positively correlated with the trait

[d] F regression statistics. *Significant associations

knee (5.53%) and cannon circumferences (HCC 5.5%, FCC 3.6%), as well as a significantly higher chest, thorax and barrel scoring (13.2%). The CC genotype of the intergenic SNP at position Ch6 81,481,064 (near the *HMGA2* gene) accounts for an increase in the score of fore and hind conformation of 29.4 and 21.9%, respectively, when compared with the TT genotype, whereas the TT genotype increases the fore cannon circumference by 6.9%. The TT genotype of the SNP rs68599858 located near the 3′ UTR region of *USP31* causes a significant increase in the knee perimeter of 3%, when compared with the CC genotype. This genotype also showed associations near the significance threshold with height at withers (1.7%) and hock perimeter (2.6%). The *MECR* SNP g.30801 G>T was significantly associated with back, withers and forelimb score, with the CC genotype displaying a scoring increase of 18%.

## Discussion

Using a candidate gene approach, ten genes, *LCORL*/*NCAPG*, *HMGA2*, *USP31*, *MECR*, *COL24A1*, *MGP*, *FAM184B*, *PTH1R*, *KLF3* and *SGK1*, were found to be associated with different conformational and locomotive traits in the Spanish Purebred horse. Among the four mutations previously associated with size variation in horse (Makvandi-Nejad et al. 2012), only those located near the *LCORL*/*NCAPG* and *HMGA2* loci have been validated in this breed. The SNP that showed the strongest association signal was at 129 Kb from *LCORL*/*NCAPG*, which was also recently associated with body conformation in Yili horses (He et al. 2015b). The ligand-dependent nuclear receptor corepressor-like (*LCORL*) gene has been associated with height in humans and carcass weight in cattle (e.g. Weedon et al. 2008; Carty et al. 2012; Nishimura et al. 2012). Another interesting gene in that region is the *NCAPG* non-SMC condensin I complex, subunit G (*NCAPG*), implicated in mitotic cell division (Dej et al. 2004) and previously associated with prenatal growth in cattle (Eberlein et al. 2009). The region that harbours the *NCAPG* and *LCORL* genes has also been associated with body weight in sheep (Al-Mamun et al. 2015) and feed intake, gain, meat and carcass traits in cattle (Lindholm-Perry et al. 2011, 2013). Finally, Orr et al. (2013) described an association of this SNP with OC susceptibility in the Dutch Warmblood, which links the prevalence in the development of OC with conformational traits. We also found significant and opposed associations between the SNP located near the high mobility group AT-hook 2 (*HMGA2*) gene, which influences cell growth, proliferation, differentiation and death (Cleynen and Van de Ven 2008), and hind and fore conformation scores (C allele) and fore cannon circumference (T allele). However, the association of this mutation with size variation described by Makvandi-Nejad et al. (2012) has not been confirmed in the Spanish Purebred horse.

The ubiquitin-specific peptidase 31 (*USP31*) gene, implicated in the control of immune and inflammatory responses, developmental processes, cellular growth and apoptosis (Tzimas et al. 2006), the mitochondrial trans-2-enoyl-CoA reductase (*MECR*), involved in the synthesis of fatty acids, cellular proliferation and differentiation, and immune and inflammatory responses (Kim et al. 2014), and the Krüppel-like factor 3 (*KLF3*), a repressor of transcription in processes including adipogenesis, B cell development, erythropoiesis, muscle cell development and cardiovascular development (see Xue et al. 2015 for revision), were found to be associated with morphometric characters. These three genes have been associated with the development of OC lesions in horse (Dierks et al. 2010; Corbin et al. 2012; Teyssèdre et al. 2012), and have been described as being implicated in immune and inflammatory responses and cellular development, which can explain their influence on the development of immune and inflammatory modifications that lead to the onset of OC lesions, as well as their effect on conformational traits through their role in cellular growth.

Although further validation is needed for the associations slightly under the significance threshold, they support the implication of six more genes (Table 3), -*KLF3* (included in the previous paragraph), *COL24A1* (effect of the non-synonymous SNP rs69511701 on walk scoring), *MGP* (fore cannon circumference), *PTH1R* (walk scoring), *FAM184B* (body length and hock perimeter) and *SGK1* (gallop scoring), in both OC and morphological traits. The collagen type XXIV alpha 1 (*COL24A1*) gene was previously associated with the development of OC in Hanoverian warmblood horses (Lampe et al. 2009). This gene, along with the matrix Gla protein (*MGP*), has also been associated with OC lesions in pigs and with ossification processes (Matsuo et al. 2008; Laenoi et al. 2010). The parathyroid hormone 1 receptor (*PTH1R*) gene, which regulates cartilage growth and chondrocytic apoptosis (Harrington et al. 2004), has been implicated in OC lesions in pigs (Rangkasenee et al. 2013). The implication in cartilage and ossification processes of these genes can explain their influence on OC onset through the disturbance of ossification and on the general body conformation, which can determine the ability of a horse to perform certain gaits and the thickness of bones such as the cannon, where the scarce muscular coverage do not bias the measurement of bone dimensions.

The function of the family with sequence similarity 184 member B (*FAM184B*) gene has not yet been characterised; however, this gene was previously associated with OC lesions in French Trotters (Teyssèdre et al. 2012) and with adult height in humans (He et al. 2015a). Finally, the serum/glucocorticoid regulated kinase 1 (*SGK1*) gene plays an important role in cellular stress response, cell survival and ischaemic necrosis (Nishida et al. 2004), which, in turn, seems to

play an important role in the aetiopathogenesis of OC (Ytrehus et al. 2004; Olstad et al. 2008), as was also suggested by the association found between OC prevalence and the SNP rs68863106 in Norwegian Standardbred (Lykkjen et al. 2010).

In conclusion, we identified ten loci, *LCORL/NCAPG*, *HMGA2*, *USP31*, *MECR*, *COL24A1*, *MGP*, *FAM184B*, *PTH1R*, *KLF3* and *SGK1*, as being significant or suggestively associated with different conformational and locomotive traits in a sample of 144 Spanish Purebred horses. Except for *HMGA2*, all SNPs seem to influence both the prevalence of OC lesions and morphological characters, supporting the link between conformation and OC. Also, the implication of most genes in either immune and inflammatory responses and cellular growth, or ossification processes, reinforces the role that these mechanisms have in the aetiology of OC, as well as their reflection on the general conformation of the individual. Except for the non-synonymous *COL24A1* SNP, all polymorphisms are located in introns or intergenic regions nearby candidate genes; thus, further resequencing of these loci is needed to elucidate the causal mutations behind these associations. In the meantime, these SNPs could be used in marker-assisted selection (MAS) programmes to improve desirable conformational traits, but taking into account their possible detrimental effect on OC prevalence.

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**Authors' contributions** N.S. carried out both experimental and computational work and drafted the manuscript. S.D. and J.C. conceived and supervised the study. J.C. helped to draft the manuscript. A.B. collected the phenotypic data. All authors read and approved the final manuscript.

**Compliance with ethical standards**

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**Ethical approval** All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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