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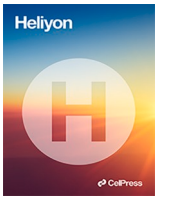

Research article

The markers of the predictive DNA test for canine hip dysplasia may have a stronger relationship with elbow dysplasia

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ABSTRACT

Canine hip and elbow dysplasias, which are prevalent orthopedic conditions rooted in developmental and hereditary factors are yet to be comprehensively assessed. This study aimed to address this gap by exploring the prognostic significance of five markers linked to canine hip dysplasia using available genome-wide association studies (GWAS) data. The influence of these markers on both hip and elbow dysplasia was examined in dogs exposed to standardized environmental conditions. We made a groundbreaking discovery using custom primers, qPCR assays, and evaluation of fluorescent resonance energy transfer (FRET) probes. Three specific SNPs previously associated with the risk of canine hip dysplasia demonstrated a potentially stronger correlation with elbow dysplasia. Notably, the SNP at nucleotide position 22691322, located near the canine *CHST3* gene, displayed significance as a marker in multivariable logistic regression analysis. Surprisingly, none of the initially targeted SNPs showed a direct association with hip dysplasia. The genomic positions of these SNPs reside within a region conserved across mammals. *In silico* analyses suggested that the relevant variant might be positioned in a region linked to bone and muscle structures. Our findings revealed a remarkable relationship between SNP2 genotypes and methylation patterns, shedding light on the underlying mechanism that partially explains the genotype-phenotype correlation in canine *CHST3*. These groundbreaking findings offer essential insights for future, more extensive investigations into canine orthopedic health. This research significantly contributes to our understanding of the molecular foundations of hip and elbow dysplasia in dogs by charting a course for advancements in veterinary medicine and the overall well-being of canine companions.

1. Introduction

Canine hip dysplasia (CHD) and canine elbow dysplasia (CED) are prevalent developmental and hereditary orthopedic disorders in

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dogs. Each of these dysplasias is characterized by arthritis and/or severely deformed joints that limit movement and thus negatively affect the well-being of dogs [1]. The development of these disorders is complex. Physical and environmental factors, such as body size, growth rate, nutrition, endocrinological features, and muscle structure, affect pathogenesis at different levels [2–4]. It is well known that genetic background is crucial in the formation of these disorders [1,3]. Genetic evaluation is a swifter and more effective approach for monitoring canine hip and elbow dysplasia than relying solely on phenotype-based analysis, which is a trend observed in many other diseases.

CHD is a hereditary condition that predominantly affects medium- and large-sized breeds. It arises from an irregular hip joint formation caused by defects in bone tissue development. Notably, CHD is frequently found in well-known working and service dog breeds such as German Shepherds, Belgian Malinois, and Labrador Retrievers [5–7]. It is recognizable by a malformation of the coxofemoral joint, resulting in instability and partial hip dislocation. These symptoms eventually lead to osteoarthritis and lameness [8]. A standardized grading system has been established and adopted by countries affiliated with the Fédération Cynologique Internationale (FCI) in response to the prevalence and significance of CHD. This system categorizes CHD into five distinct classes, A to E; A signifies normal hips, and E represents the most severe form of CHD [9,10]. The FCI score is derived from various hip "sub-traits," including joint congruency, Norberg angle, degree of joint subluxation, acetabulum shape and depth, and visible signs of osteoarthritis in the joint. Collectively, these components contribute to a comprehensive assessment of hip health in dogs, allowing for a more accurate and detailed evaluation of CHD severity [10]. Each category helps veterinary clinicians and breeders assess and communicate the degree of hip dysplasia in dogs, facilitating informed decision-making in breeding programs and health management. By applying this grading system, breeders can reduce the incidence of CHD in specific breeds, ultimately promoting better hip health in canines. The FCI score plays a crucial role in guiding responsible breeding practices and ensuring the welfare of dogs susceptible to this hereditary condition. Heritability estimates for CHD range from 0.1 to 0.68 in breeds where this disorder is common, including German Shepherd, Golden Retriever, Labrador Retriever, Newfoundland, Rottweiler, Bernese Mountain Dog, and Saint Bernard [11]. Genetic correlations caused by random fixation, linkage disequilibrium (LD), and pleiotropy have led to inadvertent selection of disease-risk variants within breeds. The high prevalence of certain diseases in a breed indicates the enrichment of disease-risk alleles during modern breed creation and domestication [12].

CED is an intra-articular degenerative lesion caused by the inability of the *trochlea humeri* to be fully covered by the *incisura trochlearis* due to joint incompatibility. It includes fragmented medial coronoid process, osteochondrosis of the humerus, an ununited anconeal process, articular cartilage injury, and incongruity of the elbow joint [3,13,14]. These conditions can occur alone or in combination [14]. The International Elbow Working Group (IEWG) determined a standard grading system based on the extent of osteophyte formation [15]. CED development involves three primary mechanisms: osteochondrosis, various joint incongruities, and biomechanical force mismatch across the elbow joint. These mechanisms are thought to arise from a genetic predisposition, with secondary environmental factors, such as high-energy diets, rapid growth rates, or excessive exercise, influencing their manifestations [3,16,17]. Recent evidence supports various forms of joint incongruity as the most likely mechanism, although osteochondrosis appears to play a role in some dogs. The biomechanical force mismatch hypothesis is currently being investigated; however, the evidence to substantiate this hypthesis remains limited [3]. As with CHD, there is polygenic inheritance in CED formation and it exhibits a multifactorial etiology [14]. Although dysplasia control programs have been implemented in dogs, the prevalence of dysplasia remains high. Nonetheless, molecular genetic studies of CED are scarce.

In recent years, studies have provided undeniable evidence that the orthopedic symptoms observed have hereditary characteristics [12,18]. Lately, a commercial high-density SNP genotyping microarray called the "CanineHD Whole Genome Genotyping BeadChip," manufactured by Illumina (Illumina Inc, San Diego, CA, USA), has been introduced to the market. This microarray offers genome-wide coverage, consisting of 172,115 SNPs evenly distributed throughout the dog genome (https://emea.illumina.com/products/by-type/microarray-kits/caninehd.html). In 2015, Bartolomé et al. [19] published a comprehensive study to develop a genetic prognostic test for the early diagnosis of hip dysplasia in 775 purebred Labrador retrievers from 64 Spanish veterinary clinics. The test relies on specific SNPs discovered through a genome-wide association study (GWAS) using the Canine HD BeadChip (Illumina) and by sequencing particular candidate genes (768 custom SNPs with the Illumina Golden Gate genotyping platform) in a population of Spanish Labradors. The authors suggested that the developed prognostic genetic test is a helpful tool for choosing the most appropriate therapeutic approach once the genetic predisposition to hip dysplasia is known. The test demonstrated favorable prediction accuracy, with an average sensitivity/specificity of 80/78 %. The test, previously known as the Dysgen DNA test, was introduced to the commercial market by Progenika Biopharma. In 2020, another genetic study validating this test in Danish Labrador Retrievers was published [20]. However, these results did not confirm the test results proposed by Bartolomé et al. [19]. Bruun et al. [20] reported no significant correlation between Dysgen test results and radiographic hip status, indicating that Dysgen test results have no prognostic value in Danish Labrador Retrievers.

Numerous efforts have been made to identify the genetic loci and variants responsible for the relative risk of developing CHD [10,12,20–24]. Although the importance of genetic background in CHD and CED is indisputable, there are some essential reasons for the emergence of controversial results in genetic testing and association analyses. First, human selective pressures have led to an unparalleled diversity of morphological traits and breed-associated behaviors in domestic dog breeds [25]. This intensive selection has influenced the morphological characteristics and important physiological and metabolic properties. Accordingly, dog breed stands out as a striking distinguishing factor, indicating that some breeds are more susceptible to the disorder than others [1,4,10,18,19,21,23,24]. However, remarkable variations were evident in studies conducted on the same breeds. It is important to note that the current consensus is that CHD is polygenic, and its genetic contribution to the phenotype can vary from small to moderate [1,4,10,18,21,23]. Recently, genetic marker analysis has predominantly led to the adoption of GWAS approaches to avoid overlooking the effects of numerous markers that may not be statistically significant but still exhibit minor genetic impacts. Nevertheless, markers originating

from loci with distinct mechanistic characteristics associated with various pathways have been proposed in these studies [10,12,22]. The applied statistical and bioinformatic perspectives also exert a significant influence. In genetic studies, besides the sample size, the quality of phenotypic data is one of the most crucial factors. The optimization of environmental characteristics is an essential factor that directly affects the reliability of phenotype-genotype association studies. The importance of phenotypic data is further highlighted, especially given that CHD and CED are greatly influenced by characteristics such as nutritional and exercise statuses [3,26]. Although the sample sizes in many studies met the desired level, animal material was predominantly aggregated data sourced from various veterinary clinics. These dogs were bred by different owners show variations in their nutritional intake as well as movement patterns, frequency, and intensity. In this study, we investigated five markers proposed to have prognostic value for CHD based on the GWAS data reported by Bartolomé et al. [19]. These dogs were bred and trained by the same trainers and subjected to the same environmental conditions from the nutrition characteristics to exercise intensity set of attributes in the same dog breeding and training center. In contrast, we hypothesized that the markers involved in the development of CHD may have alternative influences on CED development. Taxonomic assessments of genomic segments containing SNPs, along with *in silico* predictions, further supported our hypothesis. Each of these dysplasias can cause arthritis and/or severely deformed joints, thereby disabling the dog [1]. Genetic association studies on CED are remarkably limited compared with those on CHD. We report a significant locus and two other loci suggestive of CED development. These findings emphasize the importance of considering candidate genes or markers of CHD in CED.

