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The effects of polymorphisms in the CX3CR1 gene on the development of canine hip dysplasia

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Abstract: Hip dysplasia, caused by both environmental and genetic components, is a common disorder characterized by hip instability in humans and dogs. Unfortunately, the genetic mechanisms that cause the disease in both have not been fully determined. The aim of this study was polymorphisms in the exon 2 and 3′ UTR regions of the CX3CR1 gene were determined and their effects on the development of Canine Hip Dysplasia (CHD) in three dog breeds (German Shepherd, Belgian Malinois, Labrador Retriever). For this purpose, a case -control study was designed with 172 dogs in Dog Breeding and Training Center (DBTC) in Turkey. Each dog was evaluated according to the Norberg angle by the DBTC veterinarians. One hundred and seventeen dogs (32 German Shepherds, 49 Belgian Malinois, 36 Labrador Retrievers) classified as normal were included in the control group, and fifty - five dogs (24 German Shepherds, 14 Belgian Malinois, 17 Labrador Retrievers) diagnosed with CHD were included in the case group. Molecular genetic analyzes were performed with blood samples taken from each dog. Seven previously identified SNPs (g.8938599_8938600insCC, g.8937121G>A, g.8937137A>G, g.8937319T>G, g.8937441T>C, g.8937450A>G, g.8937590C>T) and a rare novel deletion (g. 8937205_ 8937206del) were identified in the 3′ UTR regions of the CX3CR1 gene. The distribution of SNPs alleles in the case and control was compared by means of statistical analysis at allelic, genotypic, haplotypic, and SNP - SNP interaction levels. Single SNP analysis revealed that g.8937121G>A was significantly associated with susceptibility to CHD in Belgian Malinois ($p = 0.00049$) in the codominant model. Five SNP - SNP interactions were identified to be associated with CHD in Labrador Retrievers and the most suggestive of these was between g.8938599_8938600insCC and g.8937450A>G ($p = 0.0004$). We found that one haplotype block, consisting of two SNPs (g.8937137A>G and g.8937319T>G) was associated with susceptibility to CHD in Belgian Malinois ($p = 0.022$). None of the detected polymorphisms was statistically significantly associated with CHD in German Shepherds.

Key words: CX3CR1, SNP, hip dysplasia, dog breeds

1. Introduction

Canine hip dysplasia (CHD) is the most prevalent developmental orthopedic disease in dogs that causes pain, functional limitation, and secondary osteoarthritis [1, 2]. CHD, a polygenic disorder with both environmental and genetic components, is characterized by hip instability, leading to hip laxity and often resulting in hip joint degeneration, painful arthritis, and lameness [3, 4].

Compared to other breeds, large dog breeds are more prone to CHD [5–7]. CHD is also common among popular working and service dog breeds such as German Shepherds, Belgian Malinois, and Labrador Retrievers [8–11]. Working dogs trained for special tasks are widely used for law enforcement and military services. Because their lengthy training time is costly, illnesses and injuries that could lead to early withdrawal from active duty are carefully avoided. However, many working dog breeds are retired before their planned retirement age due to hereditary orthopedic diseases. The most common of these are hip and elbow dysplasia [8]. In order to increase both animal welfare and to prevent economic losses, many countries have attempted to decrease the prevalence of CHD, but the success expected from breeding programs that are based on phenotypic selection against CHD has not been achieved [12]. Today, it is thought that genetic marker - assisted selection or genomic selection could be more successful in breeding programs against CHD [12, 13].

Developmental dysplasia of the hip (DDH), which shares pathways anatomical, biochemical, and clinical features with CHD [2, 14], is a common problem that causes serious impairment in the quality of life in humans. CHD and DDH tend to develop osteoarthritis in the affected joints, and both are treated in a similar manner [2,

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14, 15]. Moreover, environmental and genetic risk factors are effective in CHD and DDH formation. Over the last years, many studies have been carried out to identify the genetic background of hip dysplasia in both humans and dogs [5, 6, 16-24]. Approximately 30 genes have been reported in association with DDH [24], while over 50 have been reported in association with CHD [5, 6, 16–23]. However, very few of these have been confirmed in different populations, and none of them have been firmly correlated with hip dysplasia [13, 24].

Considering that more than 350 diseases that are often caused by the same pathways and genes are analogous in dogs and humans, co - studies with both species to detect genetic markers associated with hip dysplasia can provide important data on the genetic background of the disease. For this purpose, the relationship between the C-X3-C motif chemokine receptor 1 (CX3CR1) gene, which has been reported to be associated with hip dysplasia in humans, with CHD was investigated in three purebred dog breeds (Labrador Retriever, Belgian Malinois, and German Shepherd). The association with the CX3CR1 gene to DDH has been confirmed in three independent studies [25–27]. CX3CR1 is known to play a role in bone homeostasis and chondrocyte proliferation. It encodes a receptor of chemokine CX3CL1, which includes a G - protein coupled transmembrane domain [25]. The CX3CR1 gene (ENSCAFG00000031104), located on chromosome 23 in dogs, has 2 exons (exon 1: 77 bp, exon 2: 1101 bp) and encodes 384 amino acid residues (https://www.ensembl.org/index.html).

The CX3CR1 is an interesting target for joint disorders. Polymorphisms on the CX3CR1 gene are thought to modify the biological role of CX3CL1 and may cause many joint diseases [28]. In addition, polymorphisms in the CX3CR1 gene have been reported to increase susceptibility to hip dysplasia in humans [25, 26]. Therefore, the CX3CR1 gene was chosen as the target in this research. The goal of the present study was to investigate the relationship between the SNPs in the CX3CR1 gene and CHD disease in three purebred dog breeds (German Shepherd, Belgian Malinois, Labrador Retriever). For that purpose, genetic variations in the exon 2 and 3' UTR regions (3054 bp) of the CX3CR1 gene were investigated by DNA sequence analysis. Associations of the detected SNPs with the disease were analyzed using single - locus analysis, two - dimensional SNP analysis (interaction), and haplotype - based approaches.

