Genetic Analysis reveals Association of LGR4 Mutations with Osteosarcoma in the Irish Wolfhound

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Abstract

Background: Osteosarcoma is the most common primary malignant bone tumor in dogs, especially in Irish Wolfhounds. Developing from primitive mesenchymal stem cells, abnormal osteoblastic cell differentiation causes production of malignant osteoid which results in immature bone. A high rate of metastasis can occur early in the development of osteosarcoma, with lungs being the site of preference. On diagnosis, most affected dogs already suffer from metastases. This high rate of metastases and tumor recurrence leading to failure of therapy are the main reasons why the prognosis is extremely poor. Although research has resulted in many genes and pathways that are thought to be related to the development of osteosarcoma, a definite cause has yet to be found. This study aims to identify relevant genetic factors of osteosarcoma development in the Irish Wolfhound.
Results: LGR4 was identified as a promising gene situated on significant SNP BICF2S23146586 (P= 0.0005). On this gene multiple variations were found, one of which was predicted to lead to splice site loss and two of which resulted in a change of protein structure. The T>A variation situated on position g.21:47977601, leading to splice cite loss, could be of particular interest relating to osteosarcoma.
Conclusion: The number of genetic variations found reinforce the belief of complexity of osteosarcoma. Further research of these mutations in the Irish Wolfhound is needed to better predict the resulting effects.
Key words: osteosarcoma, genetic, dog, canine, Irish wolfhound

Introduction
In both humans and dogs, osteosarcoma (OSA) is the most common primary, malignant bone tumor.[1-4] In dogs specifically osteosarcomas make up approximately 90% of the bone tumors.[1] A prevalence rate of 7.9/10000 per year has been reported.[5] And the United States, specifically, reported 10.000 cases, yearly.[3] The prognosis, unfortunately, is extremely poor as therapeutic options are limited and ineffective. A median survival time of 8 months for 303 dogs was recorded by Moore et al. (2007).[6,7] In dogs, osteosarcomas make up approximately 90% of bone tumors.[1] This specific type of tumor develops from the primitive mesenchymal stem cell and exhibits the differentiation of osteoblastic cells which then produce malignant osteoid resulting in immature bone.[4] In dogs, a prevalence rate of 7.9/10000 per year has been reported.[5] And the United States, specifically, reported 10.000 cases, yearly.
One of the major characteristics of osteosarcomas is that it can affect all parts of the body but, in 79% of the cases, it will develop in the skeleton. These usually present as appendicular osteosarcomas or axial osteosarcomas.[3] The prognosis of appendicular osteosarcoma is worse than the axial osteosarcoma as it is locally extremely aggressive, infiltrates the surrounding soft tissue, results in bone destruction and has a higher metastasis rate.[1,3] Osteosarcoma have a high grade of malignancy and a high rate of metastasis, the most common site of metastasis (80 to 90%) being the lung. Other sites of metastases may include visceral organs, the brain, subcutaneous tissue, skin and other bones but rarely lymph nodes.[1,8] Of the dogs with osteosarcoma brought in to the clinic, 90% already have metastases occurring in their body. However, in these dogs less than 15% of the metastases are clinically detectable.[9]

For years, striking resemblances have been noted regarding similarity of the biological behavior of osteosarcoma between dogs and humans. This pertains, among other thing, to predilection sites, gender differences in occurrence, high rate of early (hematogenous) metastasis and histopathological aspects. Thereby making osteosarcoma in dogs a suitable model for human osteosarcoma.[1,10-12]
In dogs, breed-related factors have been observed to increase risk, such as increasing

standard weight, height and increasing age.[2] Possible non breed-related factors, such as occurrence at the site of metallic implants, post-orthopedic surgery, bone infarcts, bone infection, minor chronic trauma and ionizing radiation, have been observed in both dogs and humans.[3,13] A twofold risk was reported in neutered dogs.[2,14]
Surprisingly, in giant dog breeds, an odds ratio (OR) of 185 regarding prevalence has been reported while medium and small dogs showed only an OR of 1.[15] This finding, combined with the twofold risk of the front limbs, suggests that stress due to excessive body weight, causing micro traumas, may play a role in the development of osteosarcoma in large dogs.[14,16]

Diagnostic procedures play an important role in confirming the diagnosis. Fine needle biopsy, histology and cytology is often the first method used to confirm suspicion. Radiographic imaging may show bone lysis, bone proliferation of the cortex, periosteal and subperiosteal new bone formation and soft tissue swelling. Other diagnostic methods include bone scintigraphy, magnetic resonance imaging (MRI) and computed tomography (CT).[1,6]
The first line of treatment is surgery (amputation or limb-sparing). This is rarely effective enough on its own due to (sub)clinical metastases.[10,17,18] Surgical treatment is palliative and brings pain relief.[16,19] Patients treated with surgery alone have a short mean survival time, due to the occurrence of micro metastases in 90% of the cases. Therefore, patients diagnosed with metastases have a very poor prognosis.[1,7,8,19] The prognosis worsens with increasing histological tumor grade and stage of disease advancement including metastatic spread. The migration site of metastases is of significant importance to the survival time.[20-23] Adjuvant chemotherapy increases the survival time considerably. The best results are achieved when chemotherapy is combined with palliative radiotherapy and/or surgery. [2,9,22]
Despite continuous development in surgery and the various therapeutic agents, treatment continues to fail. Tumor recurrence and it's fast spreading nature are the main reasons for poor survival rates. Methods to increase survival time have been unsuccessful, resulting is a great need for more targeted therapy. Therefore, it is crucial to understand the mechanisms of tumorigenesis at molecular and genetic levels to provide insight for effective treatment strategies.