2. Materials and methods

2.1. Study population

All dogs recruited in this study were raised in a dog breeding and training center located in the South Marmara region of Turkiye. We used 119 dogs, comprising German Shepherds (n = 61) and Labrador Retrievers (n = 58). The training facility comprised a collective count of 1000 dogs. All dogs used in this study were purebred and housed under the same environmental conditions, following the same exercise and nutrition regimen. Specifically, the dogs are housed in a facility where living spaces, movement areas, and training sections are uniformly designed for each animal. Experienced trainers assist with the dogs' exercise routines. Additionally, a rigorous analysis of dog food content has been conducted to ensure the selection of food with optimal protein, fat, and carbohydrate content for dog nutrition, procured through a public tender process. In other words, the conditions regarding physical facilities, movement, and nutrition are standardized for all dogs. Moreover, they were skeletally mature and healthy dogs (no systemic diseases). Descriptions (name, identification tag, and pedigree) and history of the dogs were recorded. We performed a pedigree analysis of the dogs included in the study. Concerning inbreeding rates, we selected unrelated animals, at least at the grandparent level, as suggested by Bartolomé et al. [19]. The dogs were between 1 and 12 years old, and thus we designated dogs aged 3 years and younger as Group 1 (GR1), dogs aged 4–7 years as Group 2 (GR2), and dogs aged 8 years and older as Group 3 (GR3), to include the age effect in the statistical analysis.

Our study's inclusion/exclusion criteria are based on two key factors. Firstly, since all participating dogs are under veterinary supervision, detailed health records are available. In addition, prior to the study, each dog underwent a comprehensive general examination. This included assessments of pulsation, respiration, capillary refill time, body temperature, mucosal membrane color, and the condition of local lymph nodes. Secondly, all animals possess detailed pedigree records. Dogs that were either deemed unhealthy or related at least to the grandparent level were excluded from the study.

Dogs were categorized based on their overall joint health, considering their status in relation to CHD and CED, as demonstrated by Manz et al. [27], with some modifications to evaluate the co-occurrence of disorder. These categorizations form phenotype classes: animals without either disorder fall under phenotype class I, those with moderate hereditary joint conditions are classified as phenotype class II, and dogs exhibiting severe symptoms of both disorders are assigned to phenotype class III. Detailed information on this evaluation is provided in Table S1. A detailed diagram illustrating the experimental design of the study in sequential steps is shown in Fig. S1.

2.2. Clinical and orthopedic examinations

Initially, we performed a general examination (pulsation, respiration, capillary filling time, body temperature, mucous membrane color, and local lymph nodes) of each dog. Next, we identified dogs with suspected lameness in the fore and hind extremities during stance and walking. Lameness was graded as mild, moderate, or severe, as described by Aulakh et al. [28]. A general anesthesia protocol was applied to the dogs (further details about the anesthesia protocol are presented in the Supplementary Material and Methods section). In orthopedic examination, subluxation and Ortolani and Barlow tests were performed for hip dysplasia [29]. Joint swelling (e.g., joint capsule thickening and intra-articular effusion), muscle atrophy, pain, and crepitation were evaluated [30]. CED includes medial coronoid process disease, osteochondrosis/osteochondritis dissecans of the humeral trochlea, an ununited anconeal process (UAP), and joint incongruence [14]. Details of the orthopedic examination are presented in the Supplementary Material and Methods section. The dogs were sent to the X-ray unit for radiological examination.

2.3. X-ray evaluation

Radiographs were obtained using a fixed X-ray machine (Ajex Meditech, AJEX160H, Seoul, Korea) and an imaging system (Fujifilm, FCR Capsula X CR System, Tokyo, Japan). All radiographs were obtained by the same researcher (assisted by the same

technician) and were repeated if a positional error or technical problem (e.g., insufficient contrast, artifact, and collimation error) occurred. Further details regarding the radiological examinations are presented in the Supplementary Material and Methods. The same two experts independently evaluated all radiographs to prevent alterations and/or inaccuracies.

A standard ventrodorsal hip radiograph of each dog was obtianed with the extremities extended. Norberg angle measurements of the radiographs were made using Sante DICOM Viewer software (Santesoft, Sante Dicom Viewer Pro, Nicosia, Cyprus). The compliance of the ventrodorsal radiographs of the pelvis with the standards was determined according to Flückiger [31]. In this context, various aspects were evaluated to determine the appropriateness of radiographs. These included checking whether the hind extremities were adequately extended, ensuring the alignment of the femurs with the spine and each other, confirming the position of the patella in the sulcus femoris and at the center, assessing the adequacy of collimation, and evaluating the X-ray quality, particularly the achievement of proper contrast. Based on these considerations, necessary assessments were made to determine whether the radiographs met the required standards or not [31]. Radiographs were evaluated using the official FCI scale for hip dysplasia. This standardized scale evaluates the severity of hip dysplasia in dogs based on radiographic analysis. This diagnostic tool assesses the fit and conformity of the hip joints, detecting any signs of joint laxity or arthritic changes. The grading spans from excellent, indicating no signs of hip dysplasia, to severe, where significant joint laxity and arthritic alterations are evident [9]. The evaluation consisted of five categories: A, no signs of CHD; B, almost normal hips; C, mild signs of CHD; D, moderate signs of CHD; and E, severe CHD (Table S2). Dogs scoring A or B were classified into the control group (free of hip dysplasia), and dogs scoring as D or E were classified into the case (affected) group, as suggested by Bartolomé et al. [19]. Radiographs showing the assessment of canine hip dysplasia status according to the FCI grading are shown in Fig. 1a–e. Here, it should be noted that we included the C dogs in the case group because these dogs are not recommended for breeding purposes. Given that our study aimed to provide data for genetic-selection-driven eradication of hip and elbow dysplasias, we also considered grade C dogs (Fig. 1c). Details of the discrimination criteria among the groups are presented in the Supplementary Material and Methods section.

Radiographs of elbow joint, mediolateral neutral, mediolateral at 45° flexion, and cranio-caudal projection at 15° protonation were taken. The presence and extent of changes in the primary lesion and secondary osteoarthritis were considered to evaluate elbow dysplasia. Changes of secondary osteoarthritis (osteophyte formations) were assessed regarding the proximal surface of the processus anconeus, the cranial surface of the caput radii, the cranial edge of the processus coronoideus medialis ulnae, the caudal surface of the epicondylus lateralis, the medial surface of the condylus medialis and the medial edge of the processus coronoideus medialis. Sclerosis of the *incisura ulnaris* and osteochondritis dissecans lesions in the *condylus medialis* were evaluated. Thus, the ultimate elbow dysplasia scoring based on the suggestions of the IEWG was as follows: ED0 = normal elbow joint, ED1 = mild arthrosis, ED2 = moderate arthrosis or suspicion of primary lesion, and ED3 = severe arthrosis or an evident primary lesion [32,33] (Table S3). Illustrations of the radiographic samples used for assessment in this study are shown in Fig. 2a–d. Details of the discrimination criteria among the groups are presented in Supplementary Material and Methods.

