The genetic defect of fragmented coronoid process in Labrador Retrievers and other skeletal diseases in dogs

Jedee Temwichitr

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Moleculair genetisch onderzoek van los processus coronoïdeus bij Labrador Retrievers en andere skeletafwijkingen bij de hond

(met een samenvatting in het Nederlands)

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Part I Fragmented coronoid process in Labrador Retrievers

Chapter 1

Aim and scope of the thesis

Introduction

A number of developmental skeletal diseases have been described in companion animals, with most occurring with a high incidence in particular breeds of dogs, although some of these diseases can be caused by trauma and dietary aberrations (Hazewinkel, 1993; LaFond et al., 2002; Breur et al., 2001). Dog breeds have closed gene pools, so that dogs have shared characteristics (Patterson, 2000; Mayers-Wallen, 2003), such as behavior and physical appearance, i.e., size, shape and hair coat (Patterson, 2000; Sutter and Ostrander, 2004). The skeletal phenotype is strongly influenced by an animal's genetic make-up (Breur et al., 2001), and for this reason developmental skeletal diseases with a high incidence in particular breeding lines are considered to be hereditary, although additional environmental influences, including dietary constituents, cannot be ruled out. Hereditary skeletal developmental diseases in dogs with a known genetic basis include fragmented medial coronoid process (FCP) in Labrador Retrievers, Rottweilers, Bernese Mountain dogs, and other breeds (LaFond et al., 2002); chondrodysplasia in Alaskan Malamutes (Fletch et al., 1973), Scottish Deerhounds (Breur et al., 1989), and Labradors (Sande and Bingel, 1982); and hereditary elbow luxation in Miniature Poodles and Skye terriers (Bingel and Riser, 1977; Lau, 1977). FCP is the most important elbow dysplasia and affects 17% of the Labrador dog population and up to 50% of the Bernese Mountain dog population in the Netherlands (Ubbink et al., 1999; 2000). Chondrodysplasia and hereditary luxation of the elbow joint are disabling skeletal diseases with a low incidence and are accompanied by a disproportionately short stature. Possibly, collagen abnormalities have a role in both diseases.

Most genetic diseases of skeletal development become apparent at a time of accelerated skeletal growth, for example, between 4 and 6 months of age in dogs. The late manifestation of these diseases means that affected animals have already left the breeder and gone to their new owners. In this way, the trait can be spread in the population before it is noticed by breeders and veterinarians. As a consequence, genetic research into developmental diseases in dogs necessarily involves the cooperation of dog owners and breeders, veterinarians, and veterinary practices. Disease pedigrees can be drawn with the help of breeders clubs, and the incidence of specific traits can be investigated in representative cohorts of animals (Ubbink, 1998). However, it should be borne in mind that diseases with a recessive mode of inheritance with varying penetrance or which require specific environmental conditions for their manifestation may go unnoticed in disease-free animals.

Aim and scope of the thesis

Identification of the gene or genes responsible for a disease provides not only insight into disease pathogenesis but also a means to trace animals carrying the relevant gene or genes. The ability to trace carriers will facilitate disease detection and prevention, making it possible to intervene in an early stage of breeding.

The general concept that the physical characteristics and pathobiology of FCP, chrondrodysplasia, and hereditary elbow luxation share a common etiology prompted us to investigate the basic defect in these orthopedic diseases. This thesis is divided into two parts. Part I describes the investigation of FCP in Labrador Retrievers. **Chapter 2** provides an overview of different aspects of FCP, including the pathobiology of FCP and strategies for genetic studies to identify the gene or genes responsible for FCP. The aim of the study described in **Chapter 3** was to evaluate the involvement of various collagen genes in the development of FCP in Labrador Retrievers. We carried out a sibling-pair analysis in FCP families and expected to find collagen marker alleles shared by FCP-affected siblings. In the study described in **Chapter 4**, we attempted to localize the causative genes of FCP on the canine genome. To this end, we performed a genome-wide scan for FCP gene(s), using different sets of markers, with subsequent fine mapping of potentially relevant loci.

In the studies described in part II of this thesis, collagen markers were developed to assess the involvement of collagen abnormalities in FCP in Labrador Retrievers, and then these markers were tested in other collagenopathic diseases. The aim of the study described in **Chapter 5** was to develop markers that could be used to evaluate collagenopathic diseases, including FCP. We evaluated polymorphisms of these markers in different diseases and breeds, namely, cruciate ligament rupture (a suspected collegenopathic disease) in Boxers. We then investigated the involvement of these collagen markers in chondrodysplasia in Labrador Retrievers in the study described in **Chapter 6**. The aim of the study described in **Chapter 7** was to gain insight into the pathogenesis of the elbow abnormality in Bouviers des Flandres. To this end, we investigated the radiographic and genetic features of hereditary subluxation of the radial head in these dogs. Knowledge of the pathogenesis may help us to understand the disease mechanisms underlying FCP. The findings of these studies are integrated and discussed in **Chapter 8** and summarized in **Chapter 9**.

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Chapter 2

Fragmented medial coronoid process, a heritable disease

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Abstract

Fragmented coronoid process (FCP) is one of the main diseases associated with elbow dysplasia. FCP is often diagnosed in medium-to-large breed dogs with front leg lameness, for instance in Rottweilers, Labrador Retrievers and Bernese Mountain dogs. Dogs with FCP develop osteoarthrosis of the elbow joint despite conservative or surgical treatment. Although FCP is considered a hereditary condition, the gene or genes causing FCP have yet to be identified. This article provides an overview of different aspects of FCP, including elbow joint development, hypotheses about disease pathogenesis, the genetic background of FCP, and genetic methodology to identify gene or genes responsible for FCP.

Introduction

Elbow dysplasia, a common problem in dogs (Table I), is a syndrome in which one or more of the following conditions is present: fragmented medial coronoid process (FCP) of the ulna, osteochondritis dissecans (OCD) of the humeral condyle, ununited anconeal process (UAP), and incongruity of the elbow joint (INC). FCP is the most frequently diagnosed form of elbow dysplasia in growing dogs of different breeds (Grøndalen and Grøndalen, 1981; LaFond et al., 2002; OFA, 2009). The first clinical signs of lameness due to FCP usually occur at 4-6 months of age (Presnel, 1990), but can occur as late as 6-8 months, or even much later (>6 years). Clinical signs of FCP include exorotation of the affected legs, moderate joint distension, crepitation during movement, and decreased range of motion of the elbow joint in advanced cases (Hazewinkel et al., 1988).

The elbow joint is traditionally evaluated radiographically, using five views: a standard craniocaudal view, a craniocaudal oblique view, a mediolateral view one with the elbow extended and one with the elbow flexed, and a mediolateral view with the elbow extended and the antebrachium supinated at 15^{o}. The latter two are regarded as best for visualizing the medial coronoid process (Voorhout and Hazewinkel, 1987; Miyabayashi et al., 1995; Kirberger and Fourie, 1998; Wosar et al., 1999), while the other views are useful for detecting other forms of elbow dysplasia and the signs of osteoarthritis. In FCP, osteoarthritis develops due to medial joint instability and chronic irritation of the affected elbow joint (Grøndalen, 1979c). Computed tomography enables better visualization of the FCP than does radiography and is used to confirm the clinical diagnosis (Carpenter et al., 1993; Tromblee et al., 2007). The sensitivity of computed tomography is 88.2% for FCP and coronoid lesions (Carpenter et al., 1993). Magnetic resonance imaging (MRI) can also be used to confirm the occurrence of FCP and has a sensitivity of 83.3% for coronoid lesions and up to 100% for non-attached coronoid process (Snaps et al., 1997).

Arthroscopy has been developed as a diagnostic and therapeutic tool for elbow diseases (Van Ryssen and van Bree, 1997; Meyer-Lindenberg et al., 2006; Fitzpatrick et al., 2009) and is regarded as gold standard for the detection of cartilage defects (Moores et al., 2008). Its use leads to earlier detection of FCP lesions than is obtained with plain film radiography or computed tomography (Van Ryssen and van Bree, 1997). Arthrotomy or arthroscopy is used for the definitive diagnosis and surgical treatment of FCP (Evan et al., 2008), with conservative treatment being advocated if FCP does not cause lameness (Houlton, 1984). Surgical removal of the FCP leads to progressive improvement in dogs with FCP-induced lameness (Theyse et al., 2000; Samoy et al., 2006). The treatment prognosis depends on the extent of joint damage

caused by the FCP, although osteoarthritis will develop even with early removal of the FCP (Theyse et al., 2000). For this reason, preventive measures are needed.

Although FCP is a genetic disease (Guthrie and Pidduck, 1990; Grøndalen and Lingaas, 1991; Studdert et al., 1991), its pathogenesis has yet to be determined. Identification of the genes involved could establish the pathogenesis and make it possible to develop diagnostic tools. In this article, the developmental biology of the elbow joint is described, followed by an overview of the possible pathogeneses of FCP and the results of genetic studies.

Table I The incidence of ED in different breeds, according to OFA and others.

Rank	Breed	Percentage of dysplastic elbow (OFA)	Percentage of ED in other studies	References
1	Chow Chow	46.6	47.4%	Kirberger and Stander, 2007
2	Rottweiler	40.8	33-54.7%	Grøndalen and Lingaas 1991; Swenson et al., 1997; Kirberger and Stander 2007; Coopman et al., 2008
3	Bernese Mountain dog	29.1	20-45%	Grøndalen and Lingaas 1991; Swenson et al., 1997; Ubbink et al., 1999; Kirberger and Stander 2007: Coopman et al., 2008
6	Newfoundland	24.7	20-33%	Grøndalen and Lingaas 1991; Kirberger and Stander 2007; Coopman et al., 2008
7	Fila Brasileiro	23.5		
8	German Shepherd dog	19.4	12-22%	Remy et al., 2004;Janutta et al., 2006; Coopman et al., 2008
9	Dogue De Bordeaux	19.3		
10	American Bulldog	18.6		
11	St. Bernard	18.5		
13	Staffordshire Bull Terrier	15.7	31.3%	Kirberger and Stander, 2007
14	Bloodhound	15.7		
19	English Springer Spaniel	13.5		
20	Shiloh Shepherd	13.0		
23	Iris Wolf Hound	11.9	16.7%	Kirberger and Stander, 2007
24	Greater Swiss Mountain dog	11.6		
26	Golden Retriever	11.4	14.5-38.3%	Kirberger and Stander, 2007; Coopman et al., 2008
27	Labrador Retriever	11.2	13-20.6%	Ubbink et al., 2000; Kirberger and Stander, 2007; Coopman et al., 2008

The table shows the incidence of elbow dysplasia in rank order, adapted from Orthopedics Foundation for Animal elbow statistics (Elbow Dysplasia Statistics, OFA, 1994-2007), and references as indicated.

Growth and development of the elbow joint

The ulnar bone forms a smooth concave trochlear notch that glides along the humeral trochlea when the elbow extends. The trochlear notch flares at the lower end and is

more prominent at the medial coronoid process than at the lateral side. In addition, the ulnar bone forms a concave joint surface with the radial head, allowing for antebrachial supination and pronation (Evans and Christensen, 1979). The radial head and the lateral ulnar coronoid process articulate with the capitulum humeri, whereas the medial ulnar coronoid process and part of the radial head articulate with the trochlea humeri (Figure 1). Both radius and ulna bear the weight of the dog, with up to 50% borne by the medial and lateral coronoid processes and the rest by the radial head (Berzon and Quick, 1980; Presnell, 1990; Mason et al., 2005).

The limb bud is recognizable at 27 days post coitum (p.c.) in Beagle dog embryos (Anderson, 1970). Endochondral ossification starts in the mid-diaphysis, and cartilage proliferation starts at 35 days p.c.. To date, little is known about the genes responsible for the appearance and development of the radius and ulna, whereas a number of genes are known to be involved in limb bud development. In mice, a fibroblast growth factor 10 (FGF10), which is expressed in mesenchymal cells, induces FGF8 in the surface ectoderm layer to initiate limb buds and to sustain their outgrowth. In turn, FGF8 promotes mesenchymal cell proliferation, mediated by fibroblast growth factor receptor 2 (FGFR2), thereby sustaining FGF10 during limb bud elongation (Xu et al., 1999).