A canine genetic history
Dogs inhabit a particular place in the genetic field. Due to their breeding history, domestic dogs provide a great opportunity to investigate the genetic basis of behavioral traits, morphological variation and disease susceptibility. The close proximity of dogs and humans within the mammalian evolutionary tree enables comparative analysis between both species. In a great number of diseases that are often seen in the human population, clinical manifestation occurs in a similar fashion in dogs. The high prevalence of specific diseases within certain types of dogs makes the dog suitable for research.
The preference of man for a selective type of dogs has been the foremost reason for the historical evolution. Over the years, stringent breeding and bottlenecks have caused closely delineated morphologies to occur.[24-26]
Various breeds consistently mentioned regarding osteosarcoma are: the Irish wolfhound, Rottweilers, Great Dane, Doberman Pinchers, Scottish Deerhounds, Irish setters, Boxers and Saint Bernard. These breeds are at high risk, as large weight, height and long limbs put them at risk for the development of osteosarcoma. This predisposition for certain breeds suggests a genetic background of oncogenesis.[2,27,28]
For the Irish Wolfhound specifically, breeding programs and historical events, like the World War II, resulted in four distinctive bottleneck phenomena and an extremely high inbreeding coefficient, narrowing the genetic diversity greatly.[24] The consequential homogenous genetic pool and high frequency of certain disorders, such as osteosarcoma, combined with the fact that dogs are a good model for humans, make them especially interesting for genetic research.

Genetic mutations in canine and human osteosarcoma
The evidence of pathologic and molecular features found in the majority (if not all) of osteosarcoma tumors strongly suggests that (epi)genetic mutations, disrupting the osteoblast differentiation, might be the cause of osteosarcoma [29,30] After years of research, an extensive list of genetic factors and genes has arisen for both canine and human osteosarcoma.[4,31]
Leading research accomplished by Karlsson et al. (2013) found 33 inherited risk loci related to osteosarcoma among three high-risk dog breeds by using the Genome-wide association study (GWAS). Through these risk loci, Karlsson was able to explain 55-85% of phenotype variance in different breeds.

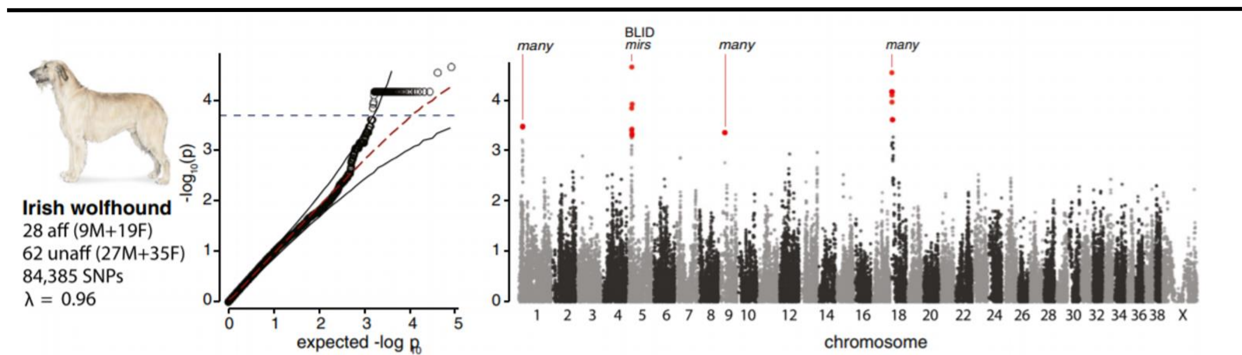

Figure 1: GWAS corrected for population structure and identification of loci associated with Irish wolfhound osteosarcoma[30]

Table 1:Osteosarcoma related loci identified by GWAS[30]

SNP	chr	Position	P	Risk allele	OR	f (A)	f (U)	Region start-end	Size (kb)	Genes
Irish Wolfhound										
BICF2S23746532	5	15264066	2.1E-05	A	1.40	0.45	0.16	14720254-15466603	746	BLID
BICF2P1466354	18	4937944	2.7E-05	C	1.36	0.56	0.21	4266743-5854451	1,588	C7orf72, COBL, DDC, FIGNL1, GRB10, IKZF1, VWC2, ZPBP
BICF2P1225386	1	17742179	3.2E-04	C	1.31	0.46	0.19	16768869-18150476	1,382	BCL2, KIAA1468, PHLPP1, PIGN, RNF152, TNFRSF11A, ZCCHC2
BICF2P1125643	9	19623231	4.3E-04	C	1.75	0.14	0.02	18896060-19633155	737	ABCA5, KCNJ16, KCNJ2, MAP2K6

Specifically pertaining to the Irish wolfhounds, as shown in Figure 1 and Table 1, four regions were found to be associated with osteosarcoma.
By using Gene Relationships Across Implicated Loci (GRAIL) analysis on these 33 associated single nucleotide polymorphisms (SNPs), Karlsson identified 5 significant genes with non-random connectivity in the Irish Wolfhound. Three of which were connected with the key word ‘bone’: VWC2, IKZF1 and BCL2.[30]
Also, high-frequency DNA copy number variations have been identified in spontaneously arising canine osteosarcomas.[32] Using cross-species comparative analysis, strong similarities have been found in the global pattern between human and canine osteosarcoma.[33] These similarities usually consist of abnormal karyotypes (as shown by the presence of translocations), rearrangements and DNA copy number changes.[34,35] The gene variations are suggested, according to Levine and Fleischili(2000), to result from faulty DNA repair, sensing mechanisms, segregation mechanisms or due to sentinel events (chromothripsis). Two of the most widely known germline mutations are variations in the p53 tumor suppressor gene and RB1 gene signaling.[4] P53 regulates genes that are involved in DNA damage response, apoptosis and the cell cycle while also encoding a transcription factor.[29] In 60-67% of all osteosarcoma cases, the cell line would contain an overexpression of the p53 protein. In 41% of the cases mutations would arise.[4]
Tang et al. (2008) found that 75-80% of the mutations found in osteosarcoma consist of allelic loss. Point mutations were observed in 20-30% and large gene rearrangements in 10-20%.[29] However a few years later on, Fenger et al. (2014) found missense point mutations in 74% and deletions in 26%.[4]
Earlier studies have questioned the importance of RB1 regarding the dysregulation in canine osteosarcoma.[36] More recent studies, however, implicate RB1 dysregulation in the formation of osteosarcoma by identification of DNA copy number loss connected to RB1.[36,37]
The RB1 gene is an essential regulator of the G1/S cell cycle progression. In 70% of the sporadic osteosarcoma cases, RB1 mutations have been reported.
Such as, as already noted by Tang et al. in 2008, p53 and RB mutations are one of the few genetic alterations which have been detected in most osteosarcoma tumors.[29]
In addition to these two genes, the PTEN tumor suppressor gene has also been documented to be related to human and canine osteosarcoma.[38] Frequently seen as homozygous gene deletions in human osteosarcoma, canine osteosarcoma cell lines have shown reduced PTEN expression and gene deletions. Recent data suggests copy

number loss relating to the PTEN gene to be not uncommon in canine osteosarcoma.[32,39]