2.4. Genomic DNA extraction

Blood samples were collected in Vacutainer test tubes (Vacutest Kima srl, Arzergrande, PD, Italy) containing K_3EDTA. Genomic DNA was extracted using the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) based on the

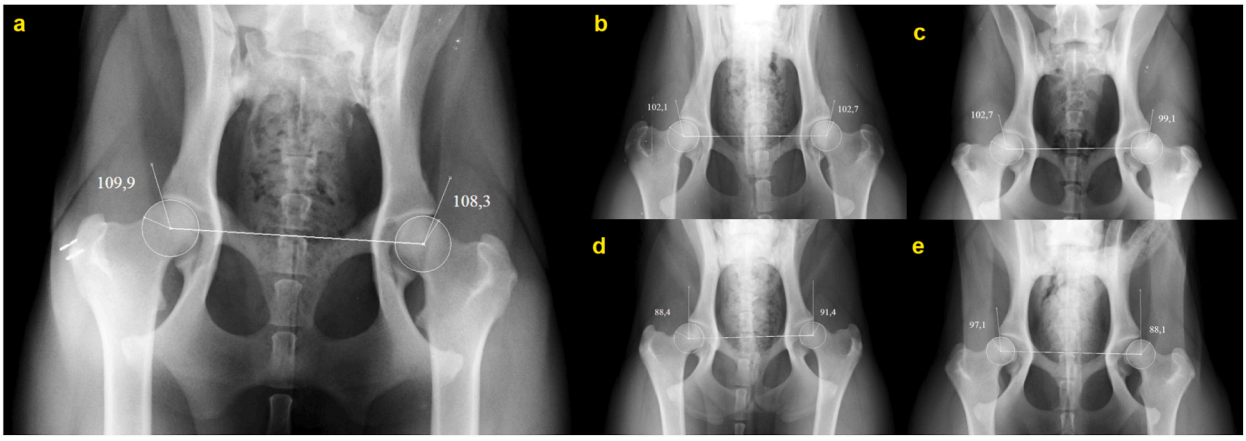

Fig. 1. Evaluation of the canine pelvis using conventional ventrodorsal radiographic projections, following the guidelines established by the Fédération Cynologique Internationale (FCI) criteria. The Norberg Angles (NA) for each joint are furnished. (a) Normal hip conformation (category A); 4-year-old female Labrador Retriever; NA-right: 109.9° -left: 108.3° (b) Almost normal hip conformation (category B); 3-year-old male German Shepherd; NA-right: 102.1° -left: 102.7° (c) Mild signs of dysplasia (category C); 2-year-old female Labrador Retriever; NA-right: 102.7° -left: 99.1° (d) Moderate signs of dysplasia (category D); 2-year-old male Labrador Retriever; NA-right: 88.4° -left: 91.4° (e) Severe dysplasia (category E); 2-year-old male Labrador Retriever; NA-right: 97.1° -left: 88.1°. Further details for FCI scoring are presented in the supplementary material and methods.

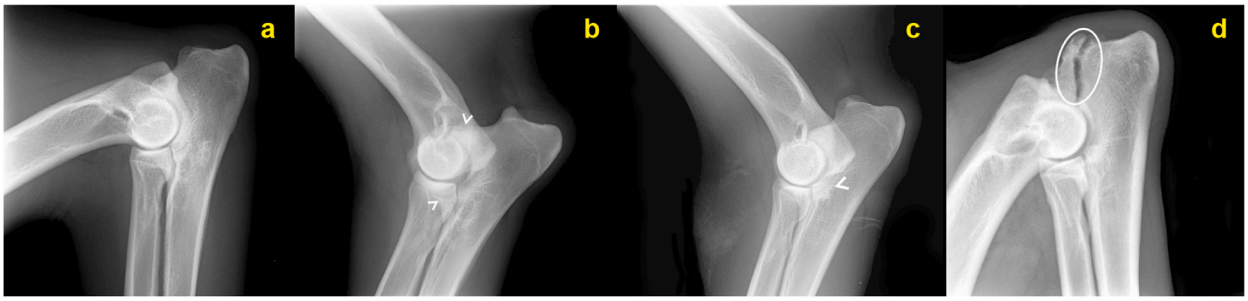

Fig. 2. Assessment of the canine elbow joint on mediolateral radiographic projections, in accordance with the International Elbow Working Group (IEWG) criteria. (a) Normal elbow conformation (ED0); 2.5-year-old male German Shepherd; no primary lesion related to elbow dysplasia and no signs of arthrosis. (b) Mild arthrosis (ED1); 5.5-year-old male German Shepherd; while there is no primary lesion in the elbow joint, there is new bone formation ≤2 mm (1st degree) around the *proccesus anconeus* indicated by the white arrow. (c) Moderate arthrosis or suspect primary lesion moderate arthrosis or suspect primary lesion (ED2); 3.5-year-old male German Shepherd; while an ostensibly primary lesion manifests within the demarcated region, concomitant therewith is an insignificantly small extent of ≤2 mm, tantamount to a condition of primary degree arthrosis, encircling the *processus anconeus* as indicated by the white arrow. (d) Severe arthrosis or evident primary lesion (ED3); 3-year-old female German Shepherd; A primary lesion (ununited anconeal process: UAP) is evident within the demarcated region marked by the white ring, accompanied by a 2–5 mm, or 2nd degree arthrosis, encircling the *processus anconeus*. Further details for IEWG scoring are presented in the Supplementary Material and Methods.

manufacturer's instructions. Briefly, 300 μL of blood was collected into 1.5 mL Eppendorf tubes and treated with Red Blood Cell Lysis Buffer (Roche Diagnostics). Following a 10-min incubation, the samples were centrifuged, and the supernatant was discarded. This lysis and centrifugation step was repeated, and the pelleted cells were subsequently resuspended in fresh lysis buffer. Binding Buffer and Proteinase K were added, mixed thoroughly, and incubated at 72 °C for 10 min. Isopropanol was then introduced to precipitate DNA. The samples underwent a series of transfers to new collection tubes with filtration, interspersed with wash steps using specific buffers and centrifugation to ensure thorough cleansing of the DNA, which was finally eluted in a pre-warmed elution buffer. The DNA was then collected, with the elution buffer facilitating the detachment of DNA from the filter surface into the collection tube.

The quantity (ng/μL) and purity (260/280 absorbance ratio) of the samples were determined using a NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA).

2.5. Real-time quantitative PCR (qPCR) assay

In this study, we genotyped German Shepherd and Labrador Retriever dogs based on five SNPs from a predictive model for canine hip dysplasia reported by Bartolomé et al. [19]. The details of the SNPs are presented in Table 1. We used custom primers for real-time quantitative PCR (qPCR) from the NCBI database https://www.ncbi.nlm.nih.gov/. The flanking sequences of the primers and probes are listed in Supplementary Table S4. The fluorescent resonance energy transfer (FRET) probes were evaluated using Light Cycler Probe Design Software 2.00 (v.1.0).

The primer and probe sequences are listed in Table 2. Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to ensure that the primer sets amplified DNA products with high specificity and avoided non-specific amplification. Genotyping was performed based on the LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany). PCR was performed using LightCycler FastStart DNA Master HybProbe (Roche Diagnostics GmbH, Mannheim, Germany). A 20 μL reaction was set up containing 0.5 μM of each primer (1 μL), 0.2 μM of the FL and LC probes (1 μL, each), 1.6 μL of 25 mM $MgCl_2$ (Roche Diagnostics GmbH, Mannheim, Germany), 2 μL of FastStart Enzyme Mix (Roche Diagnostics GmbH, Mannheim, Germany), 7.4 μL molecular grade water, and 5 μL of extracted DNA. LightCycler was programmed to automatically perform melt-curve analysis after amplification. The cycling parameters were as follows: an initial denaturation step at 95 °C for 10 min, followed by 60 cycles at 95 °C for 5 s, 60 °C for 10 s, 50 °C for 10 s (single acquisition), and extension at 72 °C for 10 s, with a 20 °C/s transition time. The melt-curve parameters were as follows: 95 °C for 20 s, 40 °C for 20 s with a temperature transition rate of 20 °C/s, and finally, a slow rise in the temperature to 85 °C at a rate of

Table 1
Brief information on the SNPs studied in the present study.

SNP[a]	SNP name[b]	Chromosome[c]	Nucleotide position	Nucleotide alteration	Notable nearby gene
BICF2G630558239	SNP1	7	36171712	A/G	*SMYD3*
BICF2P772455	SNP2	4	22691322	A/G	*CHST3*
BICF2S230609	SNP3	18	48695616	A/G	*FGF4*
BICF2G630339806	SNP4	3	40302288	A/G	*CHSY1* and *ADAMTS17*
BICF2S2452559	SNP5	10	47923623	G/A	*PKCE*

[a] SNP names based on the predictive model for canine hip dysplasia reported by Bartolomé et al.[19].
[b] SNP names designated in the present study.
[c] The chromosomal position corresponds to CanFam 3.1.

Table 2
Primer and probe sequences in the present study.

SNP name[a]	Forward primer	Reverse primer
SNP1	5'GCCAAGAACCCAAACCTAGA3'	5'ATTGCCTATGCCTTGTGTAATG3'
SNP2	5'TCAGCTCAGGAGCATCACT3'	5'AGAGTGGGTCTGGGTTTCT3'
SNP3	5'CATCCATACACGACCTGTCC3'	5'CTGGTCCTAACGTCCTACCT3'
SNP4	5'GGTCTGGAATTACCCACTTTCT3'	5'CTTTCATGGGTAGAGCCAAGT3'
SNP5	5'GGTTCCTATCCTAGGTGTACT3'	5'TGACCTAATTACTCACATTGCT3'
	FL probe	**LC probe**
SNP1	5'-CCGGAGGTGAAGAACACAACAGTT-FLU-3'	5'LC640-AGGGGAGTTAAC**g**GGTC-Ph-3'
SNP2	5'-GTGGGGGACACCCTTGTCCTGTG-FLU-3'	5'LC640-AGAGACC**g**GTGGTCAGA-Ph-3'
SNP3	5'-CCAATCAACGTCATTTGCCATGAACAGAATTC-FLU-3'	5'LC640-GCGTCGT**g**ACTCACCCA-Ph-3'
SNP4	5'-GTGAGGCTTTCCAGAATACTATTTACATATTGAAAC-FLU-3'	5'LC640-CTCTCA**g**TAACTTGTAGATACTCATCT-Ph-3'
SNP5	5'-CCTCAGGTGAGGGGGATCTCTGCCATGG-FLU-3'	5'LC640-GTTTT**g**GTGAACATGTACTCTACAG-Ph-3'

[a] SNP names designated in the present study. The counterparts of the SNP names based on the predictive model for canine hip dysplasia reported by Bartolomé et al.[19] are presented in Table 1.