2. Materials and methods

2.1. Sample collection

Permission was obtained from Trakya University Animal Experiments Local Ethics Committee to collect blood samples from dogs. For two years (2018–2019), a total of 172 blood samples were collected that purebred dogs (56 German Shepherds, 63 Belgian Malinois, 53 Labrador Retrievers) raised in Dog Breeding and Training Center (DBTC) in Turkey. Norberg angles were measured in ventrodorsal hip - extended radiographic views by the DBTC veterinarians. The radiological analyses were performed in the veterinary clinic in the DBTC. One hundred and seventy - two purebred dogs were divided into two groups as the case (< 105, with CHD) and the control (≥ 105, normal) according to Norberg angles. Fifty -five purebred dogs were classified as the case (24 German Shepherds, 14 Belgian Malinois, 17 Labrador Retrievers) and 117 purebred dogs as the control (32 German Shepherds, 49 Belgian Malinois, 36 Labrador Retrievers). The case group consisted of one - year - old dogs diagnosed with CHD and the control group consisted of dogs older than one-year old that have normal hip joints. For the case group and the control group, blood samples were collected in EDTA - tubes and stored at –18 °C in a deep freezer until molecular studies were performed. All cases and controls were selected to be unrelated at the parental level. The privacy rights of DBTC were maintained. The environmental impact factors were eliminated because of dogs included in the study were in similar feeding and sheltering conditions.

2.2 Genotyping

Genomic DNA was extracted from the blood samples using the Quick Blood DNA Purification Kit (Eurx, Poland) according to the manufacturer's instructions. Five primer pairs were designed based on ENSCAFG00000031104 to amplify exon 2 and 3' UTR regions in the CX3CR1 gene using the Primer 3 program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The information for oligonucleotide primers, PCR product sizes, and annealing temperatures are shown in Table 1. PCR amplifications were carried out in volumes of 25 µL using; 12.5 µL PCR 2X Master Mix (Thermo Fisher Scientific, USA, cat no. K0171), 50 ng genomic DNA, 5 pmol each primer, and ddH_2O. The temperature cycling protocol on T100 Thermal Cycler (Biorad, USA) consisted of 3 min incubation at 95 °C followed by 35 cycles at 95 °C for 30 sec, annealing for 30 sec (annealing temperatures for each primer pairs are given in Table 1) 72 °C for 45 sec, and a final extension at 72 °C for 10 min. Five microliters of the PCR products were subjected to electrophoresis on 2% agarose gel (Bioshop, Canada) and then visualized by the WGD30S Molecular Imager (Wisd, USA). The PCR products were purified using the GeneMatrix (EURx, cat no. E3520 - 02) in accordance with the manufacturer's protocol. The purified PCR products were sequenced in the forward direction of the primers in Table 1. To confirm the results, bidirectional DNA sequencing was performed with randomly selected samples from each genotype.

Table 1. Primer names, sequences, annealing temperatures, PCR product sizes, and primer locations in the CX3CR1 gene.

Primer name	Primer sequence (5′ > 3′)	Amplification region*	Location	Annealing Temperature (°C)	PCR Product (bp)
CX1	F:TACAGGAAAACAGTGTCCGC	8939331-8940080	Exon2	60	750
	R:TGTGGGACCAAATAGAATCCCAA				
CX2	F:GTTGTTTTTGGGCATTCGGTT	8938803-8939394	Exon 2	60	592
	R:CTGCTCCCCCTGCTCATTAT				
CX3	F:AGGTTGGTGGGCATCTCTTG	8938293-8938921	3′ UTR	66	629
	R:TACCAAAGCCTTGTCCCCAC				
CX4	F:GCTCTGTCAACACTGCTCCT	8937757-8938312	3′ UTR	60	556
	R:CAAGAGATGCCCACCAACCT				
CX5	F:CCAGAGGATATCATTGAAGAGGAG	8937026-8937658	3′ UTR	62	663
	R:CTGACCAAGCCCTCCAGTTG				

* The location on the reference genome accession ID: NC_006605.3. bp; base pair.

Sequencing reactions were performed by using the DTCS - Quick Start Sequencing Kit and T100 Thermal Cycler (Biorad, USA) with the following program: 96 °C for 20 s, 50°C for 60 s, 60°C for 4 min for 40 cycles. Sequencing was performed by using the GenomeLab GeXP Genetic Analysis System (Beckman Coulter, USA). The sequences were analyzed using the BioEdit v7.0.5.3 software [29] and Chromas v2.6.6 (http://www.technelysium.com.au).

2.3. Statistical analyses

Hardy Weinberg equilibrium (HWE) P - values for each SNP were calculated for each dog breed using the HardyWeinberg-1.6.3 [30] package with R Software version 3.6.2 [31]. HWE in control groups was assessed using Pearson chi - squared test or Fisher exact test when necessary.

The genetic data were analyzed using single - locus analysis, two - dimensional SNP interaction analysis, and haplotype - based approaches. The associations between the single -SNPs and the risk of CHD were evaluated by calculating the odds ratios (OR) with 95% confidence intervals (CI) using SNPassoc under codominant genetic models. These analyses were performed by the SNPassoc package [32] in R software –3.6.2 [31] and SPSS (Version 20.0, Chicago, IC, USA). Differences were considered statistically significant when P - value was less than 0.05. Bonferroni corrections were applied for all multiple tests.

Linkage disequilibrium (LD) and haplotype frequencies, as well as an association between haplotype and CHD, were determined with the Haploview [33] software. Haplotypes were analyzed within blocks defined by the default confidence - interval method according to the criteria defined by Gabriel et al. [34]. The protocol by default ignores markers with minor allele frequency below 0.05. This method sorts the list of all possible blocks, and after starting with the largest one and keep adding blocks as long as they do not overlap with an already declared block.