Several studies have reported the presence of cancer stem cells (CSCs) in various types of cancer as the main cause of drug resistance, tumor recurrence and metastasis. CSCs have been known to share similar properties with regular stem cells, such as self-renewal, high proliferation rates, differentiations potential and multi-drug resistance. To maintain CSCs, it has been agreed that alterations in the Wnt/β-catenin signaling pathway are crucial.[40]
Normally, the Wnt pathway is fundamentally important to healthy bone development and regeneration.[41] Variations occurring in the Wnt/β-catenin pathway have been thought to result in tumorigeneses, tumor development and the maintenance of CSCs.
This hypothesis has been confirmed in mouse models where the variations in the Wnt/β-catenin signaling pathway have been reported to be extremely tumorigenic.[40] Overexpression of Wnt ligands and receptors as well as epigenetic silencing of genes have been found to result in osteosarcoma development and progression.[41]
SP cells, a side population of CSCs, are considered enriched CSCs and after further research were proclaimed to have upregulated protein levels of B-catenin and cyclin D1 leading to less tumor suppression.
Furthermore, this research noted elevated expression levels of various stem cell proteins (Cd133, SOX2, Nanog and nestin Oct-4) in the same osteosarcoma SP cells.
Besides the Wnt signaling pathway, additional pathways have been found by Selvarajah et al. (2009) relating specifically to aggressive phenotypes of canine osteosarcoma.[31]
Unfortunately, up until now, none of the genetic variants found in association with canine osteosarcoma have been conclusively verified. Karlsson et al (1013) remains the leading study in this field. Conformation of variants may to lead to better therapeutic possibilities and prolonged survival-time. If these variants were able to predict an increased risk of osteosarcoma in a consistent fashion, the prevalence of the disease might ultimately be diminished.[42]
The fact that large number of pathways and genes relating to the development of osteosarcoma have been considered relevant, suggests a highly complex etiology. In addition matters are complicated by the variations found in the gene expression within the tumors themselves and by the presence of the many generally accepted risk factors.

The aim of this study is to develop further insight into the genetic background of the Irish wolfhound, as is believed that multiple genetic components play a part in the development of osteosarcoma. To address this issue, this research searched for significant loci using PCR sequencing techniques in blood samples of Irish wolfhounds with and without osteosarcoma. These loci and their responsible gene mutations were then mapped. This study is part of ongoing research regarding osteosarcoma in the Irish wolfhound at the University of Utrecht at the department of Small Animal Medicine.

Materials and methods

Animals

DNA data samples were obtained by Dr. Andrea Vollmar (Austerlitz, Germay), who performed routine screening for cardiomyopathy in Irish Wolfhounds in The Netherlands, while simultaneously collecting blood samples for research purposes by the Veterinary Faculty of Small Animal Medicine of Utrecht university and the Hannover institute. The samples have been collected with informed consent given by the owners of the dogs.
For research purposes regarding the genetic background of osteosarcoma in Irish wolfhounds, DNA from 44 dogs suffering from osteosarcoma was sent to the company Neogen for genotyping of 170.000 SNP markers. Using GenABEL software, the genotypes from the cases were compared to the genotypes of control dogs not suffering from osteosarcoma.
With the database consisting of 800 Irish Wolfhounds of all ages and families, selection of the control group was made using 2 criteria. Included were solely dogs who did not have shared parents and who were 6 years of age and older. This with the aim to decrease the influence of family connections and the possibility of false-negatives. No other inclusion or exclusion criteria were incorporated into this study.
134 client-owned Irish Wolfhounds, age of 6-12 years (mean age of 7.5 years) were used in this study, with 90 dogs making up the control group and 44 dogs in the case group.
The significance of differences found in allele frequency was based on the chi-square test. The results were corrected for multiple testing and underlying family connections with statistical significance determined at $P \leq 0.05$.
After statistical analysis to determine the most significant SNPs, the first SNP to be sequenced was: BICF2G630550898.

Table 2: Selected SNPs with accompanying primers (KASP PCR)

SNPs	Forward (F) or Reverse (R)	Primer ('5 → 3')
BICF2G630550898	F	GTTGGATGCTCTGGCACTTG
	R	GAGAAGCGTTCTGTATGTTA
BICF2S23146586	F	CTTTGCCTAGATTCCTAATTGG
	R	TAAGATAAAGCTCTGRGRCATCCC

Table 3: Selected SNPs with accompanying primers (KASP PCR)

SNPs	Forward (F) or Reverse (R)	Primer ('5 → 3')
BICF2G630550898	F	TGGGTTAAGGTGTGGATCAATGAG
	R	TAGGGAAGGAGGAGATAAGCAGAG
BICF2S23146586	F	GCTGATACATGTTTAAGGTGTG
	R	CTCTGTGTCATCCCAGACTC

Primer design (KASP PCR)
Using the KASP™ genotyping assay (a competitive allele-specific PCR), a number of primer sequences were constructed by hand (Table 2). The first SNP primers were constructed using Primer.exe after applying the following criteria: Tm: 80-90ºC;
GC: 50-75; TmΔ: max 10-15ºC.
The second SNP primers were constructed using the program Perlprimer.