0.2 °C/s with continuous acquisition of fluorescence data. We used the LightCycler 480 Gene Scanning software to analyze the melting temperature (Tm calling) of each amplicon and convert the melting data to a derivative plot. Thus, we determined different genotypes/alleles with melting curve analysis provided by the LightCycler 480 software.

2.6. Sanger sequencing

To confirm this, we sequenced representative samples from each genotype targeting the studied SNPs. The DNA samples were examined using primers designed for specific SNP regions and the FastStart High Fidelity PCR System DNTPack kit (Roche Diagnostics GmbH, Mannheim, Germany). The amplified PCR amplicons were purified using the Zymo DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA, USA, #D4013). After purification, the samples were sequenced using an ABI 3500 automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing outcomes were examined and edited using the BioEdit software (v8.1.0). Finally, the findings were cross-referenced and validated using the dog genome available on the Ensembl genome browser (https://www.ensembl.org/index.html) and the results of the real-time qPCR assays.

2.7. Evaluation of the genotypic data

The genotypic/allelic distributions of all SNPs were calculated using a standard procedure [34]. Next, we estimated heterozygosity (He), effective allele numbers (Ne), and polymorphism information content (PIC) based on population genetics indices, as described by Nei and Roychoudhury [35] and Botstein et al. [36]. Hardy–Weinberg equilibrium (HWE) for each SNP was assessed using the chi-square (χ^2) test. The fixation index (FIS) was estimated from the theoretical (Hthe) and experimental (Hexp) heterozygosities, according to Crow and Kimura [37].

2.8. In silico analysis

The iDNA-MS tool was used to predict the 5'-hydroxymethylation of cytosine variants [38]. Although originally developed for the study of *Homo sapiens* and *Mus musculus*, we postulated that the overlapping results for these species might also be relevant to Canis species, considering their intermediate evolutionary positioning. To further substantiate our hypothesis, we investigated nucleotide conservation using resources such as the Ensembl database (https://www.ensembl.org/index.html) and the Clustal OMEGA tool for multi-sequence analysis (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.9. DNA methylation analysis

After conducting *in silico* analysis to predict gene regulation via potential methylation in the genomic region of SNP2 within the *CHST3* gene, we profiled the methylation status in this region. Genomic DNA was subjected to bisulfite modification using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA) following the manufacturer's recommended protocols. Primers intended to assess the methylation status of the *CHST3* gene region were designed using MethPrimer Software (https://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), with reference to the NCBI and ENSEMBL databases. These primers were tailored to target the CpG islands of the gene (Fig. S2), ensuring specificity for both the methylated and unmethylated states. Below are the primer sequences (5'-3' direction) utilized in the study for both methylated and unmethylated states:

Left methylated primer GATTTAGAATGAGCGAGTAATTTTC.
Right methylated primer CTTACCTAATACAACCCCTAACGTA.
Left unmethylated primer TTTAGAATGAGTGAGTAATTTTTGT.
Right unmethylated primer CTTACCTAATACAACCCCTAACATA.

The LightCycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany) was employed to analyze the methylation profile of

bisulfite-treated DNA samples. Determination of whether each sample was methylated or unmethylated involved assessing the difference in Tm between the methylated and unmethylated primer sets. The methylation status of each sample was verified using positive control DNA (methylated).

2.10. Statistical analysis

The data were initially explored using frequency distributions. Each variable and potential interactions between variables were examined using univariate logistic regression. Based on the results of the univariate approach, variables with a $P < 0.20$ association were determined as candidate variables for multivariable analysis [39]. The determined variables were modeled using multivariate logistic regression analysis with a backward stepwise elimination procedure. The Hosmer–Lemeshow goodness-of-fit test was used to test the fit of the logistic model. The logistic model included the effects of sex, breed, age, and genotype. Data analyses were conducted using the Stata 16 statistical software.

3. Results

The distributions according to the FCI grades for the CHD and IEWG scales for the CED are shown in Tables 3 and 4 for Labrador Retrievers and German Shepherds, respectively. The tables also show the age, breed, and sex of the dogs included in this study.

No lameness was detected in 48 (40.34 %) cases in the orthopedic examination for CHD. Mild, moderate, and severe lameness were noted in 17.6 %, 14.2 %, and 27.7 % of the patients, respectively. Upon palpation of cases displaying lameness, the hip subluxation test yielded positive results in 50 cases, and the Ortolani test yielded positive results in 33 cases. Among the positive cases, 24 (48 %) were Labrador Retrievers, while 26 (52 %) were German Shepherds.

During the orthopedic examination for CED, 57.1 % of cases exhibited no signs of lameness. Among the cases without lameness, 25 (36.7 %) were Labrador Retrievers and 43 (63.2 %) were German Shepherds. Among the patients clinically assessed for elbow dysplasia, moderate-to-severe lameness was detected in 52 instances. Palpation of the elbow joint in cases of lameness revealed crepitation, pain, and joint swelling in 43.6 % of the cases.

The melting peaks derived from the real-time PCR assay proved to be definitive and reliable markers for each genotype (Fig. 3a–e). The sensor probe was meticulously designed to correspond to the wild-type sequence within the target DNA, yielding a comparatively high temperature. Conversely, the presence of a mismatched mutant sequence in the target DNA reduces the stability of the sensor aligned with the wild-type sequence, leading to a notable reduction in the melting temperature.

Genotypic/allelic distributions, population genetic indices, and the HWE test results are presented in Table 5. In the SNP analysis, we observed all three possible genotypes of the diallelic locus except for SNP4. The frequency of the GG genotype was notably elevated in SNP4. The minor allele frequency (MAF) values ranged spanning from 0.06 to 0.48. The uneven distribution of genotypic frequencies within SNP4 leads to diminished genetic diversity. In this context, we identified the most undesirable population genetic parameters associated with this variant. When considering all the SNPs investigated in the study, the values of He ranged from 0.11 to 0.50; Ne values displayed variability ranging from 1.13 to 1.99; and the calculated PIC values spanned from 0.11 to 0.38. HWE testing revealed that SNP1, SNP2, and SNP5 distributions deviated from the expected HWE proportions. Additionally, negative values of F_{IS}

Table 3
Distribution according to the FCI and IEWG scales for canine hip and elbow dysplasia, respectively, sex, and age groups of the Labrador Retrievers ($n = 58$) in the present study.

FCI Grade[a]	Sex		Total
	Female	Male	
A	3	7	10
B	8	5	13
C	5	6	11
D	5	3	8
E	11	5	16
IEWG Grade[b]			
ED0	11	9	20
ED1	3	2	5
ED2	9	8	17
ED3	9	7	16
Age[c]			
Group1	17	17	34
Group2	11	5	16
Group3	4	4	8

[a] A = no signs of dysplasia, B = almost normal hips, C = mild signs of hip dysplasia, D = moderate signs of hip dysplasia, and E = severe hip dysplasia.
[b] ED0 = normal elbow joint, ED1 = mild arthrosis, ED2 = moderate arthrosis or suspect primary lesion, and ED3 = severe arthrosis or evident primary lesion.
[c] The dogs aged 3 years and younger: Group1, the dogs aged 4–7 years: Group2, and the dogs aged 8 and over: Group3.

Table 4
Distribution according to the FCI and IEWG scales for canine hip and elbow dysplasia, respectively, sex, and age groups of the German Shepherds ($n = 61$) in the present study.

FCI Grade[a]	Sex		Total
	Female	Male	
A	2	4	6
B	11	8	19
C	9	1	10
D	6	3	9
E	9	8	17
IEWG Grade[b]			
ED0	18	12	30
ED1	6	7	13
ED2	8	2	10
ED3	5	3	8
Age[c]			
Group1	16	14	30
Group2	13	6	19
Group3	8	4	12

[a] A = no signs of dysplasia, B = almost normal hips, C = mild signs of hip dysplasia, D = moderate signs of hip dysplasia, and E = severe hip dysplasia.
[b] ED0 = normal elbow joint, ED1 = mild arthrosis, ED2 = moderate arthrosis or suspect primary lesion, and ED3 = severe arthrosis or evident primary lesion.
[c] The dogs aged 3 years and younger: Group1, the dogs aged 4–7 years: Group2, and the dogs aged 8 and over: Group3.

were noted for SNP3 and SNP4, whereas the highest Fɪs value was observed for SNP5, as shown in Table 5. The frequencies of each variant in healthy dogs and those with the disorder are shown in Table 6.