Potential interactions between pairs of variants on CHD were estimated using the SNPassoc package within R statistical software performing log - likelihood ratio tests. The microRNA (miRNA) target sites on the CX3CR1 gene were predicted using the online miRDB program (www.mirdb.org).

3. Results

The DNA samples from three different dog breeds (Belgian Malinois, Labrador Retrievers, German Shepherds) were amplified (Figure 1) and sequenced for the exon 2 and 3' UTR in the CX3CR1 gene. Eight variations (6 SNPs, 1 insertion, 1 deletion) were identified by sequencing analysis of the 3' UTR region. There was no polymorphism in the exon 2 region. Seven variations detected in each of three dog breeds have been previously described in the dbSNP, Ensemble (https://www.ensembl.org/index.html). However, the g.8937205_8937206del was not found in the dbSNP. The rare novel deletion (g.8937205_8937206del) was identified only in Belgian Malinois. Three genotypes of g.8938599_8938600insCC were observed in Belgian Malinois, but CC/CC homozygote genotype was not found in Labrador Retriever and German Shepherd. The AA genotype of g.8937121G>A SNP was not found in all three purebred dog breeds. Three genotypes of g.8937319T>G were detected in Labrador Retrievers, while the GG genotype was not observed in Belgian Malinois and TG genotype in German Shepherd. The GA genotype of g.8937137A>G SNP was only found in Belgian Malinois, but other genotypes (AA and GG) were observed in all three dog breeds. The TT and CC genotypes of g.8937590C>T SNP were observed in all three dog breeds but CT genotype

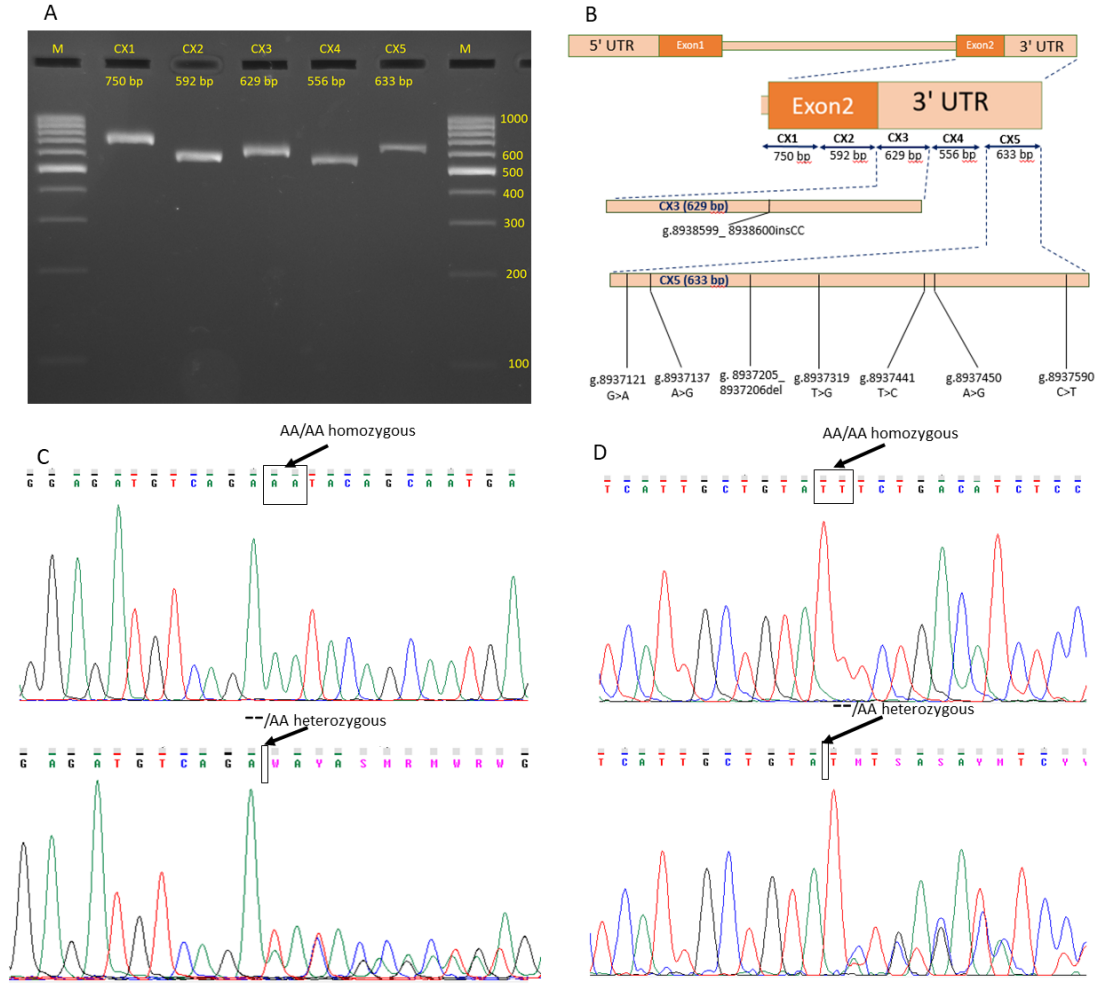

Figure 1. A- Agarose gel electrophoresis results of PCR products. M; marker (100-1000 bp). B- Graph of the CX3CR1 gene and position of amplified DNA fragments on the gene C- The electropherogram of DNA sequencing (forward strand) showing the AA deletion (g. 8937205_ 8937206del) in the 3′ UTR region of the CX3CR1 gene. D- The AA deletion was confirmed using DNA sequencing the reverse strand.

was not only found in German Shepherd (Table 2). Six of the seven SNPs detected in German Shepherd dogs were observed to deviate from HWE for the control group. In the German Shepherd, only one SNP (g.8937121G>A) was found to be in HWE for the control group. Two SNPs were found to deviate from HWE for the control group in Belgian Malinois (g.8937441T>C, g.8937590C>T) and Labrador Retriever (g.8937137A>G, g.8937590C>T) (Table 3). Details of all variants are presented in Table 2.