KASP PCR
Standard KASP PCR protocol was followed. Starting with a 10μM primer solution, the KASP assay mix consisted of 4μL of both allele-specific forward primers, 15μL common reverse primer and 27μL Milli-Q resulting in a mix of 50μL. The KASP Master mix used was a universal Master mix.
Using a 384 well plate, to every 2μL of DNA sample, 0.055μL KASP assay mix and 2μL KASP Master mix were added.
The PCR started with a 15 minutes hot-start activation at 94ºC after which 10 cycles followed consisting of 20 seconds at 94ºC and 60 seconds at 61ºC. Every cycle ended with a drop of 0.6ºC. The next step consisted of 30 cycles of 10 seconds at 94ºC and 60 seconds at 55ºC. The last 10 cycles consisted of 20 seconds at 94ºC for denaturation and 60 seconds at 57ºC for annealing and elongation. After completion of all cycles, the program ended with 5 minutes at 20ºC.
The primers were optimized in three different ways:

1. Substitution of 2μL DNA by 4 μL.
2. Adding 1.25μL Betaïne solution to every sample.
3. Changing the temperature from 61ºC/ 55ºC/ 57ºC to respectively 68ºC/ 62ºC/ 64ºC.

After completion of primer optimization, the procedure was changed to sequencing the samples.

Primer design (sequencing)
Utilizing the National Center for Biotechnology Information (NCBI) to retrieve the genomic DNA sequence of the canine Boxer, primers were designed using the Boxer sequence in combination with PerlPrimer (Table 3).
After primer design, the first SNP was sequenced; 9 samples (consisting of 5 cases and 4 controls) were run as a preliminary test followed by running four batches of 24 samples each, resulting in 105 samples. As this SNP was not significant, the remaining samples were not sequenced.

Sequencing PCR
A primer work solution of 10μM was used. For every 2μL genomic DNA, the desired PCR solution included 1.5μL 10x PCR buffer, 0.6μL $MgCl_2$ (50mM), 0.3μL dNTP (10mM), 0.75μL of both the forward and reverse primer, 0.15μL Platinum Taq polymerase, 3.75μL Betaïne (4M) and 5.2μL Milli-Q.
The PCR program commenced with a 5 minute initial denaturation at 95ºC followed by 35 cycles each consisting of 30 seconds at 95ºC, then 30 seconds at 55ºC (or higher depending on the required specificity of the primer) and ending with 30 seconds of 72ºC. The final extension stage following the completion of all 35 cycles lasted 10 minutes at 72ºC. The holding temperature was set at 20ºC.
The products were made visible on a 2% Agarose ethidium-bromide gel, adding 2,0μL dye and 3,5μL 100bp ladder to every 7,5μL DNA.

Purification
Before starting the tercycle, purification of the samples is advised by using Exonuclease I. This study purified the samples using 10μL PCR product + 1μL Exo (2units/μL). The samples were placed in a thermal cycler with heated lid and run for 45 minutes at 37ºC, 15 minutes at 75ºC and were held at 4ºC.

Table 4: LGR4 exon primers

SNPs	Forward (F) or Reverse (R)	Primer ('5 → 3')
1	F	TGCACCCGGAGCCGCA
	R	GCAGCGCCCCGTCCCT
2	F	TTTGACTATCCCTGTGTCTG
	R	GAAAGTAAGCTATCTGAATGCC
3	F	CTCATTGGGAGAATCTTGGG
	R	AAAGAAACTGTGCTTACCCT
4	F	GTGCTTTCCACCTTGACCTC
	R	CATGTCCTTGTCCCTCACCA
5	F	AAAGAAGGAATGCCACGACTC
	R	AGTATCTCTTCAACAGCACCC
6	F	CTATTTGCTGTTTCCACCAGTC
	R	TAGTGAGCCAAACTCAAGACC
7	F	ACTATGGTTGGGAAGGAGAG
	R	CATCCAGTCTGTATGAGCCT
8/9	F	AACTTCTGTTTCCTCACGCT
	R	TGTCTTCTGGAGCAACAATGAG
10	F	GAACACTTAGCCTCATTATCAC
	R	AAAGCACTGCCTCAAATCTG
11	F	GCAAATGAGACCCATAAAGGA
	R	TTGAAATATACATGACCGACCC
12	F	GATCTTCTTACCTGGACTCTC
	R	CAGAATGCCCGATAAGTACC
13	F	CGAGATCAATCAGTGTTAATCAGG
	R	TTTCACCTACCAATGTACGG
14/15	F	GAGGGATTGAACATAAACACGA
	R	TACCAAATTTGTGAGGACTGAG
16/17	F	GTTGTCATCTGACACCAGAG
	R	CTGGAGAGCTTATGAACACC
18 (1st half)	F	GCCTAGTCCAATGACTAGAATAGC
	R	GGAGATTGCAGTGATCAACGG
18 (2nd half)	F	ACGCCCTCGTTAGGATTTACTG
	R	ACAAATTGGTTCCAAAGAGACCAC

Tercycle

The tercycle reaction proceeded the sequencing analysis and was completed for both the
forward and reverse primer. 2μL PCR product of every sample was used, after which 1μL Big Dye Terminator Cycle Sequencing Ready Mix, 1μL forward or reverse primer (3.2μM), 2μL 5x sequencing buffer, 1.5μL Milli-Q and 2.5μL Betaïne was added, resulting in a total amount of 10μL.

Following standard protocol, the tercycle reaction consisted of 25 cycles, each lasting 30 seconds at 96ºC, 15 seconds at 50-55ºC (depending on the annealing temperature in the PCR, this being 5ºC below the PCR temperature) and 30 seconds at 60ºC.

Sequence analysis

The tercycle product was purified using a 96-well Multiscreen MAHV Sephadex plate. 15μL Milli-Q was added to every sample, after which everything was pipetted to the center of the Sephadex columns and the plate was centrifuged at 1900rpm for 5 minutes. If necessary, another 10μL Milli-Q was added to individual wells, after which the centrifuge phase was repeated. The samples were analyzed by placing them in a 3500XL Genetic Analyzer and running the program appropriate for the length of the product.

The resulting DNA sequence was analyzed using Seqman Pro software

The next SNP selected for analysis was BICF2S23146586. After finding this SNP to be significant, the remaining samples were sequenced, resulting in a total of 134 samples. The PCR ran at an annealing temperature of 55ºC. The tercycle ran at 50ºC, resulting in a significant finding after applying the chi-square test. Following this finding, surrounding genes were examined and the most likely candidate gene was selected, using keywords cancer and bone.