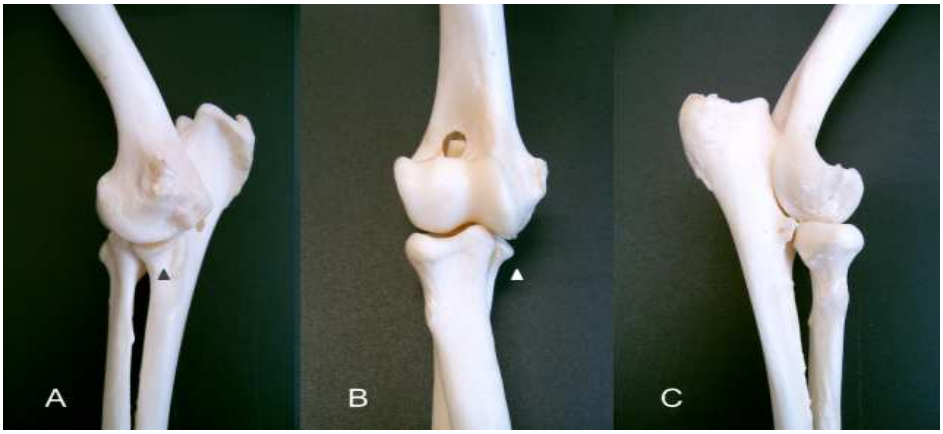

Figure 1. Anatomy of elbow joint.
The pictures show that the radius articulates with the lateral humeral condyle on top and with the coronoid process on both sides. Fig 1A, arrow head indicates apex of medial coronoid process which can be seen as sclerosis. Fig 1B, osteophyte can be seen at arrow point where the humero-ulnar articulation is. Fig 1C, the lateral view of elbow joint is illustrated.

At birth, ossification of the diaphysis has extended to the metaphysis, whereas the epiphyses of the long-bones have yet to ossify (Anderson et al., 1970). Secondary ossification centres in the proximal radius and ulna can be seen on radiographs at 21-35, and at 35-64 days of life, respectively (Voorhout and Hazewinkel, 1987; Olsson and Ekman, 2002). The growth plates of the proximal radius, olecranon and anconeal

process close at the age of 20-44, 20-40 and 12-20 weeks, respectively (Dyce et al., 2002; Olsson and Ekman, 2002). The coronoid process is not a secondary ossification centre and growth occurs by interstitial growth of the cartilaginous anlage (Olsson and Ekman, 2002). Ossification of the cartilaginous template of the medial coronoid process occurs via endochondral ossification from the base towards the tip, except for the articular cartilage layer. The metaphysis is composed of trabecular bone and is the most metabolically active portion of the growing bone. The orientation of the trabeculae in the metaphyseal area reflects the direction of maximum stress exerted on the bone and can change under the influence of mechanical forces acting on the bone (Summerlee, 2002). Ossification of the coronoid process is complete at 16-20 weeks of age; it is completed earlier in small breed dogs than in large breed dogs (Breit et al., 2004). Remodelling of the trabeculae of the ossified coronoid process occurs as a result of loading of the humero-ulnar joint and radio-ulnar joints and of stress exerted by the annular ligament attached to the process. These forces change during the early postnatal period (4 to 24 weeks of age) (Wolschrijn and Weijs, 2004). Joint cartilage is formed during the initial stage of joint development and is not renewed during life, unlike subchondral bone, which has a regular turnover (Roughley, 2001; Eyre, 2002).

Pathogenesis of FCP

There are different hypotheses concerning the pathogenesis of FCP and most of them involve abnormal endochondral ossification or abnormal mechanical forces. These possible causes are described below.

Disturbed development of endochondral ossification

Olsson (1977) and Tirgari (1974) suggested that FCP is caused by a disturbance of endochondral ossification of the medial coronoid process, and thereby proposed that OCD and FCP are members of the same group of developmental bone diseases. The cartilaginous template of the coronoid process, like growth plate cartilage, matures as a result of endochondral ossification (Ekman and Carlson, 1998). The process includes chondrocyte division, maturation, and apoptosis with matrix maturation, mineralization, and ultimately ossification. If endochondral ossification does not proceed normally, osteochondrosis, including OCD and FCP, may occur. This aetiology is supported by the high incidence of concurrent OCD and FCP seen in dogs (Grøndalen, 1979a, 1979b; Mason et al., 1980; Hazewinkel et al., 1988; Studdert et al., 1991; Wolschrijn et al., 2005). Chondromalacia (i.e., abnormal softening and degeneration of the cartilage), which is occasionally detected at the site of the medial coronoid process (Van Ryssen and van Bree, 1997), also provides support for FCP being a disturbance of endochondral ossification.

Abnormal bone

Abnormality of trabecular bone

Danielson et al. (2006) reported that the histomorphometric abnormalities seen in the FCP lesion apparently originate in the subchondral trabecular matrix, not in the covering layer of cartilage, as seen in osteochondrosis. Microscopy of the medial coronoid process from a patient with FCP revealed diffuse fatigue microdamage in trabecular subchondral bone, including loss of osteocytes and increased porosity of the trabecular bony matrix. These changes are possibly the result of force-related abnormalities that lead to fracture of subchondral bone and cartilage and ultimately to fragmentation of the medial coronoid process.

Sclerosis of subchondral bone

Sclerosis of subchondral bone, seen on radiographs as an area of dense opacity at the trochlear notch, is one of the first signs of FCP (OFA, 2009; IEWG, 2009). The proportion of bone in trabecular structures is higher in dogs with FCP than in normal dogs of the same age (Wolschrijn and Weijs, 2004). Sclerosis may be due to an imbalance, of unknown origin, in the rates of bone apposition and resorption or to an increased rate of endochondral ossification during the development of cartilage and bone. Dequeker et al. (1995) have suggested that the stiffness or density of subchondral bone may influence its deformability in response to impact, which in turn would make cartilage more vulnerable to damage as more force is transmitted to the overlying cartilage. These authors concluded that primary osteoarthritis is initiated by subchondral bone stiffness rather than by primary cartilage damage. Similarly, sclerosis of subchondral bone in the coronoid area may lead to fissures and fractures of the joint cartilage in this area, ultimately causing fragmentation of the medial coronoid process.

Mechanobiology of FCP

These possible aetiologies are supported by findings regarding the origin of forces acting on the medial coronoid process.

Tension forces arising from the annular ligament

Wolschrijn and Weijs (2004) suggested that the tensile force arising from the annular ligament could cause an avulsion fracture of the medial coronoid process because at an age when fragmentation occurs (i.e., 4 months of age), the trabeculae along the cranio-caudal axis have the same orientation as the annular ligament. Similar observations have been made in the human elbow joint, where the sagittal orientation of trochlear notch trabeculae reflects a functional adaptation to tension and bending forces acting on the elbow joint (Eckstein et al., 1999). This possible aetiology is supported by the

frequent observation, during surgical removal of the FCP, of the coronoid process attached to the annular ligament.

Force arising from incongruity of radius, ulna and humeral condyle

A. Pressure force due to incongruity of the ulnar trochlear notch

A typical form of incongruence of the elbow joint is the elliptical semilunar notch, which is thought to be a possible cause of FCP and UAP. This incongruity is the consequence of abnormal development of the trochlear notch of the ulna (Wind, 1986). The oval shape of the trochlear notch does not properly fit around the humeral condyle, which could lead to the development of abnormal forces at the coronoid and anconeal processes. The observation that FCP and UAP often co-occur in the elbow joint supports this hypothesis (Tirgari, 1974; Hazewinkel and Voorhout, 1986; Meyer-Lindenberg et al., 2006).

B. Shearing force due to incongruity of radio-ulnar articulation

An incongruity of radio-ulnar articulation could cause FCP in many dog breeds, especially Labrador Retrievers, Golden Retrievers and Rottweilers. The FCP lesion often affects the lateral portion of the medial coronoid process rather than the apex of the coronoid process (Figure 2). This form of FCP can be considered as a chip fracture, where the radius forces a superficial part of the ulna at the radio-ulnar joint to detach. Since pronation and supination still occur in young dogs, the rotation of the radius could cause FCP, either alone or in combination with inadequate remodelling. Computed tomographic studies of growing dogs could provide insight into this possible aetiology.

C. Overload due to short radius

Presnel (1990) has suggested that a short radius might cause overloading of the medial coronoid process, leading to FCP. Preston et al. (2001) showed that incongruity between the proximal level of the radius and the ulna initiated by a short radius may lead to fragmentation along the radial facet of the medial coronoid process (Figure 2). Künzel et al. (2004) reported a similar finding and suggested that, in the case of a short radius, a weight-bearing force might cause fissure lines with the same pattern of alignment as subchondral bone.

A survey of Bernese Mountain dogs in the Netherlands in 1999 revealed that more than 70% of the dogs had elbow dysplasia due to FCP, and that 86% of these cases had a combination of FCP plus elbow incongruity (Hazewinkel and Ubbink, 1999). Other breeds, such as Labrador and Golden Retrievers, rarely have a short radius.

D. Pressure force due to long radius

Schulz (2006) hypothesized that, if the radius is relatively longer than the ulna, the humerus rotates around the anconeal process due to pressure of the radius against the capitulum humeri. Because of the rotational force, the medial coronoid process would be overloaded, resulting in its fragmentation. Although this hypothesis cannot be substantiated on the basis of radiological findings, it is worthwhile investigating further.

While these suggested pathogeneses are different, they all explain the occurrence of FCP. Although there is not detailed information in the veterinary literature about the different forms of FCP occurring in different dog breeds, clinical experience suggests that a short radius occurs mainly together with fragmentation of the apex of the medial coronoid process in Bernese Mountain dogs, fragmentation of the apex of the coronoid together with UAP occurs together with an elliptical ulnar notch in German Shepherd dogs and other breeds (Wind and Packard, 1986), and lateral fragmentation of the medial coronoid process is seen mainly in Rottweilers and Retrievers (Grøndalen and Grøndalen, 1981).

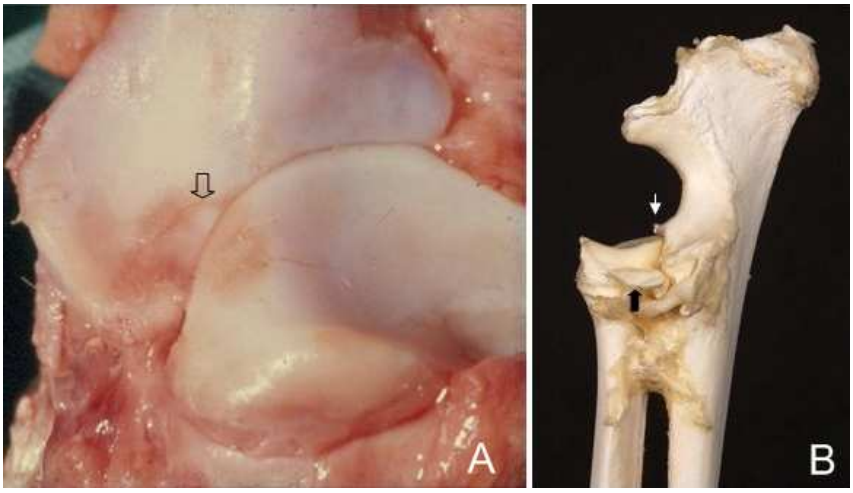

Figure 2. Lesions of FCP.
A fragmented coronoid process can be seen in different positions and characteristics, according to their possible pathogeneses. Figure 2A shows a FCP lesion (arrow) in the lateral portion of the medial coronoid process, believed to be the consequence of shearing force between radius and ulna. Figure 2B shows a lesion at the apical portion (black arrow) of the medial coronoid process, believed to be the consequence of a short radius, due to incongruity (arrow) of the elbow joint with a too long ulna.

Genetics of fragmented coronoid process

A breed can be considered a subgroup of the canine species (Parker et al., 2004) because favour characteristics have been selected by breeding among a small pool of dogs (Patterson et al., 1989). The existence of breed related diseases suggests there being a genetic cause and many diseases are the consequence of stringent selection (Patterson, 2000). Many studies have considered a genetic cause of FCP (Guthrie and

Table II Comparison of the incidence of ED in male to female ratio in different studies.

Breeds	Number of dog	ED specified	An aselective or Selective group	Male: Female ratio	References
Bernese Mountain dogs, Boxer, German Shepherd dog, Golden Retriever, Labradors Retriever, Newfoundland, Rottweiler and Saint Bernard	5406	ED	Aselective	1.37:1	Malm et al., 2007
Bernese Mountain dog	162	FCP + INC	Aselective	1.13:1	Ubbink et al., 1999
Labrador and Golden Retriever	5130	Osteochondrosis (FCP and OCD)	Aselective	2.2:1	Guthrie and Pidduck., 1990
Saint Bernard, German Shepherd and Basset hound	284 cases from 1381 dogs	ED (unclassified UAP and FCP)	Selective	1.6:1	Hayes et al., 1979
Labrador Retriever	1247	ED (FCP and OCD)	Aselective	2:1	Studdert et al., 1991
Rottweiler, Bernese Mountain dog and Newfoundland	1423, 414 and 209, respectively	Osteoarthrosis	Selective	1.25:1 to 2:1	Grøndalen and Lingaas, 1991
Labrador Retriever, Golden Retriever, German Shepherd, Bull mastiff and Rottweiler	31 cases in 30 months period	ED (FCP and OCD)	Selective	5:1	Houlton, 1984
Labrador Retriever, Rottweiler, Saint Bernard, German Shepherd and Collie	68 dogs during 10 years period	FCP and OCD	Selective	1:1	Mason et al., 1980

Pidduck, 1990; Grøndalen and Lingaas, 1991; Studdert et al., 1991; Ubbink et al., 1998; Everts et al., 2000). Ubbink et al. (1998) reported the clustering of FCP in a particular cohort of Labrador Retrievers rather than its dissemination throughout the entire population, with affected animals descending from a few common ancestors. Ubbink et al. (2000) reported the genetic risk of FCP to be 18% in the Labrador population, with a higher risk (28-50%) occurring in certain families. The high prevalence of FCP and elbow dysplasia in male dogs is thought to be weight related (Table II). We found that, of 1194 dogs (Labradors, Golden Retrievers or their mix; 570 males and 585 females) bred as guide dogs for the blind in the Netherlands, 14.7% suffered from FCP, with male dogs being affected with FCP 1.6 times more often than bitches (unpublished data).