A comprehensive analysis of how factors such as breed, sex, age, and genotype influenced CHD risk is shown in Table 7. The findings indicated no significant difference between German Shepherds and Labrador Retrievers in terms of CHD risk ($P > 0.05$). Furthermore, the effects of sex and age on CHD were negligible. A noteworthy outcome of this investigation was the absence of an association between CHD and five reported SNPs known for their predictive potential. This outcome was derived from an extensive examination of GWAS results. The details of the influence of each SNP within the formulated logistic regression model are outlined in Table 7.

During the course of this study, we made a significant observation, suggesting that three specific SNPs (SNP1, SNP2, and SNP3) previously linked to the risk of CHD might be more closely associated with CED. The exhaustive findings of the logistic regression analysis are presented in Table 8. Among these findings, SNP1 and SNP3 emerged as suggestive markers according to the univariate mixed logistic regression model, whereas SNP2 was a significant marker in the final multivariate logistic regression assessment. Detailed information regarding the impact of SNP2 in the conclusive logistic regression model is presented in Table 9. Upon reviewing the outcomes, it became evident that the GG genotype was a favorable variation in terms of CED. Similar to the findings regarding CHD, our observations indicated that factors such as breed, sex, and age did not have a substantial impact on CED (Table 8).

Using the iDNA-MS tool, we identified that cytosine in SNP2, a variant in the promoter region of the *CHST3* gene, is predicted to be 5'-hydroxymethylated (Fig. 4) in both *Homo sapiens* and *Mus musculus*. This tool was developed to predict modifications to Homo or Mus. As Canis species are evolutionarily intermediate, we predict that this modification will also be present in this cytosine variant. Fig. 5a–f illustrates the relevant SNP sequences in this genomic region and the assessment of their taxonomic significance. Supplementary material (Phylogenetic Tree Data) includes extended sequences of the studied SNPs from genomically related species.

We next explored the significant association between SNP2 genotypes and methylation patterns (Fig. 6a), thus providing molecular insights into the mechanism governing the genotype-phenotype correlation of canine *CHST3*. This served as an experimental validation for the *in silico* predictions.

4. Discussion

Incorporating genomic screening into breeding programs holds significant promise for mitigating the effects of hip and elbow dysplasia in dogs. Adopting genetic insights can aid in identifying breeding pairs with diminished genetic risk, thereby decreasing the chances of harmful allele transmission to their progeny. Variations in individual environmental elements like diet, physical activity, and living circumstances amplify the intricacy of these disorders. Consequently, it is vital to recognize that disorder susceptibility is not solely governed by genetic predisposition, as environmental factors also have a significant influence. CHD manifests as an inherited ailment prevalent in breeds with substantial stature, such as Labrador Retrievers and German Shepherds. This condition culminates in compromised mobility and functionality, significantly undermining the quality of life [19]. Recent research indicated that CHD can be predicted using genetic markers [10,19,20,27]. However, the credibility of findings derived from cohorts belonging to various dog owners and/or frequently distinct clinics, where diverse environmental variables exert significant influences, might remain constrained despite the utilization of suitable statistical techniques in the analysis. While an accumulating understanding of the genetic

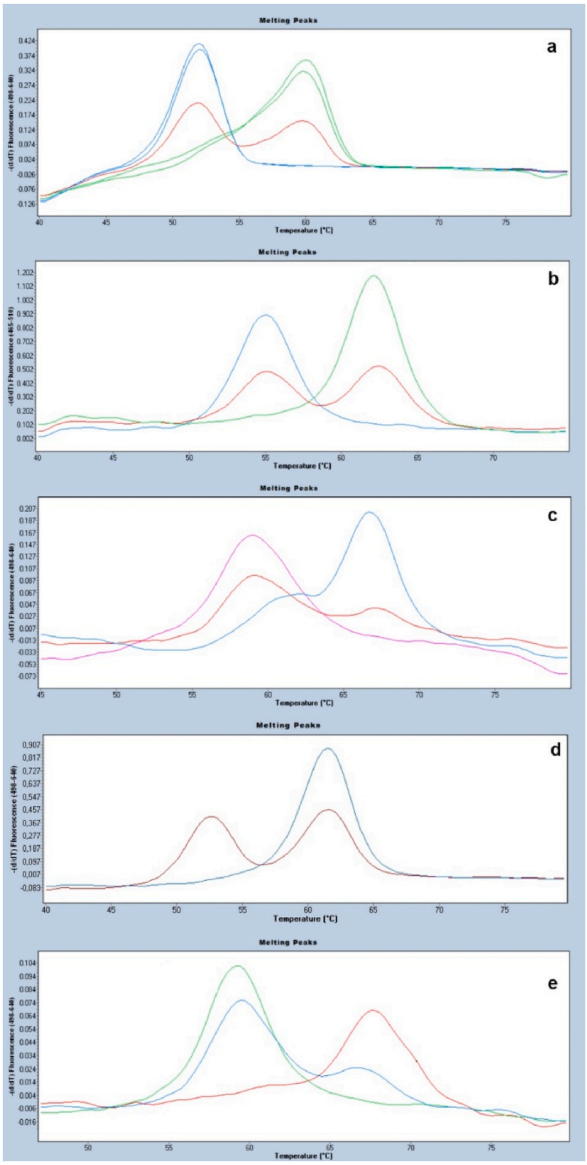

Fig. 3. Melting peaks for the detection of three genotypes of the SNPs identified in the Real-time PCR analysis with the specific fluorescence resonance energy transfer (FRET) hybridization probes. For each SNP, the initial singular peak corresponds to the wild-type variant, while the subsequent singular peak represents the mutant-type variant. The presence of the heterozygous variant is discerned by the simultaneous presence of two peaks. (a) SNP1: BICF2G630558239, (b) SNP2: BICF2P772455, (c) SNP3: BICF2S230609, (d) SNP4: BICF2G630339806, (e) SNP5: BICF2S2452559.

underpinnings of CHD is unfolding, molecular insights accessible to CED remain limited. To the best our knowledge, no published investigation exists wherein the variant profiles of candidate genes implicated in the etiology of these hereditary disorders have been jointly and comparatively evaluated. In this study, we performed a comparative analysis of the SNPs associated with both CHD and CED. These SNPs were corroborated by GWAS findings and were identified as components of a DNA-based predictive test for CHD [19]. Our study focused on a population characterized by exceptional uniformity in terms of environmental influences. Given the shared hereditary nature of CHD and CED as bone and joint disorders [40], we hypothesized that candidate predictive variants for CHD may also be significant in the context of CED. In addition to the properties of the studied population, precise and dependable phenotyping is another critical element for investigating intricate traits. This becomes crucial when the trait consists of numerous interrelated sub-traits, collectively accounting for only a minor portion of the overall variance. Given that the evaluation of CHD depends on FCI scoring, the presence of standardized, first-class radiographs and a limited number of evaluators is paramount in curbing interobserver biases [10,41]. In this study, the phenotyping process encompassed the concurrent execution of interventional radiological procedures and X-ray assessments conducted by identical researchers. Consistent efforts were made to minimize phenotyping discrepancies.

Table 5
Genotypic distributions, population genetic indices (He, Ne, PIC), compatibility with the Hardy-Weinberg equilibrium, and fixation index values (Fɪs) of polymorphisms analyzed in this study.

SNP Name[a]	SNP1			SNP2			SNP3			SNP4			SNP5		
ID	BICF2G630558239			BICF2P772455			BICF2S230609			BICF2G630339806			BICF2S2452559		
Genotypes	AA	AG	GG	AA	AG	GG	AA	AG	GG	AA	AG	GG	AA	AG	GG
Genotype count	19	32	68	12	10	97	72	39	8	0	14	105	49	25	45
Frequency (%)	15.97	26.89	57.14	10.08	8.40	81.52	60.50	32.77	6.73	0	11.77	88.23	41.18	21.01	37.81
MAF	0.2941			0.1428			0.2311			0.0588			0.4832		
He	0.4152			0.2448			0.3554			0.1107			0.4994		
Ne	1.7001			1.3172			1.5485			1.1271			1.9968		
PIC	0.3290			0.2148			0.2922			0.1046			0.3747		
χ^{b} (HWE)[b]	14.7765[c]			51.3447[c]			0.7201			0.0269			39.9428[c]		
Fɪs	0.3524			0.6569			−0.0974			−0.2647			0.5794		

MAF: minor allele frequency, He: heterozygozity, Ne: effective allele number, PIC: polymorphism information content, HWE: Hardy-Weinberg equilibrium, χ^2Fɪs: fixation index.

[a] SNP names based on the predictive model for canine hip dysplasia reported by Bartolomé et al.[19].

[b] If one of the observed genotype counts is smaller then 5, chi-square and *P* value with Yate's correction for continuity were considered.

[c] $P < 0.001$.

Table 6
The prevalence of genotypes in healthy and dysplastic dogs ($n = 119$).