Primers were constructed for every exon in the LGR4 gene (Table 4) utilizing the same method as described previously.

Standard PCR protocol, with an annealing

temperature of 55ºC, was used to sequence LGR4 genes following the previously described steps. A few minor but important differences regarding the annealing temperature were implemented.
For exon 1, an annealing temperatures of 55ºC, 60ºC, 65ºC and 75ºC was used. After this, a Q5 PCR was performed. To every 2µL genomic DNA (1:10 diluted), 3µL 5x Q5 buffer, 0.3µL dNTP's (10mM), 3µL Q5 GC enhancer, 0.75µL of both primers (10µM), 1.3µL Milli-Q, 0.15µL Q5 hot start HF taq and 3.75µL Betaïne(4M) was added. The initial denaturation was run for 30 seconds at 98ºC, followed by 35 cycles starting with 10 seconds at 98ºC, 30 seconds at 60ºC and ending with 30 seconds at 72ºC. The final extension stage was run for 2 minutes at 72ºC. The samples were held at 20ºC.
Exon 5, 16, 17 and 18 were run at 60ºC.

The results of the LGR4 sequence were blasted in NCBI to check for variations between the Irish wolfhound and the universal gDNA of the boxer. The variations found were analyzed to ascertain potential protein change. The program Polyphen-2 or Splice Site Prediction (by Neural Network, Berkeley Drosophila Genome Project: BDGP) was used to predict the influence of these proteins.

Results

This study investigated the genetic background of Irish wolfhounds with osteosarcoma. After the results of the case samples were received from Neogen, after selecting a control group based on age and after excluding parental overlap, an analysis was set up with 90 control and 44 cases. The analysis using GENable software found the most significant SNPs (Figure 2), based on the chi-square test. The

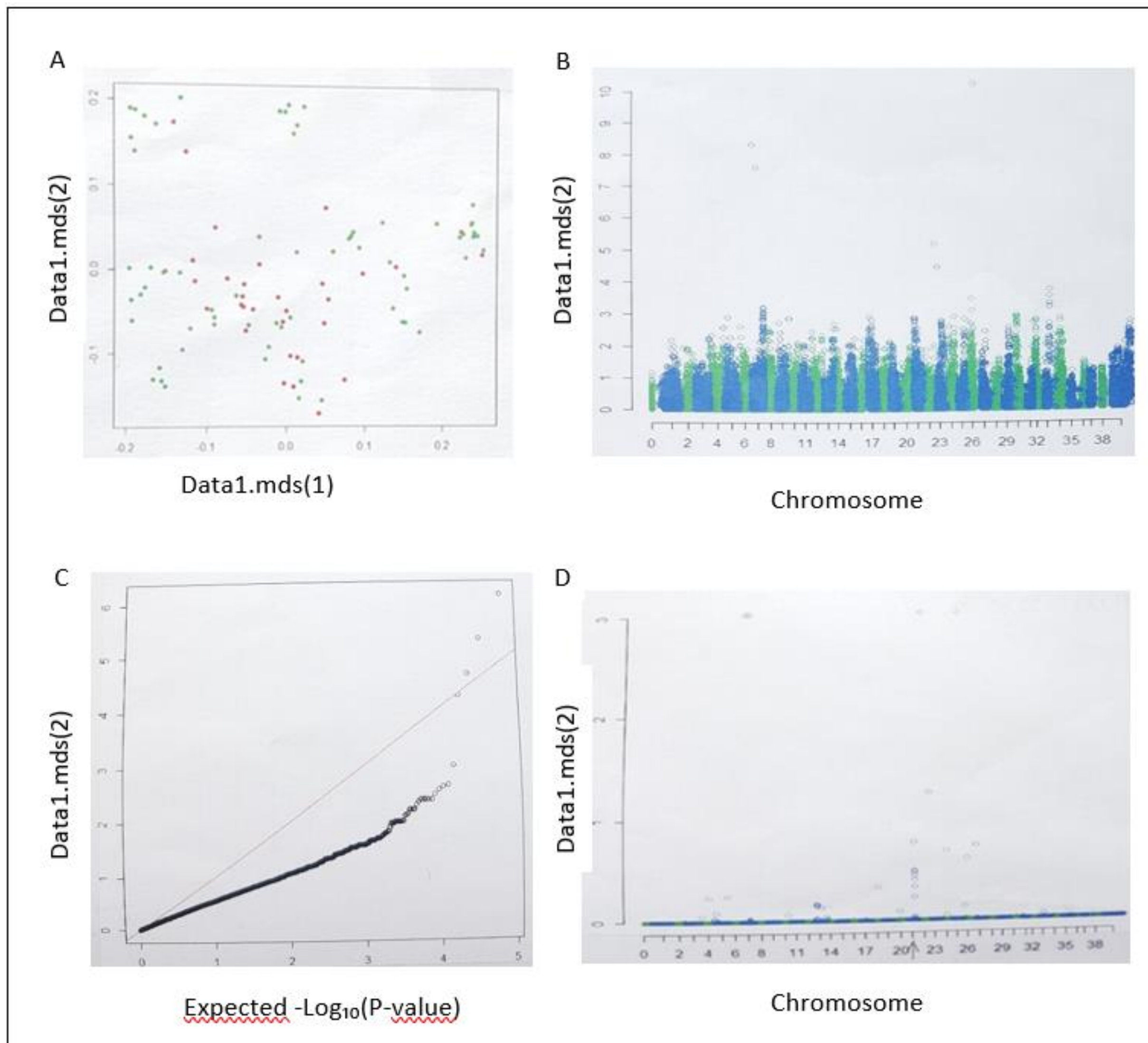

Figure 2: Data analysis of SNPs corrected for population and multiple testing. A, MDS plot: green dots being controls; red dots being cases. B, Qtscore (Manhattan plot) uncorrected. C, QQ plot. D, Qtscore (Manhattan plot), corrected for familly connections and multiple testing

Table 5: 50 Most significant SNPs as found after GENable software analysis based on chi-square test