Mode of inheritance

The mode of inheritance of FCP is still unclear, although many patterns of inheritance have been ruled out (Guthrie and Pidduck, 1990; Studdert et al., 1991). A radiographic investigation of the elbow joints of complete litters of Labradors failed to demonstrate an autosomal-dominant trait (Ubbink et al., 1998; Everts et al., 2000). A classical pattern of X-linked inheritance was suggested by Guthrie and Pidduck (1990). They also suggested a multifactorial pattern of inheritance of osteochondrosis, which included OCD and FCP in their study, with a genetic and an environmental effect. However, Guthrie and Pidduck (1990) concluded that elbow dysplasia (OCD and FCP) is a polygenic trait, this is not necessarily true for FCP alone. A model based on a major causative gene determined by Mäki et al. (2004) is likely to explain elbow dysplasia in Rottweilers, but not in Labrador Retrievers. Another gene model may be relevant for Dutch Labradors (Everts et al., 2000).

Heritability of FCP

Heritability (h^2) is an indirect indicator of the variance in a certain phenotype that can be explained by genetic variance. It should be noted that heritability is defined only if a multifactorial model is used. The heritability of elbow dysplasia (i.e., OCD plus FCP) in the study of Guthrie and Pidduck (1990), in a group of Labrador and Golden Retrievers bred as guide dogs for the blind in the UK, was 0.77 for male dogs and 0.45 for bitches. However, a lower h^2 of 0.27 for OCD plus FCP was reported by Studdert et al. (1991) in a group of Labrador Retrievers trained to be guide dogs in Australia. Since Labrador and Golden Retrievers have a different genetic background (Parker et al., 2004), it might be more informative, in terms of establishing the contribution on inherited factors, to determine the h^2 of a particular disease in a single breed. The h^2 for FCP is 0.31 in Rottweilers (Mäki et al., 2004), 0.18 in German Shepherd dogs (Janutta et al., 2006) and 0.29 in Labradors Retrievers (Hazewinkel, personal communication).

Methodology to study the genetics of FCP

Different approaches, such as linkage and association analysis, can be used to identify genes that cause disease. Linkage analysis compares segregation of DNA marker data with segregation of the phenotype in families, whereas association analysis determines the frequency of marker alleles in groups of affected and unaffected dogs. Both linkage analysis and association studies can be performed by means of either preselected candidate gene approach or by way of a whole-genome scan (Hirschhorn and Daly, 2005).

Two types of polymorphisms are used as markers for gene mapping. Microsatellite markers are known for their high level of variability (Ellegren, 2004; Nussbaum et al., 2004). A microsatellite marker is a short nucleotide sequence present on chromosomes in variable numbers of tandem repeats, and in the human genome microsatellites occur with a frequency of at least 1/30 kb (Beckman and Weber, 1992). Microsatellite markers are stably inherited by the offspring. One would expect to see the same microsatellite marker allele close to an FCP gene inherited by affected dogs of a family, and thus share one marker allele among them.

Since the whole genome sequence in the dog was published in 2003 (Kirkness et al., 2003), numerous single-base variations, known as single-nucleotide polymorphisms (SNPs), have been identified. Currently, more than 2.5 million SNPs have been found with an average of 1/900 base pairs along the canine genome (Lindblad-Toh et al., 2005). A particular advantage of SNPs is that large-scale genotyping can be accomplished with high-throughput techniques. The SNPs are mostly used in association studies (see below). The frequent presence of a particular allele of bi-allelic SNPs in dogs with FCP could be utilized to identify the gene causing FCP. In addition, a combination of SNPs that are located close to each other can mimic a multi-allelic marker that can be analysed in the same way as microsatellite marker when observing linkage in a family.

Linkage analysis

Linkage analysis can be performed by using a combination of informative family data and genetic markers. This methodology is promising when applied to Mendelian phenotypes, rare diseases, or diseases with strong genetic effect (Risch, 2000; Hirschhorn and Daly, 2005). Moreover, a large number of samples are not necessary and only a limited number of informative markers are needed. Linkage analysis identifies chromosome regions that are co-inherited with a trait more often than expected by chance if they are far apart on the same chromosome or on different chromosomes (Terwilliger and Ott, 1994; Kruglyak et al., 1996), (Figure 3). Linkage analysis can be performed either with a number of markers across the genome (whole genome scan or genome-wide scan) or with one close to a candidate gene. Linkage analysis is suited for a family-based study. Either specified model analysis (parametric

linkage analysis) or model-free linkage analysis (non-parametric linkage analysis) can be performed. Parametric linkage analysis is appropriate for testing when the inheritance pattern fits an expected genetic model, whereas non-parametric linkage analysis is more appropriate if the inheritance pattern is deviant or unclear (Kruglyak et al., 1996). The disorder FCP could benefit from non-parametric linkage analysis, since the mode of inheritance is unclear. Based on the finding that there is probably not full penetrance of FCP in affected dogs (Everts et al., 2000) and the estimate of sensitivity of registration FCP with regular screening (i.e., plain radiography) is less than 80% (Snaps et al., 1997), it can be concluded that it is hard to identify dogs genetically unaffected with FCP. Sibling pair analysis, a non-parametric linkage analysis, would be appropriate for investigating dogs affected by FCP.

Collagen is the main component of articular cartilage and cancellous bone (Eyre, 2002). Because collagen protein abnormalities could play an important role in a disturbance of bone and cartilage development that underlies FCP, a number of collagen genes are candidate genes for FCP. Polymorphic microsatellite markers close to a number of collagen genes have been developed (Temwichitr et al., 2007). An investigation in Labrador retrievers using non-parametric linkage analysis found that these collagen genes were probably not involved in FCP (Salg et al., 2006), therefore, mutations of collagen genes are probably not responsible for FCP. Another approach would be to investigate genes possibly associated with other pathogenetic mechanism underlying FCP. However, whole genome linkage might be more appropriate for further molecular genetic studies of FCP because predetermined candidate genes are not required and non-parametric analysis can be applied.

Association analysis

FCP is a multifactorial disease in some breeds, such as Labrador retrievers (Guthrie and Pidduck, 1990; Mäki et al., 2004). Association analysis is a powerful tool for analysing a common disease or complex trait. This method can be performed by either whole-genome association or candidate-gene association studies. The method relies on the difference in the frequency of alleles or genotypes between a case and a control group. Since a number of markers across the genome are used, the analysis is called a genome-wide association study. Such studies can make use of at least 10,000 SNPs (Lindblad-Toh et al., 2005) and do not require knowledge of a disease model or casual variants (Hirschhorn and Daly, 2005). Association analysis might be the best approach for FCP, given the unclear mode of inheritance of the disorder, the lack of known candidate genes, and frequent inability to use family information.

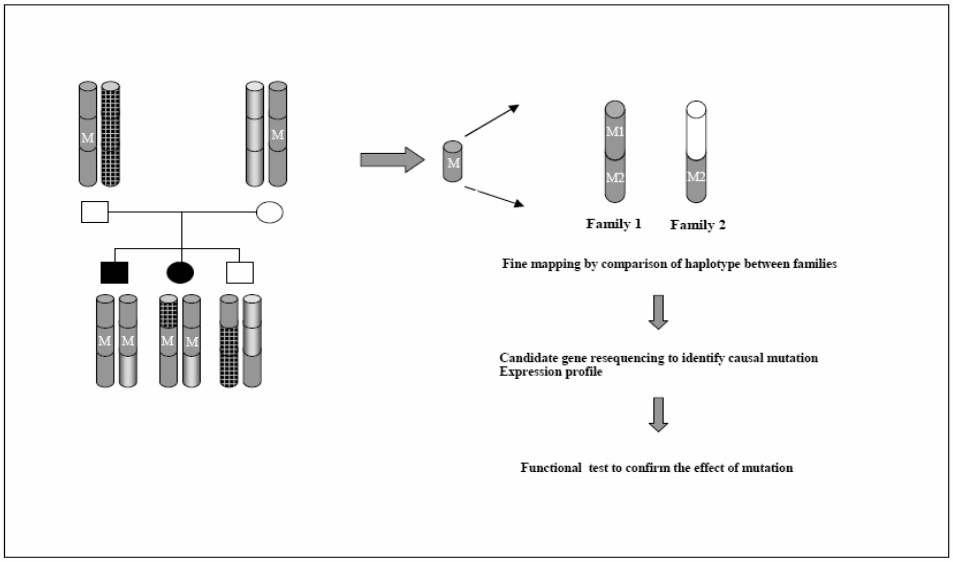

Figure 3. Localization of the gene of interest by linkage analysis.
In this example, the parents share a chromosomal region with a recessive disease gene which is inherited from a common ancestor. The two affected offsprings (filled symbols) have received different parts from the region from both parents. The differences are the result of recombination events and exchange of DNA between homologous chromosomes during the formation of the gametes of the parents. The only region they share homozygously (M) is the location of the causative mutation. The region can be recognized by genotyping of polymorphic DNA markers. The location can be refined by comparison of patients from other families and related breeds.

Conclusion

In conclusion, despite numerous studies, the aetiology of FCP and its causative gene have not been elucidated. A diagnostic test for FCP is needed, to be used instead of, or together with, radiography because radiography alone is not accurate enough to detect FCP in all cases. Indeed, there are reports of breeders with elbow dysplasia-positive litters originating from elbow dysplasia-negative (determined by radiographic screening) breeding stock. Since FCP is not observed in other species, identification of the genes involved by known FCP genes is not an option. Whole genome scan with a set of microsatellite markers, or whole genome linkage as well as association analysis with a number of SNPs can be performed with high throughput technology. Elucidation of the gene causing FCP will be instrumental to learning more about the aetiology of FCP, developing a sensitive diagnostic test and eradicating this disease. Because of the different presentations of FCP in different breeds, it is to be expected that breed-specific research will be necessary.

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Chapter 3

Assessment of collagen genes in development of fragmented coronoid medial processes of Labrador Retrievers as determined by affected sibling-pair analysis

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Abstract

Objective

To evaluate the involvement of various collagen genes in the development of fragmented medial coronoid processes (FCPs) in Labrador Retrievers.

Sample population

93 dogs originating from 13 litters were used in the study; FCP was diagnosed in 35 dogs, and each affected dog had at least 1 sibling that was also affected. Twelve dams and sires were included in the analysis. All dogs were purebred Labrador Retrievers except for 2 litters (offspring of a female Golden Retriever-Labrador Retriever mixed-breed dog).

Procedures

For each dog, DNA was isolated from blood samples. Polymorphic microsatellite markers adjacent to 14 candidate genes (i.e., COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1) were analyzed by use of PCR assays; genotypes were determined via automated detection of DNA products. The level of allele sharing between pairs of affected siblings was assessed.

Results

Among the 93 dogs, allele sharing of the 14 collagen genes was determined as follows: COL1A1, 45%; COL1A2, 47%; COL2A1, 37%; COL3A1, 32%; COL5A1, 43%; COL5A2, 32%; COL6A3, 36%; COL9A1, 45%; COL9A2, 49%; COL9A3, 38%; COL10A1, 46%; COL11A1, 52%; COL11A2, 47%; and COL24A1, 47%.

Conclusions and Clinical Relevance

Because siblings share 50% of their genome at random, the fact that the percentages of allele sharing among the analyzed collagen genes were not significantly > 50% indicates that these genes are not determinant candidates for FCP in Labrador Retrievers. The gene for the vitamin D receptor could also be excluded because of its proximity to COL2A1.