Variant	Healthy			Canine Hip Dysplasia		
	AA	AG	GG	AA	AG	GG
SNP1	10	13	30	9	19	38
SNP2	5	6	41	7	4	56
SNP3	31	17	5	41	22	3
SNP4	0	7	46	0	7	59
SNP5	19	10	22	30	15	23
	Healthy			**Canine Elbow Dysplasia**		
	AA	AG	GG	AA	AG	GG
SNP1	11	23	30	8	9	38
SNP2	4	4	47	8	6	50
SNP3	36	13	3	36	26	5
SNP4	0	5	50	0	9	55
SNP5	20	11	20	29	14	25

SNP1: BICF2G630558239, SNP2: BICF2P772455, SNP3: BICF2S230609, SNP4: BICF2G630339806, SNP5: BICF2S2452559.

Table 7
Associations from univariable mixed logistic regression models (Nagelkerke $R^2 = 0.896$) between risk factors related to Canine Hip Dysplasia (CHD) in German Shepherd and Labrador Retriever breeds in the present study.

Variable	Odds ratio	95 % confidence interval	*P* value
Breed			
German Shepherd	–	–	
Labrador Retriever	1.36	0.65–2.85	NS
Sex			
Male	–	–	
Female	1.71	0.81–3.61	NS
Age[1]			
Group1	1.50	0.48–4.72	NS
Group2	1.35	0.44–4.13	NS
Group3	–	–	
SNP1			
AA	–	–	
AG	1.41	0.46–4.34	NS
GG	1.76	0.63–4.95	NS
SNP2			
AA	–	–	
AG	0.36	0.06–2.16	NS
GG	1.05	0.31–3.56	NS
SNP3			
AA	–	–	
AG	1.04	0.46–2.33	NS
GG	0.42	0.09–1.92	NS
SNP4			
AG	–	–	
GG	1.41	0.46–4.30	NS
SNP5			
AA	–	–	
AG	0.90	0.33–2.42	NS
GG	0.60	0.26–1.39	NS

SNP1: BICF2G630558239, SNP2: BICF2P772455, SNP3: BICF2S230609, SNP4: BICF2G630339806, SNP5: BICF2S2452559, NS: Not significant.
[1] The dogs aged 3 years and younger: Group1, the dogs aged 4–7 years: Group2, and the dogs aged 8 and over: Group3.

Therefore, we maintain that the data acquired from this analysis possess practical validity and can contribute to forthcoming research endeavors.

Initially, we addressed genotypic distributions and population genetic parameters within the scope of this study. Among the five SNPs examined, the GG genotype exhibited the highest frequency (>80 %) of SNP2 and SNP4. Indeed, the genotypic distributions of all SNPs were conducive to enabling association analysis (MAF≤0.05) because MAF below 5 % should be excluded from association analyses to draw reliable conclusions, as indicated by Bongiorni et al. [42]. However, the absence of the AA genotype in SNP4 and the predominance of GG genotyping (n = 105) in most dogs led to diminished genetic variability. While He and Ne quantified the impact of allele variations within populations, PIC values are the most commonly used metrics for assessing the extent of marker polymorphisms [43]. The categorization of PIC follows the guidelines outlined by Botstein et al. [36]. Accordingly, PIC levels are classified as highly informative polymorphisms for PIC>0.50, moderately informative polymorphisms for 0.25 < PIC<0.50, and low informative

Table 8
Associations from univariable mixed logistic regression models (Nagelkerke $R^2 = 0.131$) between risk factors related to Canine Elbow Dysplasia (CED) in German Shepherd and Labrador Retriever breeds in the present study.

Variable	Odds ratio	95 % confidence interval	Significance
Breed			
German Shepherd	–	–	
Labrador Retriever	1.944	0.92–4.10	NS
Sex			
Male	–	–	
Female	1.040	0.49–2.18	NS
Age[1]			
Group1	0.889	0.28–2.80	NS
Group2	1.131	0.37–3.49	NS
Group3	–	–	
SNP1			
AA	–	–	
AG	1.929	0.61–6.09	NS
GG	3.117	1.07–9.06	***P* < 0.05**
SNP2			
AA	–	–	
AG	0.700	0.08–6.22	NS
GG	0.209	0.04–0.98	***P* < 0.05**
SNP3			
AA	–	–	
AG	2.503	1.07–5.84	***P* < 0.05**
GG	1.765	0.39–7.96	NS
SNP4			
AG	–	–	
GG	0.478	0.14–1.63	NS
SNP5			
AA	–	–	
AG	0.834	0.31–2.22	NS
GG	0.721	0.31–1.67	NS

SNP1: BICF2G630558239, SNP2: BICF2P772455, SNP3: BICF2S230609, SNP4: BICF2G630339806, SNP5: BICF2S2452559, NS: Not significant.
[1] The dogs aged 3 years and younger: Group1, the dogs aged 4–7 years: Group2, and the dogs aged 8 and over: Group3.

Table 9
Results from the final multivariable logistic regression model for risk factors of Canine Elbow Dysplasia (CED) in the present study.

Variable	*B*	z	Odds ratio	95 % confidence interval	Significance
SNP2*					
AA	–	–	–	-	
AG	−0.2034	−0.179	0.816	0.09–7.54	NS
GG	−1.5379	−1.891	0.215	0.04–0.96	***P* < 0.05**

* SNP2: BICF2P772455.

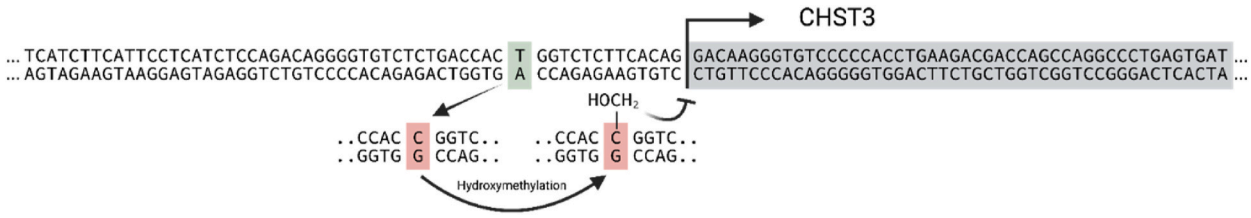

Fig. 4. The *in silico* predicted mechanism of action for SNP2 (BICF2P772455). The predicted system indicates that transcription is regulated due to C hydroxymethylation occurring within the promoter region following the mutation.

polymorphisms for PIC<0.25. In this study, all markers, except SNP2 and SNP4, exhibited moderately informative polymorphisms. Moreover, the Ne values neared 2.00 for SNP5 within the overall dog population. Regarding HWE testing, we found that the genotypic distributions observed for SNP3 and SNP4 were in accordance with HWE expectations ($P > 0.05$). In addition, these SNPs exhibited negative FIS values (Table 5). Genetic diversity parameters are valuable metrics for quantifying polymorphisms among genotypes in breeding programs. Given the population genetics parameters, especially PIC values, within our examined population and the favorable dispersion of the investigated SNP markers, we can deduce that these markers elucidate the genetic diversity in a population consisting of Labrador Retrievers and German Shepherds (the low variation in SNP4 should not be overlooked). Furthermore, they hold

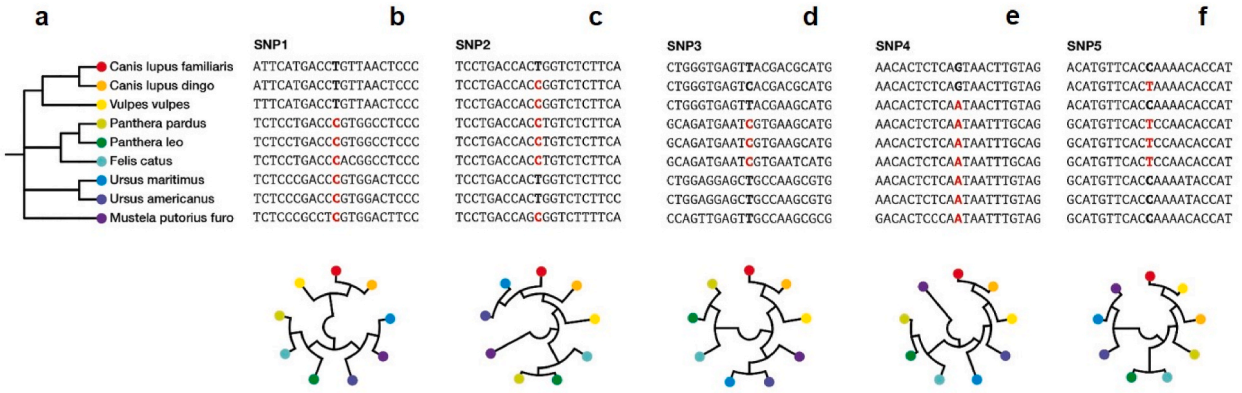

Fig. 5. Examination of the pertinent SNP sequences within this genomic region and assess its taxonomic significance. (a) common taxonomic trees of the selected species. (b) SNP1: BICF2G630558239, (c) SNP2: BICF2P772455, (d) SNP3: BICF2S230609, (e) SNP4: BICF2G630339806, (f) SNP5: BICF2S2452559.