	Chromosome	Position	Strand	A1	A2	effB	se_effB	chi2.1df	P1df	Pc1df	effAB	effBB	chi2.2df	P2df
chr5_79913933	5	79913933	+	C	A	0.022844668	0.004381724	27.18189617	0.01	0.000999001	0.023868415	0.037534657	27.427444	0.241
BICF2P778583	14	14419653	+	C	A	0.025017437	0.005065462	24.39201016	0.028	0.000999001	0.025017437	NA	24.39201016	0.433
BICF2G630737393	31	21456154	+	G	C	0.020579395	0.004640051	19.67069771	0.156	0.000999001	0.020579395	NA	19.67069771	0.962
BICF2P76108	9	34569467	+	C	T	0.017936628	0.004551592	15.52940661	0.58	0.001	0.017936628	NA	15.52940661	0.997
chr6_28159863	6	28159863	+	C	A	0.026632625	0.007792563	11.68065867	0.97	0.028	0.026632625	NA	11.68065867	1
TIGRP2P253788_rs8882281	18	44493641	+	T	C	0.021384251	0.006813296	9.850845941	0.999	0.115	0.021384251	NA	9.850845941	1
BICF2P768779	6	44406077	+	C	G	0.019815613	0.006461851	9.403752405	0.999	0.155	0.019815613	NA	9.403752405	1
BICF2P1342564	3	82148878	+	C	T	0.018689085	0.006375139	8.594028298	1	0.294	0.018689085	NA	8.594028298	1
BICF2G630639786	21	52222964	+	C	T	0.012333072	0.00435127	8.033606282	1	0.416	0.012333072	NA	8.033606282	1
TIGRP2P288741_RS8613277	21	52417122	+	G	T	0.011968368	0.004292458	7.77423656	1	0.483	0.011968368	NA	7.77423656	1
BICF2P885972	3	82143098	+	G	A	0.018009743	0.006472586	7.742111341	1	0.489	0.018009743	NA	7.742111341	1
TIGRP2P53292_RS8747144	3	82171660	+	T	G	0.018009743	0.006472586	7.742111341	1	0.489	0.018009743	NA	7.742111341	1
BICF2P59474	31	4854400	+	C	T	0.02328242	0.008381227	7.716866894	1	0.496	0.02328242	NA	7.716866894	1
TIGRP2P36875_rs8966525	3	7948282	+	A	T	0.02165421	0.007798359	7.710424026	1	0.498	0.02165421	NA	7.710424026	1
BICF2S23146586	21	52250889	+	T	C	0.012057438	0.004341943	7.711554122	1	0.498	0.012057438	NA	7.711554122	1
BICF2P1489932	21	52281183	+	G	T	0.012057438	0.004341943	7.711554122	1	0.498	0.012057438	NA	7.711554122	1
BICF2P815291	21	52331733	+	C	T	0.012057438	0.004341943	7.711554122	1	0.498	0.012057438	NA	7.711554122	1
BICF2P897184	21	52386044	+	A	G	0.012057438	0.004341943	7.711554122	1	0.498	0.012057438	NA	7.711554122	1
BICF2G630336509	22	57409740	+	A	C	0.011032399	0.004131542	7.130429303	1	0.655	0.007920886	0.03361674	9.244700635	1
BICF2G630396964	30	39453327	+	A	G	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
BICF2G630396765	30	39730030	+	A	G	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
BICF2S23526858	30	39741233	+	T	C	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
BICF2P649394	30	39759464	+	C	T	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
BICF2G630396597	30	39905583	+	A	G	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
BICF2G630394445	30	42220251	+	T	C	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
BICF2P535001	31	4650069	+	T	C	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
TIGRP2P375316_RS8865135	31	4792296	+	T	C	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
BICF2P347614	31	4833369	+	A	G	0.022677634	0.008497113	7.122831741	1	0.655	0.022677634	NA	7.122831741	1
chr28_24512648	28	24512648	+	G	A	0.01457154	0.005509338	6.995392115	1	0.692	0.01457154	NA	6.995392115	1
chr20_44931643	20	44931643	+	T	G	0.010790071	0.004088578	6.964725373	1	0.697	0.010790071	NA	6.964725373	1
BICF2P1133187	22	59529325	+	G	T	0.008209631	0.003146298	6.808436954	1	0.74	0.010195438	0.013430399	7.206735625	1
BICF2P285889	22	59553280	+	G	A	0.008209631	0.003146298	6.808436954	1	0.74	0.010195438	0.013430399	7.206735625	1
BICF2G630571722	6	77131288	+	C	T	0.010846377	0.004237367	6.552051906	1	0.798	0.010846377	NA	6.552051906	1
TIGRP2P288604_RS8796184	21	52119768	+	G	T	0.007121302	0.002785357	6.536676611	1	0.8	0.009669948	0.012368179	7.300752674	1
BICF2P587940	21	52371227	+	T	C	0.007121302	0.002785357	6.536676611	1	0.8	0.009669948	0.012368179	7.300752674	1
TIGRP2P359062_rs8799036	28	24775643	+	G	T	0.009000552	0.003520494	6.536286988	1	0.801	0.013768992	0.00132114	10.53494792	1
BICF2P1112552	28	24847275	+	C	T	0.009000552	0.003520494	6.536286988	1	0.801	0.013768992	0.00132114	10.53494792	1
chr28_24873198	28	24873198	+	C	T	0.009000552	0.003520494	6.536286988	1	0.801	0.013768992	0.00132114	10.53494792	1
BICF2G630595184	32	20059452	+	C	G	0.008129194	0.003208682	6.418618734	1	0.83	0.004586904	0.024142256	8.884101013	1
BICF2G630595309	32	19796756	+	G	A	0.008149793	0.00322734	6.376811527	1	0.84	0.004579643	0.024187127	8.866616244	1
BICF2P189412	22	47969392	+	G	A	0.006417443	0.002545292	6.356951322	1	0.841	-0.001883627	0.01328412	11.5713442	1
TIGRP2P359283	28	24685489	+	T	C	0.008795472	0.003498837	6.319327739	1	0.845	0.013310816	0.001335705	10.08024002	1
BICF2P517696	7	78580067	+	T	C	0.011600721	0.004616882	6.313531854	1	0.849	0.002711902	0.083606121	20.25915439	0.931
TIGRP2P309375_rs8890982	23	2216415	+	C	T	0.006120634	0.002440084	6.291917771	1	0.857	0.003107852	0.013800927	7.470843911	1
BICF2G630596458	32	18576836	+	G	A	0.006969795	0.002791553	6.233733434	1	0.87	0.006359312	0.014242082	6.275149965	1
chr20_44784830	20	44784830	+	C	T	0.007453614	0.002994296	6.196468187	1	0.875	0.007965409	0.014072385	6.233462398	1
BICF2P881894	4	4933612	+	C	G	0.008161137	0.00330354	6.102984179	1	0.898	0.014348925	-0.001791068	12.33811565	1
BICF2G630276267	28	12044891	+	G	A	0.013280254	0.005388345	6.074380553	1	0.903	-0.000150492	0.083405229	20.16393618	0.938
BICF2P34121	22	47989485	+	C	A	-0.007055513	0.002901082	5.914759082	1	0.926	-0.008322266	-0.012388242	6.112742325	1
BICF2P429699	21	51568652	+	C	T	0.006675361	0.002750981	5.88809075	1	0.934	0.009905156	0.012204009	6.886364815	1