3.1. Single - marker association analysis

The distribution of allele and genotype frequencies of polymorphisms determined in the CX3CR1 gene in three dog breeds are shown in Table 3. Statistical correlation analysis was not performed on polymorphisms deviating from HWE in the control groups. No statistically significant association was observed between polymorphisms and CHD in Labrador Retrievers and German Shepherds (Table 3). However, two SNPs (g.8937121G>A, g.8937137A>G) were found to be associated ($p < 0.001$ and $p < 0.05$) with CHD in Belgian Malinois (Table 4). One of them, g.8937121G>A, was statistically significant after applying the Bonferroni correction for the eight SNPs. Statistical significance level was determined as $p < 0.00625$ after Bonferroni correction for multiple tests.

g.8937137A>G, a weak association was observed in both genotype and allele distributions between CHD and control groups ($p = 0.046$ for genotype distribution and 0.01 for allele distribution). Compared to the AA genotype, the AG and GG genotypes were significantly correlated with a reduced risk of CHD (OR = 0.108; 95% CI = 0.017–0.695 for AG genotype, OR = 0.100; 95% CI = 0.010–0.975 for AA genotype). Carriers of the G allele had a 3.333– fold higher risk of developing CHD than those with the A allele (OR = 3.333, 95% CI = 1.279 –8.688) (Table 4).

Table 2. Information of eight SNPs identified in the CX3CR1 gene and genotype frequencies of the three dog breeds.

SNP name	GenBank no.	Gene region	Belgian Malinois (n = 63) Genotype / Frequencies			Labrador Retriever (n = 53) Genotype / Frequencies			German Shepherd (n = 56) Genotype / Frequencies		
g.8938599_8938600insCC	rs851814810	3' UTR	--/--	--/CC	CC/CC	--/--	--/CC	CC/CC	--/--	--/CC	CC/CC
			0.540	0.381	0.079	0.642	0.358	0.000	0.982	0.018	0.000
g.8937121G>A	rs9022770	3' UTR	GG	GA	AA	GG	GA	AA	GG	GA	AA
			0.873	0.127	0.000	0.755	0.245	0.000	0.857	0.143	0.000
g.8937137A>G	rs9022769	3' UTR	GG	GA	AA	GG	GA	AA	GG	GA	AA
			0.095	0.190	0.714	0.679	0.000	0.321	0.018	0.000	0.982
g. 8937205_ 8937206del	*	3' UTR	AA/AA	AA/--	--/--	Monomorphic			Monomorphic		
			0.889	0.111	0.000						
g.8937319T>G	rs852431030	3' UTR	TT	TG	GG	TT	TG	GG	TT	TG	GG
			0.730	0.270	0.000	0.396	0.434	0.170	0.982	0.000	0.018
g.8937441T>C	rs852569240	3' UTR	TT	TC	CC	TT	TC	CC	TT	TC	CC
			0.603	0.238	0.159	0.075	0.528	0.396	0.964	0.018	0.018
g.8937450A>G	rs851634517	3' UTR	AA	AG	GG	AA	AG	GG	AA	AG	GG
			0.587	0.397	0.016	0.075	0.604	0.321	0.964	0.018	0.018
g.8937590C>T	rs853018978	3' UTR	TT	CT	CC	TT	CT	CC	TT	CT	CC
			0.762	0.190	0.048	0.642	0.189	0.170	0.982	0.000	0.018

* It was not found in dbSNP.

g.8937121G>A was found to be more correlated with the CHD both allelic and genotypic levels (respectively, p = 0.002, p = 0.00049) than g.8937137A>G (Table 4). AA genotype could not be determined in the control and case groups. However, a higher risk of CHD was found in carriers of the GA genotype compared to carriers of the GG genotype (OR = 17.625, 95% CI = 3.011–103.181). In addition, evaluation of allele frequencies revealed that carriers of the A allele had a 13.091 - fold higher risk of developing CHD than those with the G allele (OR = 13.091, 95% CI = 2.474–69.264).

3.2. SNPs on miRNA target regions

miRNA binding prediction analysis of the 3′ UTR region of the CX3CR1 gene was performed using the miRDB (www.mirdb.org) that online miRNA database. Eight miRNA was predicted to be targeted CX3CR1 gene in dogs. These miRNAs have a target score in the range of 54 - 86 (Table 5). It was determined an SNP (g.8937590C>T) creates (C allele) or destroys (T allele) miRNA (cfa-miR-488) seed region (Figure 2). C allele creates a 7mer - A1 target site sequence for the cfa-miR-488. Interestingly, control groups of all three dog breeds deviated from the HWE for this SNP, therefore marker - association analysis could not be performed for this SNP.

3.3. Haplotype - based association study

The extent of linkage disequilibrium (LD) between the six identified SNPs was estimated separately for all three dog breeds using Haploview 4.2. Because SNPs detected in German Shepherds did not meet the data quality criteria applied by the Haploview program, no further analyses were performed. In Belgian Malinois, the two SNPs (g.8937137A>G, g.8937319T>G) were found highly linked in one haplotype block with D′= 1 (Figure 3). The haplotype block was consisted of three haplotypes, AT, GG, and GT, with frequencies of 0.810, 0.135, and 0.056, respectively. Compared with the AT, the GT haplotype had a significantly higher frequency in the case group than in the control group, indicating it could significantly increase the susceptibility to CHD in Belgian Malinois (p = 0.022, OR = 6.222, 95% CI = 1.280–30.243) (Table 6). These P - values did not remain significant after Bonferroni correction for the eight SNPs.

Two SNPs deviating from HWE in Labrador Retrievers were excluded from the analysis by the HaploView program. The three SNPs (g.8937319T>G, rs8522569240, g.8937450A>G) in the CX3CR1 gene in Labrador Retrievers formed one LD block. GGG, TAA, TGG and TGA haplotypes had frequencies 0.387, 0.340, 0.236, and 0.038, respectively. Haplotypes were not significantly

Table 3. Genotype and allele frequencies of the SNPs in the CX3CR1 gene, P values for HWE and correlation analyses.