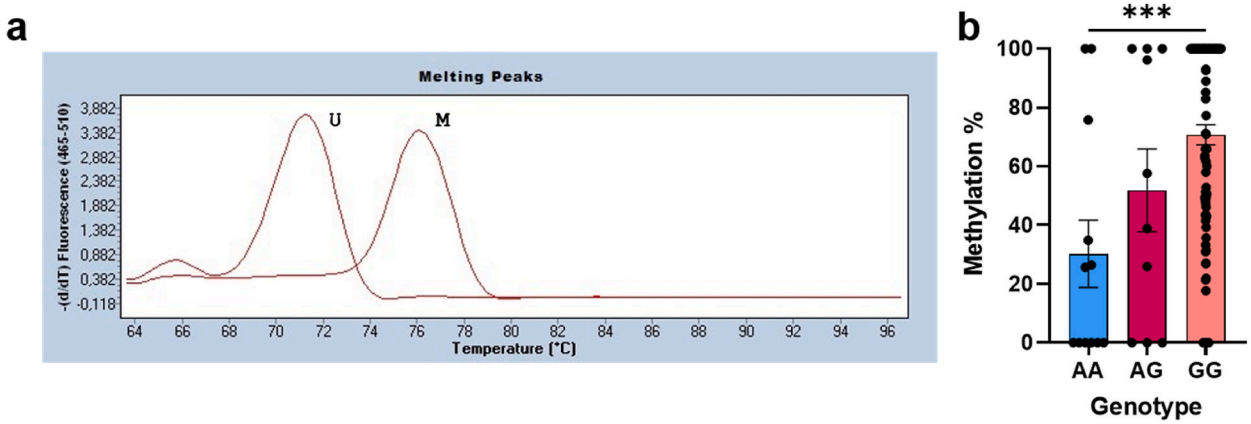

Fig. 6. Assessment of the methylation status within the genomic region containing SNP2 (BICF2P77245) in the canine *CHST3* gene. (a) sample methylation status determination involves assessing the difference in melting temperature (Tm) between methylated (M) and unmethylated (U) primer sets. (b) the association between SNP2 genotypes in the canine *CHST3* gene and methylation patterns. One way ANOVA, $^{***}P < 0.001$.

the potential for application in other genetic investigations, such as genome-wide association studies, aimed at identifying alleles governing specific traits.

CHD is the prevailing orthopedic ailment identified in dogs, exhibiting a prevalence of up to 71 % within breeds susceptible to this condition [26]. CHD development is not an inevitable outcome, even when an individual's potential genetic predisposition to CHD is evident. In this context, external factors such as nutrition, sex, age, exercise, and neutering status may have a critical impact [26]. These factors do not directly induce hip dysplasia; however, they play crucial roles in determining CHD expression and severity. Uniformity in terms of nutrition, exercise regimen, and neutering status was evident in our study. Hence, we focused on the influences of breed, sex, and age in our population. Notably, we observed no noteworthy differences in these factors between the CHD and CED patients. Breed is a significant predisposing factor for both disorders. Regarding CHD, osteoarthritis emerges as an irreversible consequence, bearing the potential for profound debilitation. Although the majority of CHD-afflicted dogs show minimal or no pronounced clinical indications, the substantial prevalence of this condition underscores its gravity. This is particularly concerning given that many breeds susceptible to CHD include highly trained working and service dogs [44,45]. Breeds with an elevated susceptibility to CHD include the Bernese mountain dog, Chesapeake Bay Retriever, German Shepherd, Golden Retriever, Labrador Retriever, Newfoundland, Old English Sheepdog, Rottweiler, Saint Bernard, and Samoyed [26,46–48]. Conversely, breeds classified as having a diminished risk include Miniature Schnauzer, Chihuahua, Maltese, Toy poodle, and Dachshund [26,48]. The primary rationale for the non-observance of breed-related effects in our study could be attributed to the selection of dog breeds that display similarities in terms of body mass and prevalence of CHD. Similar to the effect of breed, no significant sex-related effects were detected. The occurrence rate of elbow arthrosis resulting from elbow dysplasia ranges from 30 % to 50 %, typically affecting males more frequently and with greater severity [2,49]. Nonetheless, it is feasible to observe results that stand in contrast [2,50]. However, indications suggest that hormones could also play a role in the development of this condition [26]. Administration of estrogen to puppies has been shown to result in CHD. However, it is worth noting that the levels of endogenous estrogen in dysplastic puppies were not elevated compared to those in

non-dysplastic animals. A comparable promotive effect of relaxin has been documented [26]. The acetabulum is formed within the triradiate growth plate, which comprises the ilium, pubis, and ischia. A secondary ossification center appears within this plate at 4–5 months of age, while the femoral capital physis closes at 9–11 months. Dogs with CHD exhibit delayed closure of the femoral capital physis [26]. Concerning the CED, screening programs should be conducted within a standardized and narrow age range, preferably approximately 12 months, as advancing age significantly affects the prevalence and severity of elbow arthrosis. Signs of the disorder may manifest earlier (~4–10 months of age), particularly in breeds with a genetic predisposition or in animals displaying elbow pathology [2]. Notably, CHD and CED remain latent in dogs of various ages and do not exhibit visible clinical symptoms. In the present study, we did not observe any significant effect of age on either disorder. It should be acknowledged that our objective was to conduct a genotypic assessment in unconnected animals while standardizing the non-genetic variables. Consequently, this study did not adopt a demographic survey approach. Studies addressing this topic have been conducted using extensive sample sizes. However, the results of these studies were inconsistent. While we observed that sex and age had no significant effects, using a larger cohort size would be more suitable if such effects did exist.

We next performed a genotypic evaluation. In this context, Bartolomé et al. [19] investigated five markers that were reported to have prognostic significance for CHD. The dogs were reared and instructed by the same trainers to ensure consistent environmental conditions encompassing attributes such as nutrition and exercise intensity at the same training center. Additionally, we hypothesized that the markers implicated in CHD development might exert varying effects on the development of CED in Labrador Retrievers and German Shepherds. Bartolomé et al. [19] constructed a logistic model exhibiting high accuracy in predicting CHD among Labrador retrievers. This model relies on the amalgamation of SNPs (provided by genetic characterization combining GWAS and a candidate gene strategy), several of which are positioned close to genes associated with extracellular matrix processes and bone metabolism. The findings derived from Labrador retrievers provide additional support for the concept that genomics forms the foundation for the early identification of CHD [19]. Upon assessing these SNPs within our Labrador Retriever and German Shepherd cohorts, no variants displaying a significant association with CHD were identified (Table 7). Three of these SNPs exhibited potential links to CED. Among the SNPs associated with CED, SNP1 and SNP3 were suggestive variants (from the univariate logistic model), and SNP2 was a significant marker (from the final multivariate logistic model). Positioned approximately 0.5 Mb downstream from the SNP1 (BICF2G630558239), the *SMYD3* gene (SET and MYND domain containing 3) functions as a histone methyltransferase. Studies have indicated its involvement in muscle mass determination and skeletal muscle atrophy [19,51]. Given its role, *SMYD3* has emerged as a prospective candidate gene that may contribute to hind leg muscle atrophy, which is associated with hereditary muscle and joint diseases. Bartolomé et al. [19] indicated that this SNP represents a variant that directly contributes to the risk of CHD. However, we did not observe any significant effect of SNP1 on CHD. Instead, SNP1 was associated with CED in our univariate logistic model ($P < 0.05$). Our investigation provided evidence suggesting that the GG genotype may promote the occurrence of disorders (Table 8). *SMYD3* plays a role in governing the expression of myostatin, a member of the TGF-β family that negatively influences skeletal muscle growth in embryonic development as well as muscle homeostasis [51]. Hence, it is not surprising that a potential correlation exists between this genomic region and hereditary joint disorders, such as CED. Another variant that seems to be related to CED is SNP3 (BICF2S230609), which is positioned near the fibroblast growth factor 4 (*FGF4*) gene. Fibroblasts secrete components vital for the extracellular matrix, which is crucial for connective tissue structure. FGF family members with mitogenic and survival roles affect embryonic development, morphogenesis, and tissue repair [52]. *FGF4* plays a significant role in extremity growth regulation, and cumulative evidence suggests that it is associated with skeletal dysplasia, cartilage (patho)physiology, chondrodystrophy, and osteoarthritis [53,54]. It is important to highlight that these indicative markers associated with CED require additional research to establish a clear connection between SNPs and their functional implications in CED.