Table 6: Cohort results sequencing BICF2G630550898.

	Cases	Controles	Total
AA	14	13	27
AC	21	31	52
CC	6	16	22
Total*	41	60	101

*Samples lost to follow up not included (n=4: 3 cases, 1 control)

Table 7: Allele frequency BICF2G630550898

	Cases	Controles
A	0.598	0.475
C	0.402	0.525

50 most significant SNPs are shown in the table below (Table 5).
After running this SNP through the KASP PCR, the results turned out to be inaccurate. Most of the negative controls were positive, while the samples were mixed and spread all over the plot. This led to the conclusion that the results were unreliable. Consequently, a switch was made to sequencing.

Table 8: Cohort results sequencing BICF2S23146586.

	Cases	Controles	Total
CC	24	74	98
CT	14	14	28
TT	0	0	0
Total*	38	88	126

*Samples lost to follow up not included (n=8: 6 cases, 2 controls)

Table 9: Allele frequency BICF2S23146586

	Cases	Controles
C	0.816	0.920
T	0.184	0.080

First BICF2G630550898 was sequenced (Table 6). The accompanying calculated allele frequency showed an almost equal distribution of both alleles in both cohorts (Table 7).
The results showed that the P-value was 0.077. Therefore, this SNP was found to be not significant and consequently leading to sequencing of the second SNP BICF2S23146586 (Table 8 and 9).

The second SNP showed a significance with P=0.0005. In both cohorts the allele frequency showed a marked difference in allele distribution. After finding the SNP variation to be significant, attention was turned to the genes situated on and surrounding the SNP. The following genes were found: ANO3, BBOX1, BDNF, CCDC34, FIBIN, FSHB, GIF, KIF18A, LGR4, LIN7C, MRPL16, MS4A1, MS4A2, MS4A5, MS4A7, MS4A14, MUC15, SLC5A12, STX4 and TCN1. Using the keywords ‘bone’ and ‘cancer’, CCDC34, LGR4 and MUC15 were found. LGR4 was selected as the most promising gene.
The LGR4 gene was then sequenced with a standard annealing temperature of 55ºC. While making the PCR product visible, this temperature turned out to be not optimal for all exons. For exon 2-4 and 6-15 an annealing temperature of 55ºC did seem to be satisfactory. However, for 1, 5, 16, 17 and 18 this was not the case. After repetitive primer optimization (annealing temperatures were run at 55ºC, 60ºC, 65ºC and 75ºC), no optimal temperature for exon 1 was found. Based on the continuing appearance of multiple bands, the conclusion was reached that the primers were not specific enough. After the Q5 PCR was also unsuccessful, further research on exon 1 was not attempted due to a limited time frame. Exon 5, 16, 17 and 18 were run at 60ºC. However, the sequences of exon 18 were inexplicably illegible, therefore no results could be reached.
A number of variations were found in the sequencing results (Table 10).

Table 10: LGR4 gene variations

Exon	Position	Origin	mutation
2	g.21:47995407T>C	T	C
	IVS1+9	T	A
4	g.21:47977601T>A	T	A
	g.21:47977438C>A	C	A
5	IVS5	T	C
	g.21:47969745T>C	T	C
11	g.21:47963391A>G	A	G
	IVS10+36		Insertion G
13	g.21:47961306C>A	C	A
	g.21:47961308C>A	C	A
	IVS13+3		Insertion T
	IVS13+4		Insertion T
	IVS13+12	A	G
16/17	IVS16+19	G	A
	IVS17+4	T	C
	IVS17+20	T	G

*IVS: InterVening Sequence

The variation on exon 2 g.21:47995407T>C led to a protein change from Phenylalanine (Phe) to Leucine (Leu). The variation in exon 5 g.21:47969745T>C led to a change from Methionine (Met) to Threonine (Thr). Both changes were predicted by software tool Polyphen-2 as being benign. However, the variation found on exon 4 g.21:47977601T>A was predicted to result in loss of the next splice site on the gene.

Discussion

Developing osteosarcoma is one of the main reasons why Irish wolfhounds have a limited life span. Few dogs live to the age of 10 or beyond, the average lifespan being 6 to 8 years. In the last 30 years the progression of therapeutic possibilities and the improvement of survival-rate in canine and human patients has been disappointing.
The narrow gene base of the Irish wolfhound and the frequent occurrence of osteosarcoma has led to research focusing on the genetic background of osteosarcoma in the breed.[24-26] The aim of this study was to develop more insight into the genetic background of osteosarcoma in the Irish Wolfhound by identifying relevant genetic factors. To accomplish this aim 170.000 SNPs went for analysis and after identifying promising SNPs multiple PCR’s testes were ran to check for variations between case and control groups and correlations with osteosarcoma. The results showed a significant SNP housing the LGR4 gene. The LGR4 gene displayed multiple variations.