SNP code	Genotype allele	Belgian Malinois				Labrador Retriever				German Shepherd			
		Case (n =14)	Control (n = 49)	Control HWE p value	CA P value	Case (n =17)	Control (n = 36)	Control HWE p value	CA p value	Case (n = 4)	Control (n = 32)	Control HWE p value	CA p value
g.8938599_8938600insCC	--/--	0.64 (n = 9)	0.51 (n = 25)	0.730	0.537	0.47 (n = 8)	0.72 (n = 26)	1.00	0.077	0.95 (n = 23)	1.00 (n = 32)	**0.000**	**
	--/CC	0.36 (n = 5)	0.39 (n = 19)			0.53 (n = 9)	0.28 (n = 10)			0.05 (n = 1)	0.00		
	CC/CC	0.00	0.10 (n = 5)			0.00	0.00			0.00	0.00		
	--	0.82	0.70			0.74	0.86			0.98	1.00		
	CC	0.18	0.30			0.26	0.14			0.02	0.00		
g.8937121G>A	GG	0.57 (n = 8)	0.96 (n = 47)	1.00	**0.0005**	0.88 (n = 15)	0.69 (n = 25)	0.569	0.120	0.92 (n = 22)	0.81 (n = 26)	1.00	0.258
	GA	0.43 (n = 6)	0.04 (n = 2)			0.12 (n = 2)	0.31 (n = 11)			0.08 (n = 2)	0.19 (n = 6)		
	AA	0.00	0.00			0.00	0.00			0.00	0.00		
	G	0.79	0.98			0.85	0.96			0.96	0.91		
	A	0.21	0.02			0.15	0.04			0.04	0.09		
g.8937137A>G	GG	0.29 (n = 4)	0.04 (n = 2)	0.232	**0.046**	0.76 (n = 13)	0.64 (n = 23)	**< 0.001**	**	0.00	0.03 (n = 1)	**0.016**	**
	GA	0.14 (n = 2)	0.20 (n = 10)			0.00	0.00			0.00	0.00		
	AA	0.57 (n = 8)	0.76 (n = 37)			0.24 (n = 4)	0.36 (n = 13)			1.00 (n = 24)	0.97 (n = 31)		
	G	0.36	0.14			0.76	0.64			0.00	0.03		
	A	0.64	0.86			0.24	0.36			1.00	0.97		
g. 8937205_ 8937206del	AA/AA	0.86 (n =12)	0.90 (n = 44)	1.00	0.676	1.00 (n = 17)	1.00 (n = 36)	*	**	1.00 (n = 24)	1.00 (n = 32)	*	**
	AA/--	0.14 (n = 2)	0.10 (n = 5)			0.00	0.00			0.00	0.00		
	--/--	0.00	0.00			0.00	0.00			0.00	0.00		
	AA	0.93	0.95			1.00	1.00			1.00	1.00		
	--	0.07	0.05			0.00	0.00			0.00	0.00		
g.8937319T>G	TT	0.57 (n = 8)	0.78 (n = 38)	1.00	0.142	0.35 (n = 6)	0.42 (n = 15)	0.275	0.589	1.00 (n = 24)	0.97 (n = 31)	**0.016**	**
	TG	0.43 (n = 6)	0.22 (n = 11)			0.53 (n = 9)	0.39 (n = 14)			0.00	0.00		
	GG	0.00	0.00			0.12 (n = 2)	0.19 (n = 7)			0.00	0.03 (n = 1)		
	T	0.79	0.89			0.62	0.61			1.00	0.97		
	G	0.21	0.11			0.38	0.39			0.00	0.03		
g.8937441T>C	TT	0.43 (n = 6)	0.65 (n = 32)	**< 0.001**	**	0.12 (n = 2)	0.06 (n = 2)	0.276	0.715	1.00 (n = 24)	0.94 (n = 30)	**0.046**	**
	TC	0.57 (n = 8)	0.15 (n = 7)			0.53 (n = 9)	0.53 (n = 19)			0.00	0.03 (n = 1)		
	CC	0.00	0.20 (n = 10)			0.35 (n = 6)	0.41 (n = 15)			0.00	0.03 (n = 1)		
	T	0.71	0.72			0.38	0.32			1.00	0.95		
	C	0.29	0.28			0.62	0.68			0.00	0.05		

Table 3. (Continued).

g.8937450A>G	AA	0.43 (n = 6)	0.63 (n = 31)	0.666	0.390	0.12 (n = 2)	0.06 (n = 2)	0.145	0.541	1.00 (n = 24)	0.94 (n = 30)	**0.046**	**
	AG	0.57 (n = 8)	0.35 (n = 17)			0.64 (n = 11)	0.58 (n = 21)			0.00	0.03 (n = 1)		
	GG	0.00	0.02 (n = 1)			0.24 (n = 4)	0.36 (n = 13)			0.00	0.03 (n = 1)		
	A	0.71	0.81			0.44	0.35			1.00	0.95		
	G	0.29	0.19			0.56	0.65			0.00	0.05		
g.8937590C>T	TT	0.64 (n = 9)	0.80 (n = 39)	**0.025**	**	0.47 (n = 8)	0.72 (n = 26)	**< 0.001**	**	1.00 (n = 24)	0.97 (n = 31)	**0.016**	**
	CT	0.36 (n = 5)	0.14 (n = 7)			0.41 (n = 7)	0.09 (n = 3)			0.00	0.00		
	CC	0.00	0.06 (n = 3)			0.12 (n = 2)	0.19 (n = 7)			0.00	0.03 (n = 1)		
	T	0.82	0.87			0.68	0.76			1.00	0.97		
	C	0.18	0.13			0.32	0.24			0.00	0.03		

*Monomorphic ** Statistical correlation analysis was not performed because it deviated from the Hardy Weinberg Equilibrium (HWE) or monomorphic. CA; Correlation Analysis.