In our final logistic regression model, only SNP2 (BICF2P772455) was significantly associated with CED development (Table 9). This SNP is located in the promoter region of the *CHST3* (carbohydrate chondroitin 6 sulfotransferase) gene, precisely 14 base pairs upstream of the initial ATG start codon. Bartolomé et al. [19] pointed out that among 33 SNPs linked to CHD in candidate gene analysis, BICF2P772455 exhibited the most robust association with the disorder. Although no significant impact of this SNP on CHD was detected, a notable influence on CED was observed ($P < 0.05$). In this context, the GG genotype was identified as favorable. The canine *CHST3* gene is located on chromosome 4 (22,973,213–22,975,930 forward strand) and has one transcript (1519 bp) spanning two exons [55]. The *CHST3* gene encodes a sulfotransferase responsible for chondroitin sulfate biosynthesis. Chondroitin sulfate is a vital structural element in joint cartilage and is crucial for its biomechanical characteristics [19]. Thus, *CHST3* appears to be a promising candidate gene for CED. To the best of our knowledge, no previous studies have reported an association between this genomic region and CED in dogs. In addition, we found that SNP2 exerted an impact on disorder development when we categorized dogs as disorder-free, with moderate symptoms in at least one disorder, and with severe symptoms in both disorders (Table S5). Hence, focusing on this particular genomic region may yield unexpected and innovative findings. It is evident that CED often receives less emphasis on phenotypic data than CHD, particularly in studies grounded in the canine genome. Despite its prevalence, the lack of comprehensive molecular insights into CED underscores the need for more comprehensive investigations. Therefore, conducting more intricate studies centered on this specific genomic region holds promise for offering invaluable insights that contribute to both canine genetics and animal welfare.

Congenital skeletal developmental disorders are similar between humans and dogs. CHD and hip dysplasia in humans serve as compelling examples of this hypothesis. They are primarily hereditary and polygenic conditions, and both human and canine hip dysplasia follow comparable natural progression, ultimately advancing to the end stage [56]. Although comprehensive research has been conducted on numerous human genomic regions related to hip and elbow dysplasia, equivalent data concerning the same genes and markers in dogs remain scarce. For instance, while the *CHST3* gene is a prominent focus in human skeletal dysplasia studies [57–59], there is a notable absence of information concerning the interplay between this gene and its genomic region with elbow

dysplasia in dogs. However, scientific evidence underscores the profound influence of unethical breeding practices on the health and well-being of pedigree cats and dogs, thereby affecting both individuals and populations. Factors such as overemphasis on specific traits, adverse effects of inbreeding, and genetic diversity reduction have facilitated the proliferation of inherited ailments, harming animals and burdening their owners. These detrimental and unethical breeding methods can be characterized as genetic misconduct, which is no longer justifiable in light of advancements in DNA technologies, advanced diagnostic tools, and the potential for preventive measures through well-structured breeding programs [60]. The potential of molecular genetics extends beyond the manifestation of specific characteristics in breeds and can be utilized to enhance health traits and disease/disorder resistance. Hence, it is imperative to advance molecular inquiries into the genetic basis of skeletal disorders.

We utilized the iDNA-MS tool to predict 5'-hydroxymethylation of cytosine in SNP2, a variant located in the promoter region of the *CHST3* gene, in both *Homo sapiens* and *Mus musculus*. This analysis extends to Canis species because of their evolutionary intermediacy, thus presenting a broader scope for understanding genetic alterations across different species. One pivotal observation rooted in the extensive analysis of hydroxymethylomes from a spectrum of human and mouse samples is the intricate patterning of 5-hydroxymethylcytosine (5hmC) enrichment, which predominantly favors regions approximately 0.5–2 kb upstream and downstream of the transcription start sites, albeit presenting a diminished concentration in the vicinity of moderately to highly transcribed gene onset points [61]. This phenomenon aligns with the understanding that 5hmC possibly plays a suppressive role in transcription (Fig. 4), which is further supported by its enriched presence at the initiation sites of genes characterized by limited or non-existent transcriptional activity [62]. It is imperative to emphasize that these *in silico* findings represent predictive modulations and, as with all computational extrapolations, require empirical validation through rigorous wet lab experiments to confirm their biological significance. Hence, directing attention to the methylation status within the genomic locus housing the *CHST3* gene SNP2 was deemed essential. We demonstrated a substantial association between SNP2 genotypes and methylation patterns (Fig. 6b), thereby partially elucidating the mechanism underlying the genotype-phenotype correlation in canine *CHST3*.

Epigenetic modulation through DNA methylation is a complex biological process. Most CpG dinucleotides in mammalian genomes undergo methylation, although the methylation pattern is inconsistent [63]. DNA methylation regulates transcription, both directly and indirectly. CpG methylation directly represses transcription by preventing the binding of some transcription factors (TFs) to their recognition motifs [64,65]. In addition, mCpG dinucleotides can be recognized by a specific class of proteins called methyl-CpG domain–binding proteins, some of which recruit histone deacetylases and are thought to promote local chromatin condensation [66,67]. Although extensive studies have been conducted on this topic in the human genome, the available information regarding dogs is limited. This study revealed, for the first time, a regulatory mechanism involving methylation of the *CHST3* gene concerning orthopedic disorders in dogs. Furthermore, these findings clearly establish *CHST3* as a significant candidate gene for CED. To the best of our knowledge, this study represents the most comprehensive molecular genetic investigation that has comparatively assessed both disorders in dogs.

Although our study provides valuable insights into the genetic basis of canine hip and elbow dysplasias, there are limitations that warrant consideration. The complexity of these disorders suggests a multifaceted genetic architecture, necessitating further research to elucidate the complete genetic landscape. Additionally, the impact of gene–environment interactions requires more in-depth exploration to comprehend their contribution to disease expression fully. The phenotypic data in this study demonstrated remarkable uniformity and quality; however, considering the necessity of increasing the sample size and augmenting the analysis with GWAS-backed approaches remains crucial. Although the sample size used in this study was acceptable [68–70], a larger sample size would benefit from the sparsity of data between subgroups, leading to statistically significant results and narrower confidence intervals. We encountered challenges with the primers for two of the seven SNPs (BICF2G630227898 and BICG2P548082) proposed by Bartolomé et al. [19]. Resource constraints prevented us from conducting further analyses or replicating the experiments, leading to the exclusion of these two SNPs from further investigations. Furthermore, there is a substantial demand for RNA-focused investigations of CHD and CED.

Identification of candidate genes and genomic regions linked to dysplasia susceptibility further enhances our understanding of the molecular pathways involved. Our study highlights the potential genomic locations and genes associated with cartilage and joint development, shedding light on the biological processes that may be dysregulated in affected dogs, particularly those with CED. These findings suggested promising targets for further investigation, potentially leading to the development of novel molecular therapeutic interventions and early diagnostic tools. This study also underscores the potential epigenetic significance of the progression of these disorders.

5. Conclusions

Our study highlights the pivotal role of genetic elements in the development of canine hip and elbow dysplasia. By unraveling the genetic foundations and intricate molecular pathways implicated, the present results may help establish a basis for well-informed breeding methodologies and prospective therapeutic approaches. *In silico* analysis indicated that the significant genetic variations identified in our study had the potential to induce crucial changes in gene expression levels, which, in turn, could result in noteworthy alterations in musculoskeletal structural characteristics. A sustained investigation within this domain remains imperative to advance our understanding of these conditions, thereby enhancing the musculoskeletal well-being of canine populations. Genetic association studies on elbow dysplasia are significantly more constrained than those on hip dysplasia in dogs. Our study identified one locus of significance and two loci with suggestive associations with the development of canine elbow dypslasia. These results underscore the importance of examining the candidate genes and markers associated with canine hip dysplasia in the context of elbow dysplasia. Acknowledging the need to enhance this analysis using genome-wide association-supported methods is imperative. Furthermore, RNA-

centered research on hip and elbow dysplasia in dogs is required.

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Ethics statement

The present research has complied with all the relevant national and international regulations for animal welfare and institutional policies for the care and use of animals. The study was approved by Bursa Uludag University Local Research Ethics Committee (Authorization reference number: 2019–01/02).

Data availability statement

Has data associated with your study been deposited into a publicly available repository?
No.
Please select why.
Data will be made available on request.

CRediT authorship contribution statement

Sena Ardicli: Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Pelin Yigitgor:** Writing – original draft, Data curation. **Huseyn Babayev:** Writing – original draft, Validation, Formal analysis. **Dogukan Ozen:** Validation, Formal analysis. **Berkay Bozkurt:** Visualization, Validation, Methodology, Data curation. **Nursen Senturk:** Validation, Data curation. **Mehmet Pilli:** Validation, Data curation. **Hakan Salci:** Visualization, Validation, Data curation. **Deniz Seyrek Intas:** Visualization, Resources, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37716.

List of abbreviations

CHD	Canine hip dysplasia
CED	Canine elbow dysplasia
FCI	Fédération Cynologique Internationale
IEWG	International Elbow Working Group
GWAS	Genome-wide association studies
qPCR	Quantitative polymerase chain reaction
FRET	Fluorescence resonance energy transfer
SNP	Singlr nucleotide polymorphism
He	Heterozygosity
Ne	Effective allele numbers
PIC	Polymorphism information content
HWE	Hardy-Weinberg equilibrium
Fıs	Fixation index
FGF4	Fibroblast growth factor 4
CHST3	Carbohydrate chondroitin 6 sulfotransferase
OCD	Osteochondritis dissecans
UAP	Ununited anconeal process
FCP	Fragmented medial coronoid process

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