At the time of this study the American study by Karlsson et al. (2013) was the only genome wide association study to evaluate osteosarcoma in dogs. Running a GWAS analysis on 170.000 SNPs from three large dog breeds (334 Greyhounds, 166 Rottweilers and 174 Irish wolfhounds). Thirty-three loci were found to be associated with osteosarcoma but only 4 were found in the Irish wolfhound samples.[29] When comparing to these results obtained in this study, the 4 SNPs found by Karlsson do not appear in the top 50 significant SNPs found in our GWAS analysis. This would explain why the Karlsson Manhattan plot did not show any peaks similar to those produced in this study. This finding might be due to possible genetic differences between American and European Irish wolfhounds. More recently (published after the conclusion of this study) Zapata et al. (2019) published a similar study using and with the aim of expanding on the data reported by Karlsson et al. (2013).[43] The reported SNPs found are again absent from our list. This as well could be due to the same reason as Karlsson et al.(2013).
For the first SNP to be analyzed Komparative Allele Sensitive PCR (KASP) technique was first used. Although normally the KASP is a

good technique to genotype due to having a high specificity, sensitivity and high throughput, in this case the method was not effective. In various ways, an attempt was made to find the cause of failure of the KASP procedure. In order to rule out contamination the PCR was repeated on the same plate with a different disease running alongside. The results of the control disease (Von Willebrand) were correct but the osteosarcoma samples and their controls were faulty once again. This ruled out incorrect laboratory procedures. The primers are thought to be possibly implicated.

Following the well-known Hardy Weinberg principle ($p^2+2pq+q^2=1$), a normal population would contain an expected genotype frequency of $q^2= 0.25$, $pq= 0.50$ and $p^2=0.25$. Therefore, the sequencing results would be expected to show an equal amount of both alleles (allele frequency being 0.50 for p and q each).[44] In table 10, the sequencing result of the non-significant SNP reflects the almost equal distribution of both alleles. It shows the situation expected in an almost ideal population and is therefore deemed to be not significant. This was confirmed by calculating the P-value based on table 6.
However, sequencing of the second SNP resulted in a striking finding. As is shown in table 8, there is a marked difference between the cases and controls, leading to significant P-value distinctly below 0.05. This is supported by the allele frequency table (table 9) supported this, as there is an unmistakable imbalance in the distribution of both alleles in both cohorts.
After conducting an internet search the following candidate genes were selected: CCDC34, LGR4 and MUC15. As LGR4 was the only gene with a connection to the key-words this gene was chosen. CCDC34 and MUC15 were connected to other types of cancer than the Irish wolfhound was prone to having. Therefore these are not pursued any further in this investigation. LGR4 activates the Wnt signaling pathway, which directly plays a significant role in both benign and malignant bone growth and regeneration and is known to be associated with osteosarcoma development.[40,41] Interestingly enough, although Selvarajah et al (2009) reported a correlation between the Wnt signaling pathway and aggressive osteosarcoma phenotype, LGR4 was not a gene to be presented as overexpressed in their study.[31] This could be because of other genetic factors influencing the Wnt signaling pathway or possibly due to the fact that Selvarajah et al. (2009) did not include Irish Wolhounds in the study. It is possible LGR4 is a gene related to osteosarcoma development specifically in Irish Wolfhound.
After selecting and sequencing the LGR4 gene, a number of variations was found (table 10). The two variations named (exon 2 g.21:47995407T>C and exon 5 g.21:47969745T>C) led to a change in proteins that have been predicted as benign by the Polyphen-2 software tool. However, this tool only produces a prediction of the possible impact of the mutation on the structure and the function of a protein. Therefore, it cannot be said with any certainty that these variations will not have any influence on the biological behavior of genetic components.
One of the other variations (exon 4, g.21:47977601T>A) may lead to splice site loss which could lead to a change in genetic material. This change could be particularly interesting, as this variation may lead to a wide spectrum of possibilities regarding the change in genetic material and thereby the change in gene product. The effects of other variations mentioned in Tabel 10 are still unknown. Additionally variations can be seen in both case and control groups. Therefore, the dogs in the control group showing these variations deserve further future investigation to investigate whether these dogs have developed osteosarcoma over time.

In studies on canine osteosarcoma the case and control groups include relatively small numbers of dogs. This makes it more difficult to assign weight to significant findings on canine osteosarcoma in research. Progress has been slow to determine the genetic origins. It is of the utmost importance to develop and maintain a complete and up to date database. Luckily some breeders support this research to the extent that they only sell their pups with two examinations (at 2 and 4 years of age) by Dr. Andrea Vollmar, included in the selling price. The samples taken are then incorporated into the Hannover and Utrecht University databases. Despite collection of samples on an international scale our case and control groups remain fairly small and no sample size calculation was performed. Therefore it is unclear whether there is enough statistical power behind this study and if the results are reliable as this limitation could result in a type II error.[45]
Apart from the size of the cohorts being relatively small, it is also possible that our control group harbors false negatives due to a number of factors. For example, information concerning the development of osteosarcoma may not have reached the database. On the

other hand, dogs may have died due to other causes, thereby not reaching the age at which they might otherwise have developed osteosarcoma. By using a cut-off age of six years old, an attempt was made to minimize this problem.
The implications of the variations are still unknown. Understanding the ramifications could lead to a better understanding of osteosarcoma development. Future research conducted with larger study populations would be more ideal.

Conclusion
This study screened 44 cases of osteosarcoma and compared these to 90 control dogs. A significant SNP was found, containing a gene potentially related to osteosarcoma. Furthermore, multiple exon variations were found in this gene that possibly play a role in the development and progression of osteosarcoma. The extreme complexity of this disease is emphasized by the fact that multiple genes and pathways have been found over the years, now also including the multiple variations found in this study. Due to this complexity it is difficult to determine the exact role of any given genetic component(s) responsible for the development of osteosarcoma in the Irish wolfhound. Interspecies research could be mutually beneficial for both canine and human patients and lead to accelerating progression in this field.
It is concluded that further research and understanding into the genetic underpinnings of osteosarcoma is required to improve the lifespan of this short-lived breed.

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Conflicts of interest
None.

Ethics approval and consent
All data available at the start of this study was collected from a pre-existing data-base and independently collected. Therefore no Ethics committee was approached.

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