Introduction

Elbow joint dysplasia is the most common heritable disease causing forelimb lameness in dogs (Hayes et al., 1979; Mason et al., 1980; Grøndalen and Lingaas, 1991). Elbow joint dysplasia comprises different types of growth disorders including ununited anconeal process (UAP), osteochondrosis of medial humeral condyle (OCD), Fragmented coronoid process (FCP), and incongruity of the elbow joint (INC). FCP is a condition that is prevalent in several dog breeds such as Rottweiler, German Shepherd Dog, Golden Retriever, Bernese Mountain Dog, and Labrador Retriever (Olsson, 1983; Carpenter et al., 1993). In Labrador Retrievers, the first clinical signs such as elbow related lameness and signs of pain are detected between 4 and 8 months of age (Olsson, 1983; Wind and Packard, 1986; Wind, 1986). Although osteoarthritis can be diagnosed radiographically, an FCP is not always detectable (Voorhout and Hazewinkel, 1987). Visualization of the FCP often warrants additional imaging techniques or arthrotomy, the latter as part of surgical treatment (Hazewinkel et al., 1988). Although FCP has been described as an inherited disease (Hayes et al., 1979; Grøndalen, 1982; Guthrie and Pidduck, 1990; Grøndalen and Lingaas, 1991; Studdert et al., 1991; Ubbink et al., 1998), little is known about the inheritance pattern. Ubbink et al. (1998) determined that Labrador Retrievers from groups of FCP-affected dogs are more related to each other than Labrador Retrievers from groups of unaffected dogs. Those investigators were able to trace back the trait to a limited number of founders from a large group of contemporary breeding dogs and could predict the incidence of FCP among dogs on the basis of their relationship to these founders. However, this analysis could not reveal the mode of inheritance.

Results of 2 studies (Guthrie et al., 1992; Padgett et al., 1995) indicated that OCD and FCP are inherited as independent traits. For instance, in their study, Padgett et al. (1995) determined that OCD and FCP did not co-segregate in a cross and backcrosses of Labrador Retrievers affected by both traits. Another study16 revealed that FCP and incongruity of the elbow joint are independently inherited diseases in Bernese Mountain Dogs. Findings of several studies (Guthrie and Pidduck, 1990; Padgett et al., 1995) have suggested that inheritance for OCD and FCP is neither recessive nor sex linked. The reported heritability (h^2) varies from 0.27 to 0.77 (Guthrie and Pidduck, 1990; Studdert et al., 1991; Swenson et al., 1997). Results of a previous study (Mäki et al., 2004) indicated that a major gene is associated with development of FCP, but a polygenic inheritance pattern has also been suggested (Guthrie and Pidduck, 1990; Padgett et al., 1995). Also, environmental factors such as nutrition and exercise probably influence the development of FCP.

Among Labrador Retrievers, FCP is more prevalent in males than in females. The ratio of affected males to females varied from 2:1 to 5:1 in different studies (Mason et al., 1980; Hazewinkel et al., 1988; Guthrie and Pidduck, 1990; Studdert et

al., 1991; Grøndalen, 1979; Houlton, 1984; Van Ryssen and van Bree, 1997). For FCP, general failure in endochondral ossification of the ulna is suspected to be the causative abnormality (Olsson, 1983), although mechanical overloading of the medial coronoid process cannot be ruled out (Kunzel et al., 2004; Wolschrijn and Weijs, 2004). In humans, a major part of bone dysplasia disorders is attributable to mutations in genes that code for collagen proteins (Kuivaniemi et al., 1991, 1997; Olsen, 1995; Mundlos and Olsen, 1997a, 1997b).

The purpose of the study reported here was to evaluate the involvement of various collagen genes that are associated with bone dysplasias in humans in the development of FCP in Labrador Retrievers. The gene codings for collagen type I a-1 (COL1A1), COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1 were selected as candidate genes. All these candidate genes are involved in bone disorders as osteogenesis imperfecta, Stickler syndrome, or osteoarthritis in humans (Vikkula et al., 1995; Mundlos and Olsen, 1997a, 1997b; Brunner et al., 1999; Uitterlinden et al., 2000; Reginato and Olsen, 2002).

Material and Methods

Sample collection

Blood samples (8 ml) were obtained specifically for genetic studies from 93 Labrador Retrievers born in 1989 through 1999. All dogs were part of a breeding program of the Royal Dutch Foundation for Guide Dogs for the Blind and were raised under identical nutritional regimens and conditions. Part of this population has been described previously (Ubbink et al., 2000). At the age of 12 to 18 months, all dogs were examined for FCP by use of an extensive clinical and radiographic protocol (Voorhout and Hazewinkel, 1987). The disease status in all affected dogs had been confirmed by arthrotomy. Only litters with at least 2 affected siblings were used in the genetic analysis (Figure 1). The study group comprised 35 affected siblings, 46 unaffected siblings, and 12 dams and sires. The dams and sires were free of FCP except for the sire of pedigree B, in which FCP was diagnosed at a later age. A sample of DNA from this dog was not available.

The dogs were purebred Labrador Retrievers, except for 2 litters of a single dam who was a 1:1 mix of Labrador Retriever and Golden Retriever. Genomic DNA was isolated from blood leukocytes by use of the salt extraction method (Miller et al., 1988) and frozen at –20°C until used. This study was performed with consent of the owners.

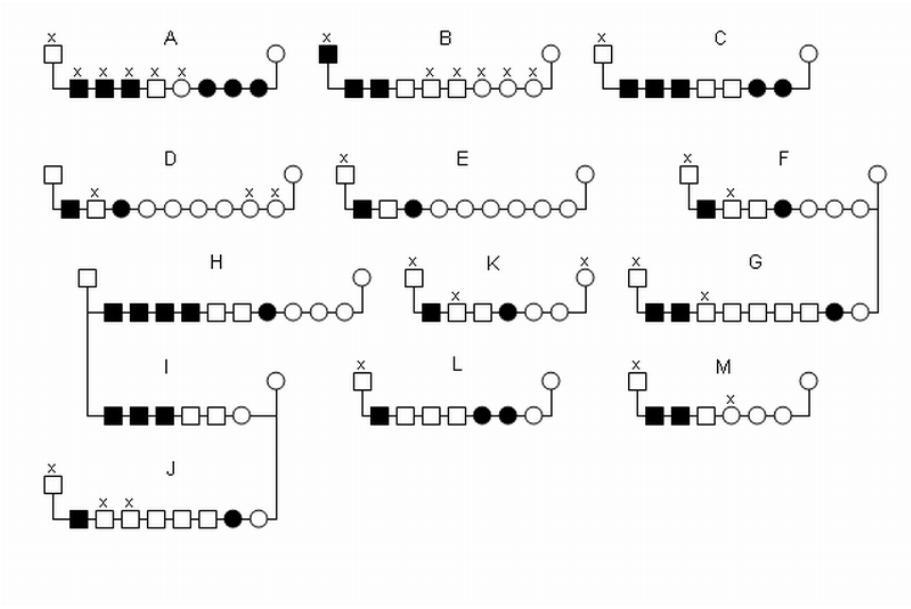

Figure 1. Schematic representation of litters of dogs, some of which were affected with FCP, used for sibling-pair analysis. All dams and sires were pedigree Labrador Retrievers except the dam of litters I and J, which was a 1:1 mix of Labrador Retriever and Golden Retriever. Male and female dogs are represented by squares and circles, respectively. Closed symbols indicate an FCP-affected individual, and open symbols indicate an unaffected individual. x = Sample of DNA not available.

RH mapping

The COL11A1 and COL11A2 genes were localized on the canine genome with the RH panel RHDF5000 (Vinnaux et al., 1999; Mellersh et al., 2000). The human coding sequence of COL11A1 (GenBank accession No. NM_00185437) and the genomic region containing exons 61 and 62 of COL11A2 (U4106837) were compared with primary DNA data from the canine genome sequence project. The primary data were accessed through the trace archives of the National Center for Biotechnology Information (www.ncbi.nlm.gov) and the comparison was made with the discontiguous basic local alignment search tool (BLAST) option. Parts of the genes were reconstituted by assembling the primary data that had high similarity to the human DNA sequences into a contiguous sequence of DNA. Oligonucleotides (primers) for amplification of specific DNA fragments were designed on the basis of these reconstituted DNA sequences. The oligonucleotide sequences for amplification of the COL11A1 fragment were 5'-TGTCCTCGTTCACCTATTGG-3' and 5'-GGAACCTGGGCTTATTGTCA-3'. The oligonucleotide sequences for amplification of the COL11A2 fragment were 5'-CAGAGCTGATGCTGTGAGAT-3' and 5'-TCTGTGTGTGCCTCCACCAA-3'.

A PCR procedure with a temperature gradient for the annealing step was performed on genomic canine DNA, hamster DNA, and a 2:1 mixture of hamster and canine DNA to optimize the PCR specificity for canine DNA.

Fragments of DNA were amplified in a thermal cycler (a) in a volume of 15 µL with 1X PCR buffer (b) 200µM dNTPs, 1.5mM MgCl2, 0.8mM primers, 0.6 units of platinum *Taq* polymerase (b) and 25 ng of DNA. The PCR reaction comprised 10 minutes at 95°C, 35 cycles of 30 seconds at 94°C, 30 seconds at the optimized temperature, and 1 minute at 72°C; to complete the reaction, a step of 2 minutes at 72°C was performed. The reaction products were stored at 4°C.

The PCR products were analyzed on a 1% agarose-gel, and the presence or absence of a copy of the canine gene was assessed for each cell line of the RH panel. The outcome was compared by use of a computer program for RH mapping (Boehnke et al., 1991; c) with the outcome of all available markers at the Centre Nationale de la Recherché Scientifique (CNRS) of the University of Rennes, France, to place the genes on the CNRS RHDF5000 map of the dog genome (Guyon et al., 2003).

Genotyping of microsatellite markers

Polymorphic microsatellite markers were selected from the RHDF5000 map (Guyon et al., 2003) for COL1A1, COL1A2, COL2A1, COL11A1, and COL11A2 (Table 1). The selected markers had a high level of heterozygosity or a location close to the gene.

The PCR reactions (15 µL) contained 25 ng of genomic DNA, 0.33µM of forward primer, 0.33µM of reverse primer, 2.5mM MgCl2, 200µM dNTPs, PCR gold buffer, and 0.3 units of a DNA polymerase (d). One of the primers was labeled by the manufacturer at the 5' end with a fluorophore (e). Several collagen genes were localized by nucleotide-nucleotide BLAST (BLASTN) searches of the completed canine genome DNA sequence at the National Center for Biotechnology Information (NCBI). The human reference complementary DNA sequences for COL3A1 (NM_000090), COL5A1 (NM_000093), COL5A2 (NM_000393), COL6A3 (NM_004369), COL9A1 (NM_078485), COL9A2 (NM_001852), COL9A3 (NM_001853), COL10A1 (NM_000493), COL11A1 (NM_001854), COL11A2 (NM_080680), and COL24A1 (NM_152890) were used for the localizations. The reconstituted COL3A1 gene was located in the vicinity of the reconstituted canine COL5A2 gene.

**Canis familiaris* chromosome.
†Position derived from DNA sequence map build 1.1 (Mb) or RHDF5000 (TSP).
‡Orientation of primers; F represents forward and R represents reverse.
TSP = Traveling Salesperson Problem. The positions were derived from reference 39, in which RHDF5000 was assembled as a solution to a Travelling Salesperson Problem.

Table 1 Genomic locations of candidate genes for FCP and microsatellite markers in Labrador Retrievers.

Gene or Marker	CFA*	Position †	O‡	Microsatellite PCR oligonucleotides 5'-3'	Source
COL11A1	6	4,630-4,710 TSP	F R	catcacaatctctaaggcgt gcatgatgagttcggttagg	Present study
COL24A1	6	63 Mb	F R	cagcgagcaccataatagta tgaggcagtcatagtcgtca	Present study
KRT9	9	13.9 Mb	F R	tcatcacttcctgctccatt tctcatgccacacggaacct	Guyon et al 2003
COL1A1	9	18.8 Mb			
FH2186	9	24.1 Mb	F R	agagggtctacatggattctgg tagagtctcaaacaatcctgtgg	Guyon et al 2003
COL5A1	9	43.5 Mb	F R	ctgtaagcctggaactctga ctgtgattctcataggccag	Present study
COL9A1	12	36.0 Mb	F R	gattaactccaggcagaatc cctaggagtgactctctgct	Present study
FH2200	12	75 TSP	F R	catgatcctggagtcccg gaaagctgcttcagtggacc	Guyon et al 2003
COL11A2	12	720-850 TSP			
REN258L11	12	1,140 TSP	F R	agagggtctacatggattctgg tagagtctcaaacaatcctgtgg	Guyon et al 2003
COL10A1	12	74.5 Mb	F R	gaagaagagagacaggtgtg cttgtctacgtggcttagtc	Present study
FH3951	14	9.3 Mb	F R	tttaaatcagaagtgttccgaag gagcttcctgtgaggagactg	Guyon et al 2003
COL1A2	14	22.4 Mb			
FH3725	14	24.3 Mb	F R	gaaagaactcactcaaaacttcc aaatgttacttcagaaaagctgg	Guyon et al 2003
COL9A2	15	5.0 Mb	F R	ctaccagcaggcaactccaa caggatgccaacgagaacaa	Present study
COL9A3	24	49.4 Mb	F R	gctccagagttcaggctcag aggaggtcggagatggagat	Present study
COL6A3	25	51 Mb	F R	gtcacttggcaccaggttag gtcaccagatgcagctcaga	Present study
FH2289	27	5.1 Mb	F R	catggtctcaggatcctagga ctaagcattctctctgatggtctt	Guyon et al 2003
COL2A1	27	9.8 Mb			
FH4001	27	9.8 Mb	F R	ctatgcaggataataccttggc ttaaatgtatcaccaagctggc	Guyon et al 2003
VDR	27	9.9 Mb			
COL3A1 and COL5A2	36	33.7 Mb	F R	ttgatacataccattggacc tatgtgtataggcattggct	Present study

The locations of COL1A1, COL1A2, and COL2A1 were already known from the RHDF5000 map (Guyon et al., 2003). Microsatellite repeats that were possibly polymorphic and informative for linkage analysis were selected from the genomic DNA sequences in the vicinity of the genes by use of a specialized software program (Benson, 1999; f).