Table 4. Allelic and genotypic association of the SNPs with CHD in Belgian Malinois.

SNP / Genotypes / Alleles	Case	Control	p value	OR	95% CI
g.8937121G>A					
GG	8	47		1	-
GA	6	2	**0.00049**	17.625	3.011 - 103.181
AA	0	0		-	-
G	22	96	**0.002**	13.091	2.474 - 69.264
A	6	2			
g.8937137A>G					
GG	4	2		1	-
AG	2	10	**0.046**	0.108	0.017 - 0.695
AA	8	37		0.100	0.010 - 0.975
G	10	14	**0.011**	3.333	1.279 - 8.688
A	18	84			

OR: odds ratio, 95% CI: 95% confidence interval.

Table 5. miRNAs and target seed location in 3′ UTR in the CX3CR1 gene.

miRNA code		Target score miRNA sequence (5′-3′)	Seed location in 3′ UTR
cfa-miR-8899	86	GUUGGGGUCCAUGCGUCUAGGAC	722; 841
cfa-miR-8836	83	AGGAAAGGAGAAGGGCCACA	825; 1053; 1125; 1300
cfa-miR-1837	73	UCUCAGAGGGACUGCGACAUCU	350; 367; 764
cfa-miR-8843	71	UUGUUUUUUUCUCUCGCCCCGCCUG	116; 1460
cfa-miR-589	64	UGAGAACCAUGUCUGAUCAGAG	1630
cfa-miR-488	58	CCCAGAUAAUGGCACUCUCAA	1104; 1332
cfa-miR-7180	57	CUGUGGCCUCUGGGUGUGUACCCU	1620
cfa-miR-8883	54	UCUGGGUGAGGAUUCGCAGGACUUU	741; 1528

miRNA: microRNA

associated with CHD after performing association analyses by the HaploView (Table 6).

3.4. Two dimensional SNP associate analysis

Two-dimensional analysis of variant pairs interactions was performed using the codominant model. Although significant interaction was not observed in both German Shepherds and Belgian Malinois (data not shown), five significant interactions were identified in Labrador Retrievers (Figure 4). It was found statistically significant interactions between g.8938599_8938600insCC with each of the five SNPs (g.8937590C>T, g.8937450A>G, g.8937441T>C, g.8937319T>G, g.8937137A>G). However, after applying Bonferroni correction for all possible combinations (n = 64, p < 0.00078) one of them remained significant (g.8938599_8938600insCC and g.8937450A>G, p = 0.00048). Other p - values were shown in Figure 4.

4. Discussion

Although CHD was first reported to originate from inbreeding 70 years ago [35], its etiology has not been fully elucidated in studies conducted so far. However, a great number of SNPs have been reported to be susceptible or protective for CHD and DDH [1, 5, 6, 17-21, 36-39]. Many human and canine diseases are thought to have the same causes and similar genetic mechanisms. Previous studies have identified variants of the CX3CR1 gene underlying hip dysplasia in humans. CX3CL1 and its receptor CX3CR1 are

Figure 2. The binding site of the cfa-miR-488 and the CX3CR1 gene was predicted by miRDB. C allele of the g.8937590C>T creates a target region for the cfa-miR-488.

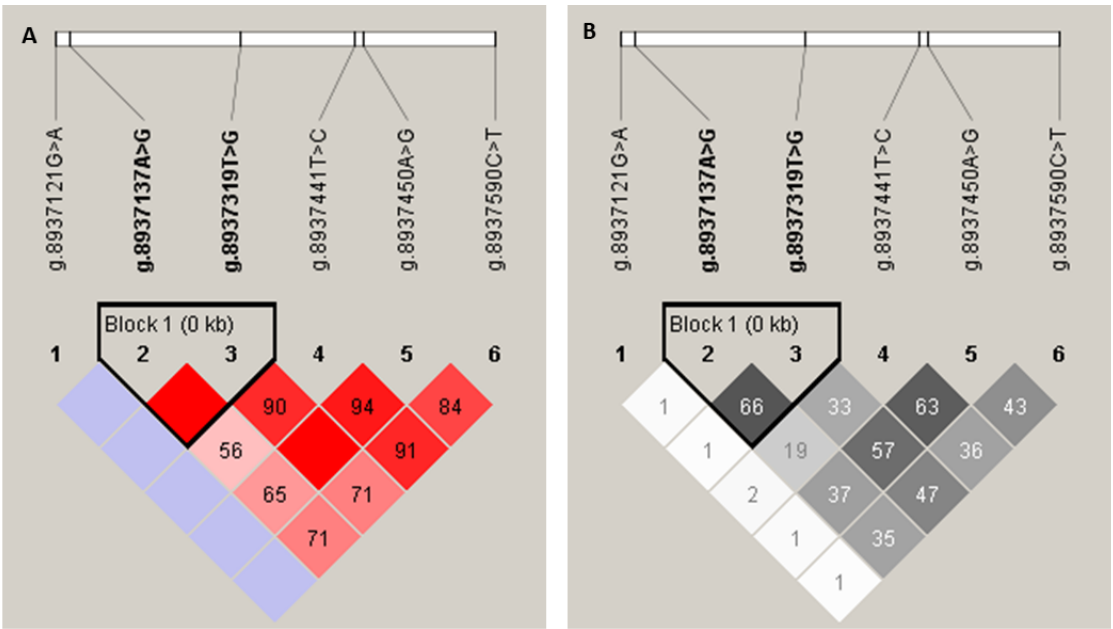

Figure 3. Linkage disequilibrium plot. A- Regions with high D' values are dark red, and regions with low D' values are lighter shades of red. Red diamond without a number corresponds to D' values of 1.0. B- Black represents high LD ($r^2 = 1$) and white indicates the absence of correlation ($r^2 = 0$).

interesting targets for joint disorders. Polymorphisms on the CX3CR1 gene are thought to modify the biological role of CX3CL1 and may cause many joint diseases [28]. The CX3CL1 / CX3CR1 axis is involved in the differentiation of mesenchymal stem cells into chondrocytes and the maturation of osteoclast cells, which are responsible for the destruction of bone tissue. In mesenchymal stem cells that will differentiate into chondrocyte cells, the CX3CR1 gene is known to be expressed at high levels [40, 41]. Therefore, it is thought that the false expression of the gene will cause the problematic development of acetabular cartilage, which may be the cause of future joint disorders [26].