Oligonucleotides (primers) for amplification of the microsatellite markers were designed with a specialized software program (Rozen and Skaletsky, 2000; g) and the 5' end of the forward primers was tailed with the M13 forward sequencing primer (5'- GTTTTCCCAGTCACGAC-3').

The PCR reactions (15 µl) contained 25 ng of genomic DNA, 1µM M13-tailed forward primer, 10µM reverse primer, 10µM M13 forward sequencing primer labeled at the 5' end with 6-FAM (e) 25mM MgCl2, 1mM dNTPs, PCR gold buffer, and 0.3 units of a DNA polymerase (d).

Thermal cycling was carried out in a thermal cycler (a) with a program as follows: 5 minutes at 95°C, followed by 10 cycles of 30 seconds at 95°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C, then another 25 cycles of 30 seconds at 92°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C. The program was completed with a step of 10 minutes at 72°C.

The PCR reactions were diluted 10- to 30-fold with H_2O, and 2 µl of the dilution was mixed with 10 µl of formamide and 0.2 µl of fluorophore-labeled size standard.(h) The products were analyzed by use of an automated DNA sequencer (i). The DNA products were sized and alleles were assigned by use of specialized software (j).

Linkage analysis

A sibling-pair linkage analysis was performed with the genotypes of FCP-affected siblings, unaffected siblings, and dams and sires when available (Kruglyak and Lander, 1995; Kruglyak et al., 1996). The allele sharing of each affected sibling pair was evaluated. Haplotypes of consecutive markers in each region with a candidate gene were constructed. Only data of informative siblings, dams and sires were taken into account; this means that only haplotypes were included in the calculations if the parental origin of the haplotype was certain and apparent recombinant offspring was discarded. The sum of all haplotypes shared by each pair of affected siblings was divided by the sum of all shared and non-shared haplotypes. According to Mendelian laws, mean allele sharing of 50% was expected for a locus not involved in development of FCP. Loci associated with sharing of significantly > 50% were suspected as being involved in development of FCP.

A software program (Rosalind Franklin Centre for Genomics Research; k; l) was used for statistical analysis of the data. The NPL option of the sibling-pair analysis was performed with this software. The level of marker allele sharing between pairs of

siblings was compared with the randomly expected level of 50%. For all analyses, a value of $P < 0.01$ was considered significant.

Results

The chromosomal localizations of the collagen genes COL11A1 and COL11A2 were determined via RH mapping with the RHDF5000 panel. The outcome of the PCR reaction for COL11A1 was compared with the outcomes of other markers on the RHDF map (Guyon et al., 2003). The lod score for COL11A1 was 13.1 and 11.0 with the markers FH3246 and EST17G5, respectively, of RHDF5000. These markers are located on CFA06 in a region that is syntenic with human chromosome 1p21, which also contains COL11A1.

Table 2 Results of the allele sharing and NPL analyses of genotype data from siblings in a study of FCP in Labrador Retrievers.

Gene	Allele sharing (%)	NPL score†	*P* value	Information content*
COL1A1†	45	0.4690	0.672	0.54
COL1A2†	47	-0.0854	0.522	0.66
COL2A1†	37	-0.2317	0.581	0.57
COL5A1	43	-0.1986	0.566	0.40
COL5A2 and COL3A1	32	-1.0338	0.866	0.57
COL6A3	36	0.0472	0.459	0.36
COL9A1	45	0.1528	0.420	0.61
COL9A2	49	-0.4035	0.644	0.57
COL9A3	38	0.3390	0.347	0.36
COL10A1	46	0.1358	0.426	0.54
COL11A1	52	0.5610	0.273	0.39
COL11A2†	47	0.3309	0.360	0.55
COL24A1	47	-0.0293	0.492	0.36

†Nonparametric linkage score from this gene was derived from multipoint NPL analysis.
*The information content is the fraction of alleles that can be assigned unambiguously.

The PCR results for the canine COL11A2 gene resulted in a lod score of 16.0 and 15.4 with the markers DLA-DQA and EST2A7, respectively. This result was also in agreement with the expected localization because these markers are located on CFA12 in a region that is syntenic with human chromosome 6p21, which also contains COL11A2. The positions of COL11A1 and COL11A2 were derived on the basis of the markers that had the maximum lod scores.

We investigated the involvement of a variety of collagen genes in development of FCP in Labrador Retrievers by measurement of allele sharing of nearby located microsatellite markers between affected littermates. In general, the genes COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1 were not associated with significantly high allele sharing (Table 2). None of the markers analyzed resulted in high allele sharing and a high NPL score with a significantly low *P* value. The highest NPL score of 0.56 was obtained for COL11A1 ($P = 0.27$); the allele sharing between affected siblings, as assessed from informative meioses, was 52% for this gene. The genes COL3A1 and COL5A2 were located closely on chromosome CFA36 and could be investigated with the same marker. The allele sharing of this marker was only 32%, resulting in a negative NPL score of –1.038 ($P = 0.87$).

Discussion

Results of the present study indicated that several Labrador Retriever and mixed Labrador Retriever- Golden Retriever litters had a high incidence of FCP, contributing to the notion that inherited factors are involved. The affected male-to-female ratio in these litters was approximately 2:1. The sex difference of the incidence indicates that the etiology of the disorder is multifactorial. Because the litters were selected on the presence of at least 2 affected siblings, a segregation analysis to establish the mode of inheritance was not feasible. To circumvent the uncertainty about the inheritance parameters, we investigated the involvement of candidate genes by use of a linkage analysis that was model free.

Collagen is the major component of the extracellular matrix of cartilage, and it is thought that abnormal development of cartilage can result in FCP (Olsson, 1983). Therefore, in the present study, collagen genes (in particular those that are involved in human bone disorders) were evaluated as candidate genes for FCP (Kuivaniemi et al., 1991, 1997; Olsen, 1995; Mundlos and Olsen, 1997a, 1997b; Reginato and Olsen, 2002; Tabor et al., 2002). To date, 42 collagen genes have been identified in humans (www.ncbi.nlm.gov). The genes COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, and COL11A2 have been implicated in human skeletal or joint disorders and were included in our study. In

addition, COL24A1 was investigated, and the genes COL5A2 and vitamin D receptor were included because of their proximity to COL3A1 and COL2A1, respectively. Most of the genes were analyzed through polymorphic microsatellite markers located in the vicinity. The inheritance of COL1A1, COL1A2, COL2A1, and COL11A2 was analyzed with markers at either side of the genes. Of these, the markers for COL1A1 and COL11A2 were not closely situated to the respective gene.

We calculated the genetic distances between the pairs of markers for each of the 4 genes from the Labrador Retriever family data, and in each case, the distance was approximately 10 cM (data not shown). Because recombinant dogs were excluded from the allele sharing calculation, this distance had no effect on the result.

In the present study, the DNA samples were obtained from Labrador Retrievers with radiographically and surgically confirmed FCP; dogs with other causes of elbow joint dysplasia resulting in osteoarthritis were excluded. By analyzing unaffected siblings, we could improve the information content of the sample. The sibling-pair analysis has the advantage that it does not require a model of inheritance (Kruglyak et al., 1996). The analysis assesses allele sharing between pairs of affected siblings that exceeds 50%. A genetic determinant of FCP carried by a phenotypically FCP-negative dam or sire is likely to be shared by affected offspring, thereby resulting in an increase from the level of randomly expected allele sharing. The affected sibling-pair approach has been used in genetic studies of human osteoarthritis (Loughlin, 2003). Results of the present study have indicated that the investigated collagen genes were not associated with high allele sharing between Labrador Retriever littermates with FCP and that none of the genes is likely to play an important role in the etiology of the disorder. It should be noted that, as in the human genome, the canine gene for the vitamin D receptor is closely located to the COL2A1 gene on chromosome CFA27, with only 80 kb between the genes. Therefore, the vitamin D receptor gene (which is involved in the regulation of bone mineral density (Uitterlinden et al., 2002) and associated with radiographically detectable osteoarthritis of the knee joint in humans (Uitterlinden et al., 2000) could also be excluded from involvement in the development of FCP in Labrador Retrievers.

The COL11A1 gene is one of the largest collagen genes. Nonparametric analysis of the gene resulted in an NPL score of 0.56, a *P* value of 0.27, and allele sharing of 52%. The COL11A1 and COL24A1 genes are located 14.1 Mb apart on CFA6. Multipoint linkage analysis of the 2 markers with the FCP phenotype resulted in an NPL score of 0.18 and a *P* value of 0.41 for the region.

A false-negative result in the present study could have been caused by a small sample size, lack of informativity of the DNA markers, and complexity of the trait. We anticipated that the risk of a obtaining a false negative result was small because the affected dogs were Labrador Retrievers or half-breed Labrador Retrievers. Given the homogeneous genetic structure of this group of dogs and dog breeds in general

(Ubbink et al., 1998, 2000; Parker et al., 2004) compared with groups of human patients, it can be inferred that the heterogeneity of complex disorders will be less and smaller numbers of affected sibling pairs are required to detect a significant deviation from 50% allele sharing. A genome-wide scan with highly polymorphic microsatellite makers should be conducted for this disease to elucidate the genetic components. The penetrance and mode of inheritance of the gene or genes should become clear after their identification. Olsson (1983) suggested that FCP frequently developed in breeds of dogs that had disturbances of endochondral ossification, which was speculated to be related to disturbed collagen synthesis. Our data have indicated that FCP is not caused by mutations of the collagen genes evaluated in the present study. It is possible that synthesis of 1 or more of those collagens is disturbed in an indirect manner (eg, through disturbed expression or altered posttranslational modification) in the etiology of FCP. It is also possible that the expression of collagens during development and growth is not synchronized or balanced with that of other bone-forming factors. Recently, a disturbance of bone modelling together with relative overload of the coronoid process has been suggested to be responsible for FCP (Kunzel et al., 2004; Wolschrijn and Weijs, 2004, 2005). Future research on the etiopathogenesis of FCP should perhaps focus on this biomechanical aspect.

Appendix

a. GeneAmp PCR System 9700 thermal cycler, Applied Biosystems, Foster City, Calif.
b. Invitrogen, Carlsbad, Calif.
c. RHMAP, University of Michican, Ann Arbor, Mich.
d. Amplitaq Gold, Applied Biosystems, Foster City, Calif.
e. HEX or 6-FAM, Eurogentec, Seraing, Belgium.
f. Tandem Repeat Software, Mount Sinai School of Medicine, New York, NY.
g. Primer3 software, Whitehead Institute for Biomedical Research, Boston, Mass.
h. GS500 LIZ or GS500 TAMRA, Applied Biosystems, Foster City, Calif.
i. Genetic Analyzer 3100, Applied Biosystems, Foster City, Calif.
j. GeneMapper software, Applied Biosystems, Foster City, Calif.
k. Web-Genehunter, Stanford University, Stanford, Calif. Available at: mozart.stanford.edu/web-genehunter.htm. Accessed October 12, 2005.
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Chapter 4

Model-free linkage analysis of Labrador retriever with fragmented medial coronoid process indicates involvement of regions on chromosome 1 and 13

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Abstract

Fragmented coronoid process (FCP) of the ulna is a disabling elbow condition often diagnosed in medium- and large-size dog breeds such as Labrador retrievers. Genetic studies have indicated that inherited factors play an important role in the occurrence of FCP, but the underlying mode of inheritance and gene mutations have not been identified. In order to localize genetic factors involved in FCP, we performed a model-free linkage analysis study, using 320 polymorphic microsatellite markers and 1536 single nucleotide polymorphisms, spread evenly on the genome, and material collected from a cohort of Labrador retrievers consisting of 35 affected and 46 unaffected dogs and 12 parents from 10 pedigrees. Candidate loci were selected and further analyzed using additional markers and a larger sample of Labrador retrievers. Data were suggestive of there being a linkage between FCP and a region on canine chromosomes 1 and 13 at positions with non-parametric LOD scores of 5.0 and 3.2, respectively.

Keyword: whole genome analysis, model free linkage analysis, non-parametric linkage analysis, fragmented medial coronoid process, dog, single nucleotide polymorphism, microsatellite marker

Introduction

Fragmentation of the medial coronoid process of the ulna can cause forelimb lameness in medium- and large-size dogs, such as Labrador retrievers. In these dogs, the clinical symptoms of fragmented coronoid process (FCP) become manifest at a young age (Olsson 1983; Wind 1986; Carpenter et al. 1993). The disease is an elbow dysplasia (ED), a classification that includes ununited anconeal process, osteochondritis dissecans of the medial humeral condyle, and incongruity of the elbow joint (IEWG 2008).

Mechanical overload of the medial coronoid process has been suggested as the main cause of FCP due to incongruity of the elbow joint or due to avulsion of the annular ligament (Kunzel et al. 2004; Wolschrijn & Weijs 2004), although disturbance of endochondral ossification is another possible etiology (Olsson, 1983; Voorhout & Hazewinkel 1987). FCP as an inheritable trait (Guthrie & Pidduck 1990; Grøndalen & Lingaas 1991; Studdert et al. 1991), with a major gene being suggested to be involved in FCP in Rottweilers (Mäki et al. 2004) and in Labrador retrievers (Everts et al. 2000), whereas a polygenic inheritance pattern of ED has been reported for other breeds (Guthrie & Pidduck 1990; Padgett et al. 1995). The heritability (h^2) of ED, including FCP, in Labradors varies from 0.25 to 0.77 (Guthrie & Pidduck 1990; Grøndalen & Lingaas 1991; Studdert et al. 1991; Swenson et al. 1997). FCP is diagnosed more frequently in male than in female Labrador retrievers, with a ratio between 2:1 and 5:1 (Grøndalen 1979; Houlton 1984; Hazewinkel et al. 1988; Gutthrie & Pidduck, 1990; Studdert et al. 1991; Van Ryssen & van Bree 1997). Thus far, the causative gene or genes of FCP are not known. Mutations of genes encoding collagen proteins have been hypothesized to cause FCP, and for this reason polymorphic DNA markers close to collagen genes have been developed and analyzed (Salg et al. 2006; Temwichitr et al. 2007). Fourteen collagen genes have been ruled out as being involved in FCP in the Dutch Labrador retriever population (Salg et al. 2006).

We consider knowledge of the genetic basis of FCP to be essential to preventing the disease, and for this reason we attempted to localize the causative genes on the canine genome. Because the mode of inheritance appears to be complex, we performed a model-free linkage analysis. This type of analysis determines whether affected related dogs share alleles at a given locus more often than would be expected by chance. We used two genome-wide sets of markers, one composed of 320 microsatellite markers and one of 1536 single nucleotide polymorphisms (SNPs) (Leegwater et al. 2007). After comparison of the results from the two analyses, the loci with suggestive linkage were fine mapped with additional markers.

Materials and methods

Samples

All the dogs in this study came from the breeding program of the Royal Dutch Foundation for Guide Dogs for the Blind (KNGF) and were reared under similar dietary, training, and housing conditions. Part of this population has been described previously (Everts et al. 2000; Ubbink et al. 2000; Salg et al. 2006). The families investigated contained litters with at least two siblings affected by FCP (Figure 1). The study group comprised 59 affected dogs (38 males) and 98 unaffected siblings (48 males) from which DNA was available, together with that of their 16 parents. In total there were 23 litters divided in 17 pedigrees. The dogs were registered by the Dutch Kennel Club as purebred Labrador retrievers except for two litters of which the dam was not a purebred Labrador and two other litters which were offspring from a Labrador and a dog from another breed (Figure 1).

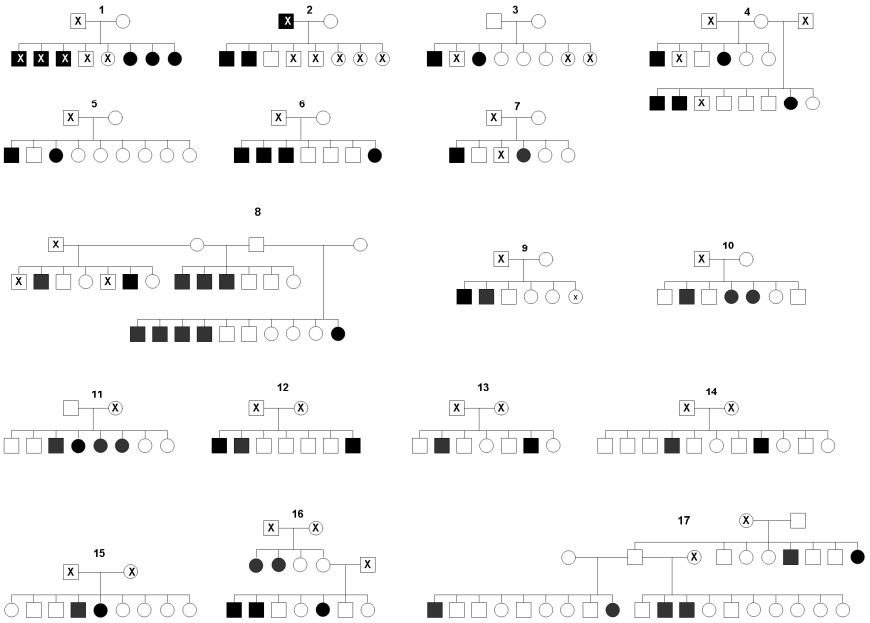


Figure 1. Pedigree diagram

Litter 1-10 were used in the analysis of both microsatellite marker set and SNPs. Litters 11-17 were included for fine mapping. Only litters with at least 2 affected siblings were used. Open circles are normal female dogs, closed circles are FCP female dogs, open squares are normal male dogs and close squares are FCP male dogs. X indicates dogs for which DNA was not available. Dogs were purebred Labrador retrievers, except for a mother of one litter in pedigree 8 (Labrador x Golden retriever), a mother of pedigree 12 (Labrador x German Shepherd), and a father of pedigree 14 was Golden retriever.

The elbow joints of the dogs were routinely screened by radiography when the dogs were 12–16 months old (Voorhout & Hazewinkel 1987; Hazewinkel et al. 1988). The

diagnosis of FCP was based on radiological signs according to the criteria of the International Elbow Working Group (IEWG, 2008) and was confirmed by surgery in all cases. Dogs without clinical and radiographic evidence of FCP in both elbow joints were considered as being FCP negative. Blood samples were collected from the jugular vein from all dogs. Genomic DNA was isolated from blood samples using the salt extraction method (Miller et al. 1988) and frozen at -20°C until use.

Marker sets

Microsatellite markers were selected from a radiation hybrid map and meiotic linkage maps (Mellersh et al. 1997; Neff et al. 1999; Guyon et al. 2003). Thirteen markers were chosen from other published reports (Ostrander et al. 1993; Werner et al. 1999; Jouquand et al. 2000; Hoffmann et al. 2008) and 7 markers were developed for this study to fill in gaps. The set comprised 320 markers and is described in the appendix of the online supplement. The position of each marker was based on alignment with the assembly of the genome reference sequences of *Canis lupus familiaris*, Build 2.1. The distance between adjacent markers was 8 Mb on average. A set of 1536 SNPs, which has been described earlier (Leegwater et al. 2007), was investigated in parallel.

Genotyping

The polymorphic DNA fragments of the microsatellite markers were amplified in a 10-µl reaction mixture containing 25 ng of genomic DNA, 10 µM reverse primer, 10 µM forward sequencing primer labeled at the 5'end with Fluorophore 6-FAM (Eurogentec, Maastricht, The Netherlands) VIC, NED, or PET (Applied Biosystems, Foster City, CA, USA), 1X PCR gold buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.3 U Amplitaq Gold (Applied Biosystems, Foster City, CA, USA). DNA samples and PCR reaction solutions were dispensed into 384-well PCR plates by a Genius pipetting robot (Tecan, Männedorf, Switzerland).

Thermal cycling was performed in the GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA) with the following program: 5 minutes at 95°C, followed by 10 cycles of 30 seconds at 95°C, 15 seconds at the annealing temperature, 30 seconds at 72°C, then another 25 cycles of 30 seconds at 92°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C. The program was completed with 10 minutes at 72°C.

Up to four PCR products were analyzed in a single run. Marker combinations were selected based on fluorophor labeling and DNA fragment size. No more than two products of the same label were used in the same reaction. GSLIZ500 was used as a size standard with 0.2 µl per 10 µl of Hi-di formamide and the DNA fragments were separated and detected automatically with an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). GeneMapper software (Applied Biosystems, Foster City, CA, USA) was used for allele assignment. The set of 1536 SNPs was

genotyped with the GoldenGate assay and scanned with a BeadArray Reader (Illumina, San Diego, CA, USA). The genotypes were clustered automatically with the BeadStation (Illumina, San Diego, CA, USA). The genotype of each SNP was replicated 30 times on average, and only the genotypes that had call and concordance rates of at least 90% were accepted. We excluded SNPs that displayed Mendelian inconsistencies according to Mega2 software (Mukhopadhyay et al. 2005).

Linkage analysis

Sibling pairs share on average 50% of their genome by chance. Genomic regions that are shared by affected sibling pairs more often than this could harbor a causal allele, regardless of the mode of inheritance. The significance of the allele sharing of both genome wide sets of DNA markers among affected sib-pairs was addressed separately with non-parametric linkage analysis of the Genehunter software package (Kruglyak et al. 1996). The genome-wide analysis with the two marker sets was performed on the genotypes of the available dogs from families 1-10 (Figure 1). We assumed that a physical distance of 1 Mb between markers corresponds to a genetic distance of 1 cM.

Fine Mapping

Genomic regions that displayed suggestive linkage with the microsatellite and the SNP sets were analyzed with densely spaced SNPs (see Appendix). These were genotyped with the Golden Gate assay, first for families 1-10 and then for families 11-17 (Figure 1). In addition, SNPs were selected for a region on chromosome 28 that showed a highly significant non-parametric LOD score (NPL) with the SNP set but which was not covered by the microsatellite markers.

Results

Because the mode of inheritance of FCP in Labrador retrievers is not known and is probably complex, we chose a model-free approach to search for the genomic regions involved. Marker sets of microsatellites and SNPs were used separately to evaluate allele sharing among affected sibling pairs. One set consisted of 320 microsatellite markers, of which 303 were informative and used for multipoint linkage analysis based on allele sharing between affected siblings. Suggestive NPL scores were obtained for canine chromosomes CFA01, 05, 06, 13, and 34 (Figure 2, Table 1).

Of the second marker set of 1536 SNPs, 1328 were informative. The multipoint NPL score for CFA01 displayed two closely spaced peak regions at position 55.4 Mb and 59 Mb, with values of 3.9 and 3.7, respectively (Figure 2). These positions were consistent with the position of the peak observed with the microsatellite markers. The NPL score for the entire CFA01 was relatively high and did not drop below 1 with either marker set.

The peak region observed with microsatellite markers at about 10 Mb on CFA05 was not seen with the SNP set. For CFA06 we obtained an NPL score of 1.85 at 29.5 Mb with the SNPs, and a score of 1.82 at position 31.3 Mb with the microsatellites. With both marker sets we obtained peak values close to position 50 Mb on CFA13, while with CFA28 we only observed a peak NPL score with the SNP set (Table 1). For CFA34 we obtained broad and overlapping peak regions with the two marker sets.

Table 1. Comparison of non-parametric linkage scores in peak regions obtained with two independent marker sets

	Microsatellite				SNP			
CFA[1]	Mb[2]	NPL[3]	p value	Inf[4]	Mb	NPL	p value	inf
1	59	3.13	0.0038	0.68	54.7- 60.6	3,92	0,0010	0,89
5	10.35	2.09	0.0247	0.81	19.23	1.67	0.0520	0.91
6	43	1.97	0.0300	0.70	29.5	1.81	0.0400	0.91
13	49	2.77	0.0071	0.75	47.5	1.83	0.3900	0.85
28	3.86	0.79	0.2020	0.58	30.7- 31.1	3.72	0.0014	0.92
34	34.91	1.84	0.0391	0.78	24.1- 27.1	3.30	0.0028	0.91

The table shows non-parametric scores obtained from whole-genome linkage analysis with microsatellite markers and SNP. The scores are for the peak region of each chromosome.
[1] canine chromosome
[2] position on the chromosome in Megabase
[3] non-parametric linkage score
[4] information content, when inf= 1, the chromosomes of all offspring can be traced to the parents.