The two SNPs (rs3732378, rs3732379) reported to be associated with the development of hip dysplasia in humans are located in the exon 2 region of the CX3CR1 gene [25, 26]. Both of these polymorphisms are the missense that leads to amino acid substitutions (T280M for rs3732378 and V249I for rs3732379) in the transmembrane domain of the CX3CR1 protein [26]. However, genetic variation was not detected in the exon 2 region of the dog breeds used in the present study. According to NCBI, there are 326 reference SNPs in the exon 2 region of the human CX3CR1 gene and one reference SNP (rs851299957, synonymous variant) in the same region in dogs according to Ensembl. Given that the minor allele frequencies of diseases-causing SNPs are generally low, new variations may be found in the exon 2 region by working with larger populations.

In the present study, six SNPs, a deletion, and an insertion in 3′ UTR regions of the CX3CR1 gene were determined. The novel deletion (g. 8937205_8937206del) was only detected in Belgian Malinois (Table 2). The minor allele frequency of the deletion was 0.05 in the control group and 0.07 in the case group. The number of SNPs deviating from HWE was found to be 6, 2, and 2 for control groups in German Shepherd, Belgian Malinois, and Labrador Retriever, respectively. An SNP (g.8937121G>A) was found to be in HWE for control groups in all three dog breeds (Table 3).

Table 6. Haplotypes in Belgian Malinois and Labrador Retriever.

Breed	Haplotypes			Total frequencies	Case frequencies	Control frequencies	p value	OR	95% CI
Belgian Malinois	A		T	0.810	0.643	0.857	**0.011**	1	-
	G		G	0.135	0.214	0.112	0.163	-	-
	G		T	0.056	0.143	0.031	**0.022**	**6.222**	**1.280 - 30.243**
Labrador Retriever	G	G	G	0.387	0.382	0.389	0.949	-	-
	T	A	A	0.340	0.382	0.319	0.523	-	-
	T	G	G	0.236	0.176	0.264	0.322	-	-
	T	G	A	0.038	0.059	0.028	0.434	-	-

OR: odds ratio, 95% CI: 95% confidence interval.

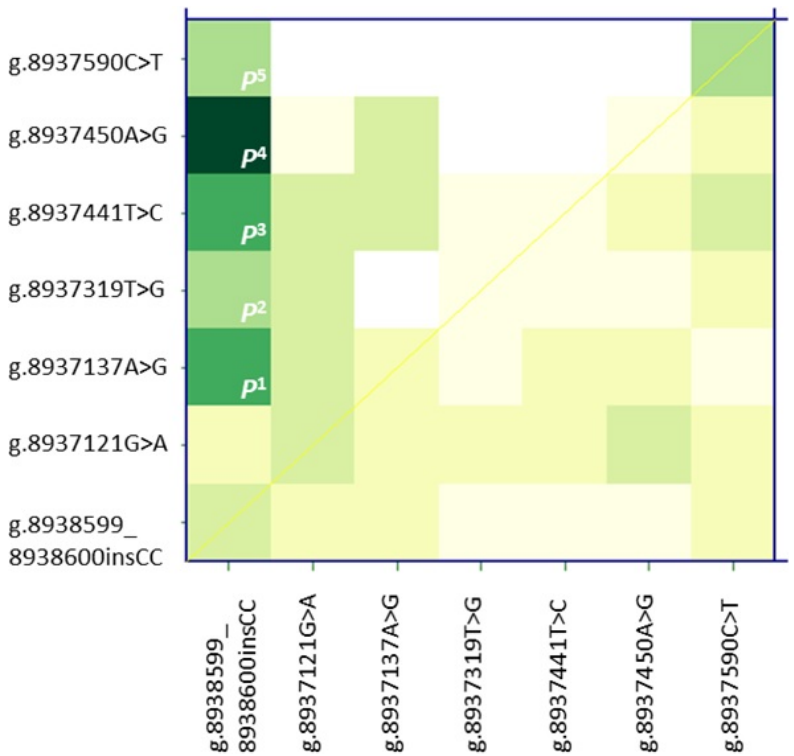

Figure 4. SNP - SNP interactions plot in Labrador Retriever dogs. The plot shows the significance levels of SNP - SNP interactions and CHD in the codominant model. Each small square represents the p-values obtained from different likelihood ratio tests. Different gradient colors indicate different statistical significance levels (the darker shades represent smaller, more significant p-values). Along the diagonal, as indicated by a line, are the p-values of the single SNP tests. The upper triangle in the matrix contains the p-values for the interaction (epistasis) log - likelihood ratio test. the lower triangle contains the p-values from the likelihood ratio test comparing the two - SNP additive likelihood to the best of the single - SNP models. p-value of interactions: $p^1 = 0.003$, $p^2 = 0.013$, $p^3 = 0.001$, $p^4 = 0.0004$, $p^5 = 0.014$

As a result of single SNP association analysis, the G allele of the g.8937137A>G and A allele of g.8937121G>A were found to increase susceptibility to CHD in Belgian Malinois (Table 3). It was observed that the statistical correlation of g.8937137A>G (OR = 3.333, 95% CI = 1.279 - 8.688) with CHD was weaker than g.8937121G>A (OR = 17.625, 95% CI = 3.011 - 103.181) (Table 4). The g.8937121G>A was statistically significant after applying the Bonferroni correction for the eight SNPs. In previous studies, it has been reported that SNPs in genes involved

in the pathways that regulate bone - cartilage development and homeostasis are associated with CHD [1, 17, 19, 38]. The two intronic SNPs in the CTIF (rs24413139 G>A) and TSPAN4 genes (rs22634232 G>T) have been reported to be associated with CHD in the Belgian Malinois and the Labrador Retriever [38]. In addition, a downstream gene variant (MMRN2 gene, rs24111003 C>T) has been found to be associated with CHD [38]. The two SNPs in the 5′ UTR and 3′ UTR region of the CHST3 gene have been reported to be associated with the development of CHD in Labrador Retrievers [1]. It has been reported to be associated with two intergenic (BICF2S2367279, BICF2P1086886) and the one intronic SNPs (BICF2P281364, KSR2 gene) the development of CHD in German Shepherd [17].