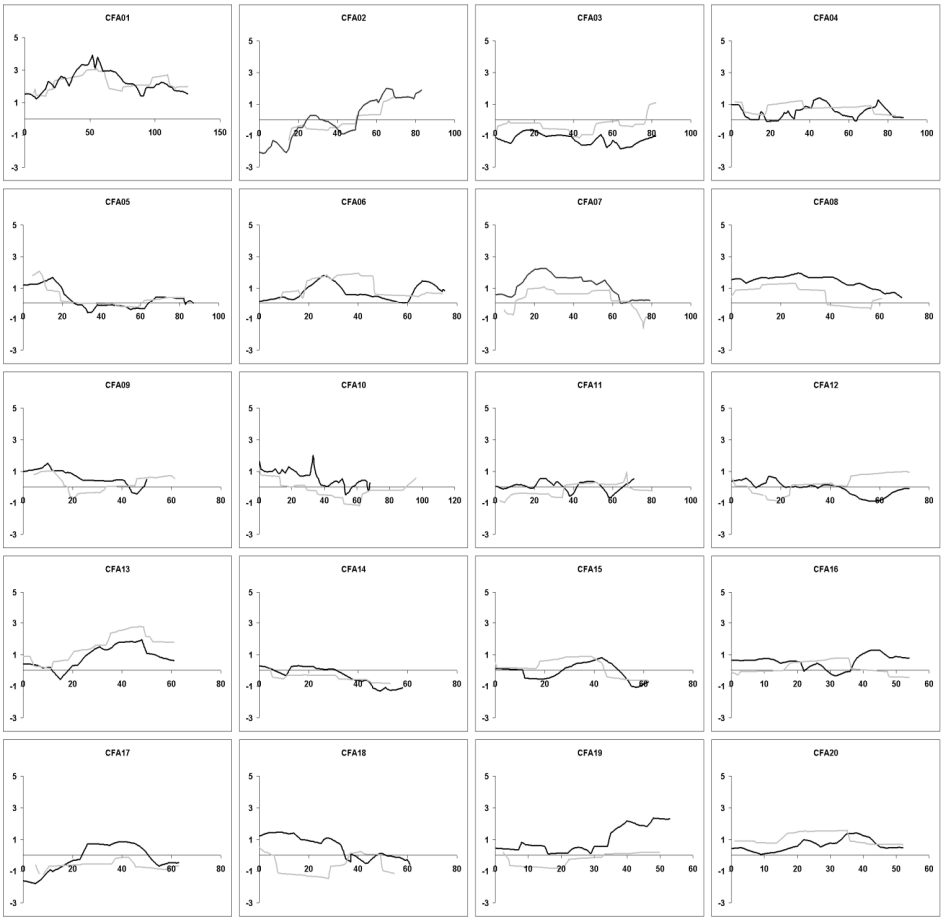
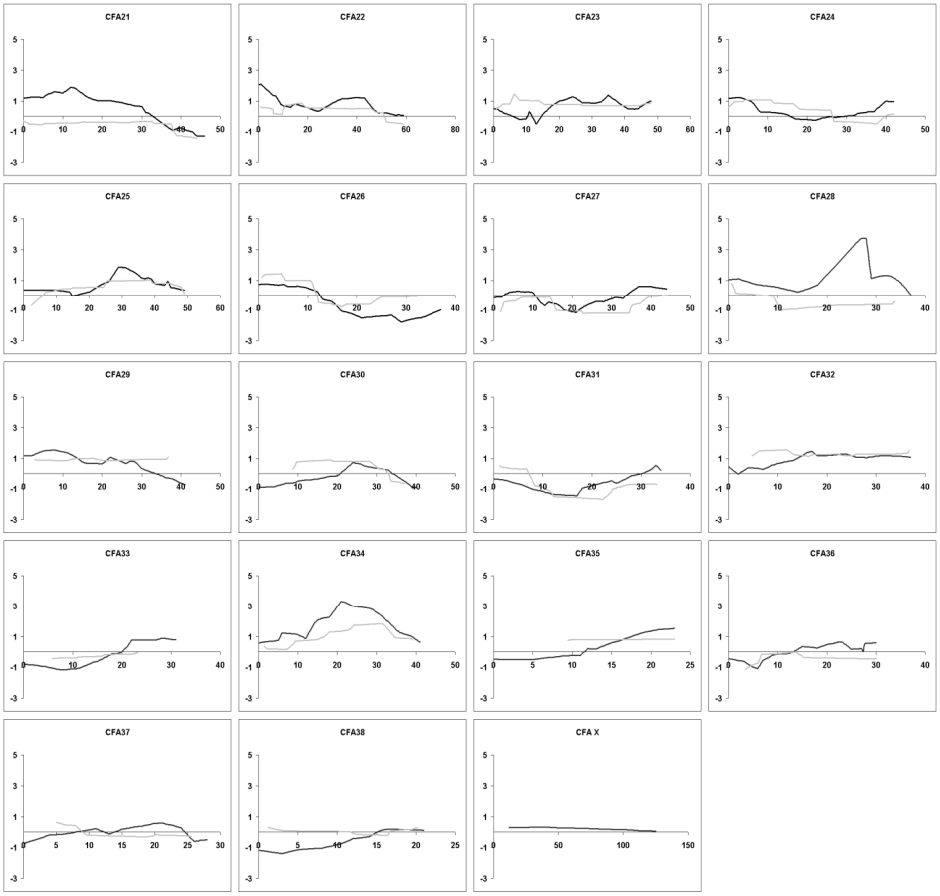


Figure 2. NPL score obtained from whole genome linkage analysis obtained with two markers sets

Dark lines represent NPL obtained from SNPs set, while light lines represent NPL from microsatellite markers set. The position on each chromosome is in Mb. NPL of CFAX was obtained only from microsatellites.



On CFA01, the NPL score of the peak region shifted fractionally to a distal position and increased to 5.0 (Figure 3). The NPL of the second region on CFA01 also increased, from 2.2 to 3.5. The region on CFA06 was excluded by fine mapping because the NPL score decreased from 2.0 to 1.1. The NPL score for the region on CFA13 increased to 3.2 from 2.8, whereas the score for the region on CFA28 decreased from 3.7 with the genome wide SNP set to 2.1 with the densely spaced SNPs. The NPL score of the peak region on CFA34 decreased from 3.3 to 1.9 upon fine mapping. We concluded that the two peak regions on CFA01 and the region on CFA13 were the most likely locations of genes involved in FCP in Labrador retrievers.

For the peak regions on CFA01, 06, 13, 28, and 34, we typed additional SNPs in more pedigrees (Figure 3). The expanded sample and the high density of typed SNPs increased the power of the sibling pair analysis and allowed us to compare haplotypes between affected dogs from different pedigrees.

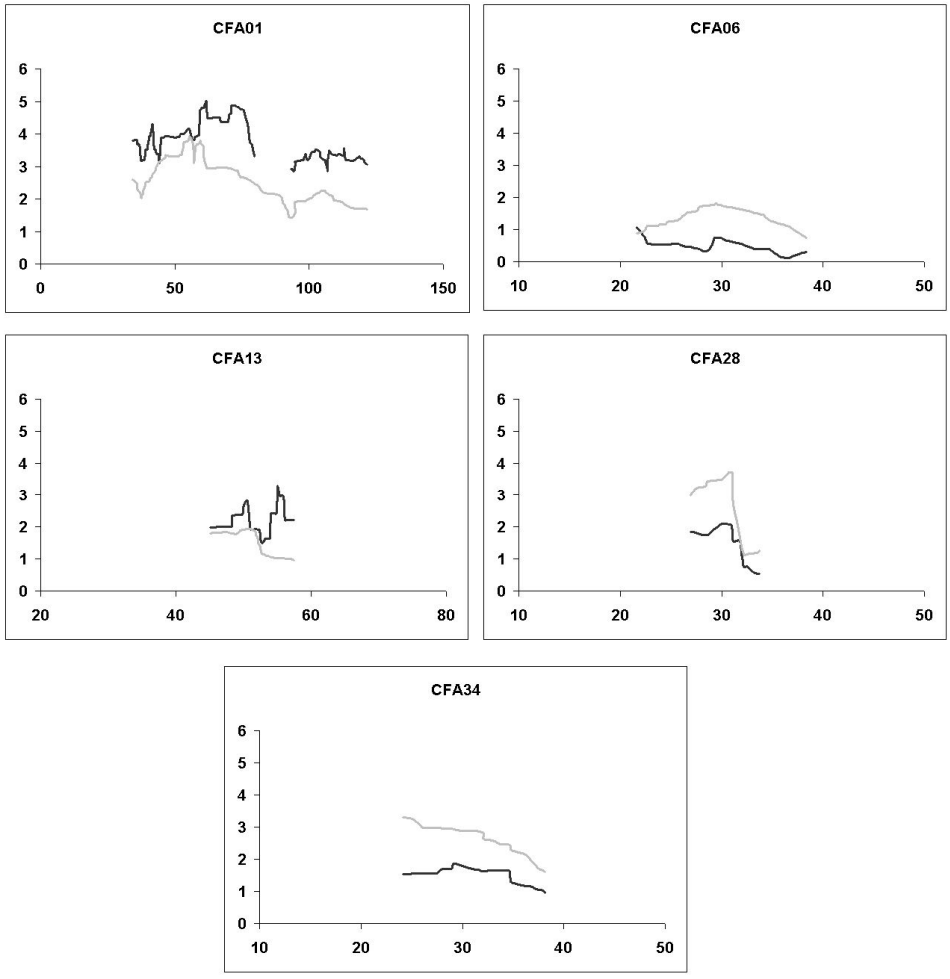


Figure 3. NPL scores after fine mapping
NPL scores after fine mapping were obtained for CFA01, 06, 13, 28, and 34. Dark lines represent NPL score after fine mapping whereas gray lines represent the NPL of whole genome with SNPs set. NPL scores for CFA01 and 13 were increased up to NPL of 5, as seen for CFA01, but were decreased for CFA06, 28, and 34.

Discussion

FCP is a multifactorial, inheritable disorder in Labrador retrievers (Guthrie & Pidduck 1990; Studdert et al. 1991; Ubbink et al. 2000). Association studies, by which the allele frequencies of huge numbers of markers are compared in large groups of patients and controls, is currently the method of choice to locate genes involved in complex

disorders. Such association studies require that the individual cases are not closely related in order to avoid overrepresentation of families in either group, which would be a source of false-positive results. The basic assumption of association studies remains that patients share causative genes inherited from a common ancestor. Thus in association studies there is the paradox that patients should be related but not closely related. This condition is difficult to meet with dog breeds. Moreover, we think that knowledge of relationships between patients can be used to improve the power of genetic studies, although this approach necessarily increases the computer time required for analysis of multiple markers simultaneously in complex pedigrees. The family material that we collected allowed us to compare affected siblings in a pairwise manner.

Siblings share 50% of alleles on average but can be expected to share more than 50% of alleles that are located close to loci that are involved in the phenotype, regardless of the mode of inheritance. This method has been used before to exclude the involvement of a number of candidate genes in FCP in Labrador retrievers (Salg et al. 2006). We used two intrinsically different sets of markers. The microsatellite markers were widely spaced but because of their high informativity they can cover large chromosomal regions in linkage analysis. The SNP set was more densely spaced, but the informativity per marker is limited because there are only two alleles per SNP and several SNPs are required to define a region effectively. Because of the different nature of the sets, we chose to analyze the data separately and to compare the results after data analysis. In this way, we limited the number of candidate regions for FCP loci to three, two on CFA01 and one on CFA13. Fine mapping pointed to there being two FCP loci on CFA01: the region with an NPL score between 4 and 5 (the drop 1 region) spans positions 53-76 Mb of the chromosome. The SNP data suggested that there could be two closely situated FCP loci in the region, but the resolution between the peaks was insufficient to conclude that they corresponded to separate loci. The minimum p-value obtained for the region was 3.9×10^{-5}, a value 2-fold higher than the threshold value for significant linkage and should be considered suggestive of such a linkage (Lander & Kruglyak 1995). Fine mapping led to an increase in the NPL score in the region around 110 Mb on CFA01 and in the region around 55 Mb on CFA13. The p-values in these regions were also considered to be suggestive of linkage with FCP.

Comparison of haplotypes between affected dogs of different families did not reveal a common haplotype in the peak regions (data not shown). This could be a reflection of the complex, multifactorial etiology of the disorder. Large-scale DNA sequence analysis and genetic studies of other dog cohorts are needed to confirm the involvement of the loci identified in this study.