miRNAs play critical roles in chondrocyte proliferation, differentiation, apoptosis, and endochondral ossification [42-45]. In addition, miRNAs control the activity of many genes required for bone metabolism [46]. Abnormal miRNA expression has been reported to play a role in many diseases, including hip dysplasia, osteoarthritis, and osteoporosis [43, 47-49]. In the present research, the potential effects of SNPs in miRNA binding sites in the CX3CR1 gene were investigated in silico using miRDB. Eight miRNAs binding sites were determined in the 3′ UTR regions of the CX3CR1 gene (Table 5). An SNP (g.8937590C>T) was identified in the core binding site of the cfa-miR-488 (Figure 2). The T allele of g.8937590C>T destroys the core binding site and can cause the CX3CR1 gene overexpression by abolishing control of gene expression. In addition, miR-488 has been reported to play a role in chondrocyte differentiation, regulation osteoblast function, bone formation, and cartilage development [50-52]. Because the control groups deviated from the HWE in all three dog breeds for g.8937590C>T, a single - marker associated analysis was not performed. However, when the SNP - SNP interaction was evaluated, it was found that an interaction g.8937590C>T with g.8938599_8938600insCC in Labrador Retrievers was significantly correlated ($p = 0.014$) with the development of CHD. This correlation may be due to changes in the expression status of the CXCR1 gene. It is known that non - coding disease - associated SNPs within 3′ UTR regions of the genes can result in gene dysregulation at the posttranscriptional level [53].

The extent of LD between the identified SNPs was estimated in Belgian Malinois and Labrador Retriever. In Belgian Malinois, the two SNPs (g.8937137A>G, g.8937319T>G) were found highly linked in one haplotype block with D′= 1 (Figure 3). The haplotype block was consist of three haplotypes, AT, GG, and GT in Belgian Malinois. Haplotype GT increased susceptibility ($p = 0.022$, OR = 6.222, 95% CI = 1.280 - 30.243) to CHD disease in Belgian Malinois (Table 6). These P - values did not remain significant after the Bonferroni correction for the eight SNPs. In the Labrador Retriever, the three SNPs (g.8937319T>G, rs8522569240, g.8937450A>G) formed one LD block. GGG, TAA, TGG and TGA haplotypes had frequencies 0.387, 0.340, 0.236 and 0.038, respectively (Table 6). Haplotypes were not significantly associated with CHD after performing association analyses by the HaploView in Labrador Retriever.

Although individual SNPs are popular markers for determining susceptibility to diseases, SNP - SNP interactions help to understand the complex mechanisms underlying diseases. An SNP, which does not affect disease formation alone, can interact with different SNPs and have a strong effect on disease formation [54–56]. In the present study, interactions of SNPs detected at the 3′ UTR region of the CX3CR1 gene were found to be associated with the development of CHD in Labrador Retrievers ($p < 0.001$). It was determined that the interaction of the CC insertion (g.8938599_8938600insCC) with 5 SNPs (Figure 4) had statistically significant effects with CHD development in Labrador Retrievers. However, after the Bonferroni correction one of them remained statistically significant (g.8938599_8938600insCC and g.8937450A>G, $p = 0.00048$). No statistically significant correlation was found between SNP - SNP interaction and CHD in Belgian Malinois and German Shepherd ($P > 0.05$). These results support the opinion that alternative genetic factors associated with the development of CHD may play a role in different breeds or even populations [18].

Until recently, more than 200 marker candidates associated with CHD have been proposed in genome-wide association studies [1, 5, 6, 17-21, 36-39]. Only one of them is in the coding gene region (rs24610835 A>C) [39], the others are in the non - coding gene regions or the intergenic region. Similarly, in the current study, statistically significant associations were found between SNPs in the 3′ UTR regions of the CX3CR1 gene and CHD ($p < 0.001$ and 0.05). The UTRs play an important role in the control of gene expression. The 3′ UTR regions are targeted by miRNAs and regulatory molecules and are involved in the cytoplasmic localization, stability, and translation efficiency of mRNA. Polymorphisms in the 3′ UTR region can cause diseases by altering these mechanisms [57].

There are some limitations of this study that should be considered. First, the sample size is comparatively small. After the Bonferroni correction for multiple testing, only one SNP (g.8937121G>A) in Belgian Malinois and, one SNP - SNP interaction (g.8938599_8938600insCC and g.8937450A>G) in Labrador Retriever remained associated with CHD. A small sample size might be a reason for this observation. Second, only SNPs in the exon 2 and 3′ UTR regions of the CX3CR1 gene were analyzed. Future studies should be designed to large sample sizes for

various dog breeds and analyze all SNPs on the CX3CR1 gene. After these genetic marker candidates are confirmed in different dog populations, they can be used in the early risk assessment of the disease.

In conclusion, a novel deletion, insertion, and six SNPs were identified in the 3' UTR region of the CX3CR1 gene by DNA sequencing in the three pure dog breeds. Using different statistical approaches, susceptibility polymorphisms for CHD have been identified in the Labrador Retriever and the Belgian Malinois. Future studies should focus on the effects of these polymorphisms on gene expression.

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Contribution of authors
SA contributed as an investigator in the study, collected samples and conducted experiments. SK guided the experiments and aided in the statistical analysis of the data. Both authors contributed to the writing of the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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