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Appendix

Table I Microsatellite markers set

Chromosome	Position (Mb)	Marker name		Chromosome	Position (Mb)	Marker name	
1	10,34	FH2452	Werner et al. 1999	17	8,53	DTRCN1	
1	11	FH2016		17	12	FH2321	
1	20	FH2313		17	26	REN294E18	
1	25	FH3413		17	40	FH4023	
1	37	REN162B09	#	17	45	FH3995	
1	48	REN112I02		17	63	PEZ8	
1	59	C01.424		17	67	FH2869	
1	64	C01.251		18	4	FH4060	
1	78	REN159f24		18	8	FH3944	
1	85	FH2309		18	11	REN186N13	
1	97	AHT138		18	41	FH3815	
1	108	FH2326		18	47	FH3824	
1	115	FH2294		18	52	REN47J11	
1	124	CFA01_124	Hoffmann et al. 2008	18	61	FH2429	
2	18,017	FH2087U	Neff et al. 1999	18	65	AHT130	
2	27,48	REN280B15		19	4	FH3969	
2	36,858	REN150M24		19	18	FH2206	#
2	44,16	FH2613		19	25	FH3313	
2	52,12	FH2237		19	36	FH3491	
2	65,5	FH2608		19	46	REN213G21	
2	72,55	C02.894		19	54	FH3299	
2	78,3	AHT111		20	5	PEZ19	
2	78,9	C02.30		20	9	REN55P21	
2		REN149E24	#	20	17	FH2951	
3	4,56	FH3396		20	25	REN100J13	
3	13	REN161A12		20	37	REN93E07	
3	14,1	FH2137		20	47	FH3771	
3	30	FH2131		20	56	REN114M19	
3	45	FH2320		21	3	FH2312	
3	50	REN92B17		21	10	FH3624	
3	57	FH3464		21	20	FH3823	
3	68	REN260I04		21	33	FH2441	
3	70	FH2316		21	39	REN118B15	
3	77	REN216N05		21	43	FH3880	
3	83,7	FH4076		21	49,7	FH3803	
3	84	FH2107		22	4	REN49F22	
3	92	FH2302		22	7,3	REN128H16	
4	5	REN298N18		22	12,9	FH3355	
4	9	FH2773		22	22,2	REN158p08	
4	15	REN171H02		22	40	FH3411	
4	20	FH3310		22	50	REN245C13	
4	40	FH2412		22	55	FH3274	
4	70	REN160J02		22	64	FH3853	
4	75	AHT103		23	6	FH2508	
4	88	FH2457		23	11	FH4033	
4		FH2142	#	23	12	FH3078	
5	9,39	FH3928		23	19	FH2626	
5	10,35	FH2594	Werner et al. 1999	23	31	REN113M13	
5	12,68	FH2140		23	38	FH2227	#
5	16,19	REN42N13		23	50	FH2001	
5	22,8	ZUBECA6		23	55	REN181K04	
5	32,9	FH3702		24	7	FH3023	
5	40,35	DTR05.8		24	12	FH2159	
5	49,37	REN262G24		24	17,6	REN209L11	
5	51,74	REN192M20		24	23	FH2495	
5	65,4	FH2383	Werner et al. 1999	24	32	FH3083	
5	76,16	FH3450		24	44	FH3287	
5	82,48	REN122J03		24	50	CFA24_50	Hoffmann et al. 2008
5	89,32	CPH14		25	6	FH3977	
6	7,9	FH2525	#	25	14	FH2318	
6	11,05	FH2576		25	17	FH2324	
6	19,98	CFA06_20	*	25	28	FH3979	
6	20,42	AHT109		25	42	FH3627	
6	31,3	REN149M14		25	49	FH4027	
6	32,15	FH2335	Neff et al. 1999, #	25	49,04	AHT140	Neff et al. 1999
6	34	CFA06_34	*	25	54	CFA25_54	Hoffmann et al. 2008
6	38,65	CFA06_38	*	26	4	CFA26_4	Hoffmann et al. 2008
6	43	CFA06_43	*	26	8	REN87o21	

6	49,2	FH2119	
6	67,42	C06.636	Neff et al. 1999
6	70,55	FH3303	
6	80	CFA06_80	
7	8	FH2226	
7	12	FH3972	
7	18	FH2917	
7	29	REN162C04	
7	33	REN286O18	
7	50	REN200G14	
7	62	FH2201	
7	70	FH2581	
7	79,765	FH2301	Neff et al. 1999
7	81	FH2973	
8	4	FH3241	
8	6,2	FH2149	Neff et al. 1999
8	14	FH3425	#
8	19	REN67O13	
8	28	PEZ9	Neff et al. 1999, #
8	33	C08.410	
8	45	C08.373	
8	65,4	FH2138	Neff et al. 1999
8	68	C08.618	
9	7,85	GALK1	
9	16,4	FH2263	
9	18,86	C09.173	
9	21,054	C02.342	
9	24,53	REN01E05	Jouquand et al. 2000
9	34,797	FH2186	
9	48,14	REN278L10	
9	48,2	C09.250	Neff et al. 1999
9	54,13	REN177B24	
9	60,43	FH2885	
9	63,2	REN287G01	
10	3,45	C10.404	Neff et al. 1999
10	12	FH4081	
10	16,3	C10.781	
10	23	RVC8	
10	32	FH2293	
10	40	REN181G20	
10	49	ZUBECA1	
10	56	FH2422	
10	66,3	C10.602	
10	79	FH3381	
10	87	C10.865	
11	5,8	AHT137	
11	8,62	FH3203	
11	12,5	FH4031	
11	19,2	REN242K04	
11	32,15	FH2004	
11	39,25	REN245N06	
11	46,6	FH3393	**#**
11	56,35	REN194N17	
11	65,338	C11.20	Ostrander et al. 1993
11	67,76	C11.873	
11	72,8	DGN13	
11	77,406	C11.750	Mellersh et al. 1997
12	4	FH2200	
12	10	REN258L11	
12	14	FH2152	
12	17	REN213F01	
12	23	FH3711	
12	27	FH2401	
12	43	REN208M20	
12	50	FH2707	
12	62	FH2347	**#**
12	75,48	TBP	
12	77,4	C12.852	Neff et al. 1999, #
13	4	C13.391	
13	6	FH3494	
13	15	REN307K04	
13	22	FH3619	
13	32	REN286P03	
13	37	FH2348	
13	45,4	REN227M12	
13	49	REN166I13	
26	14	AHTK211	
26	20	REN299M21	
26	26	DGN10	
26	35	FH2130	
27	5	FH2289	
27	9,8	FH4001	
27	10	PEZ16	
27	18	REN277O05	
27	27	FH2925	
27	39	REN56C20	
27	42	FH4019	
27	46	REN181L14	
27	48	REN72K15	
28	4	FH2759	
28	12	FH2208	
28	23	FH3033	
28	37	REN51I12	
29	6	FH2952	
29	15	FH3878	
29	21	REN165M10	
29	28	REN164F23	
29	40	FH2177	
30	6	FH3489	**#**
30	12	REN51C16	
30	21	REN248F14	
30	33	FH3632	
30	35,95	FH2305	Mellersh et al. 1997
30	37	FH3053	
30	41	LEI-1F11	
31	4	CFA31_4	Hoffmann et al. 2008
31	10	REN43H24	
31	14	FH2582	
31	26,7	FH2189	
31	30	REN110K04	
31	37	FH2712	
32	9	CPH2	
32	15	FH2875	
32	20	FH3635	
32	26	AHT127	
32	29	FH3294	
32	41	CFA32_41	Hoffmann et al. 2008
33	9	FH2965	
33	13	FH3608	
33	19	FH2361	
33	27	REN291M20	
33	32	FH2165	**#**
34	4	FH3721	
34	11	REN125M11	
34	19,5	CFA34_21	*
34	14	REN64E19	
34	27	REN243O23	
34	35	FH3836	
34	38	CFA34_38	*
34	43	FH4010	
35	14	REN01G01	
35	19	REN282I22	
35	22,6	REN214H22	
35	25,9	REN172L08	
35	26,4	FH3770	
35		FH3570	**#**
36	7	REN106I07	
36	9	FH2611	
36	17	REN179H15	
36	31,47	DTR36.3	
36	33	CFA36_33	Hoffmann et al. 2008
37	5	FH3272	
37	13	H10101	
37	22	REN67C18	
37	25	FH2532	
37	30	FH2587	
38	4	CFA38_4	Hoffmann et al. 2008
38	15	REN02C20	
38	26	FH3399	
38	23	REN164E17	
X	12	REN230I20	
X	17	REN101G16	

13	52	AHT121		X	30	FH2548	
13	53	REN154P17		X	35	FH2997	
13	55	C13.758		X	40	FH3027	
13	66	CFA13_66		x	60	D04614	#
14	9	FH3951		X	104	FH2584	
14	15	C14.866		X	125	REN144O22	
14	24	FH3725					
14	36	FH2658					
14	42	REN289L09					
14	47	FH2763					
14	57	PEZ10					
15	4	FH3802					
15	21	REN06C11					
15	31	FH2535					
15	43	FH2295					
15	46	REN193M22					
15	58	FH2278					
15	66	FH3939					
16		AHT131	Neff et al. 1999, #				
16	9	REN214L11					
16	10	FH2670					
16	21	REN85N14					
16	34	FH2175					
16	41	REN275L19					
16	53	FH2155					
16	57	FH3592					

Microsatellite in this analysis derived from Guyon R. et al., (2003) PNAS USA 100, 5296-5301, unless mentioned otherwise.
#Primers that have been droped from analysis according Mendelian inconsistencies, umbiguous position or unreliable alleles.
*Primers developed in this study.

Table II Microsatellite markers developed in this analysis

Primer	Forward	Reverse	Length (bp)	Tm
CFA06_20	ATCATTTGAGGCCAGGTCAC	AGCTCATGCTCCTGCACTTT	230	60
CFA06_34	TTAGAGCATGTCCCCCAAGT	GGCACCTGCATTCAGAAAAA	200	60
CFA06_38	GCCCTTGTTCAGTCACTTCC	ACAGATCATGGGTGGTGGTT	212	60
CFA06_43	TGCTCAGCAACAGGAAGTGT	GGAATTGGAGGGCAAAGTG	200	60
CFA13_66	GCAGGTATTGGAACGGAAGA	CTCCCCATGCCAAATAAAAG	200	60
CFA34_21	GCAAGTCATTCAGAAGCAACA	GGGTGCCTGGGTATCTGTTT	278	60
CFA34_38	TCAGGGCCTTTGCACTTACT	AGGTCAGACGGTGAAACTGC	230	60

Tm annealing temterature

Table III SNPs used in fine mapping, based on CanFam2

SNP_Name	Coordinate	Chromosome	SNP_Name	Coordinate	Chromosome
BICF2G630717229	34137264	1	BICF2S23741429	112582398	1
BICF2S22951503	34562055	1	BICF2S22922837	113043126	1
BICF2S23330260	34709403	1	BICF2P276955	113534551	1
BICF2S23132369	34882167	1	BICF2P1250060	114373187	1
BICF2S23331333	35357560	1	BICF2S23740953	114673266	1
BICF2S23016273	35627282	1	BICF2S2376232	115224560	1
BICF2S23346012	35639195	1	BICF2S23144587	115630525	1
BICF2G630718315	35813194	1	BICF2P1017691	115829008	1
BICF2S23712395	36031298	1	BICF2S23340986	116898107	1
BICF2S23719696	36715676	1	BICF2S23313587	117678943	1
BICF2G630719404	37230310	1	BICF2P24150	118749689	1
BICF2S2296370	37504155	1	BICF2S23540163	119212819	1
BICF2S2346732	37975796	1	BICF2S23634246	119821640	1
BICF2S23357148	38617574	1	BICF2P866721	121551324	1
BICF2P751054	38749139	1	BICF2S23740902	45097472	13
BICF2S23150450	39021250	1	BICF2P335937	45296712	13
BICF2S23747409	39105004	1	BICF2P655492	45487223	13
BICF2S23353614	39494103	1	BICF2S22930790	45553282	13
BICF2S22928500	39700197	1	BICF2S2345235	45648375	13
BICF2G630721321	39912758	1	BICF2S23317261	45858161	13
BICF2S23310200	40344501	1	BICF2S23531662	45931580	13
BICF2G630721686	40607260	1	BICF2S23646268	46078163	13
BICF2S23338970	40761080	1	BICF2S23331765	46152604	13
BICF2S23347923	40940337	1	BICF2P271250	46330364	13
BICF2P31849	41329673	1	BICF2S2377060	46474006	13
BICF2P927968	41425344	1	BICF2S23512558	46511775	13
BICF2G630722718	41537951	1	BICF2S23159913	46828290	13
BICF2S23117990	41776867	1	BICF2P874254	46835719	13
BICF2S24113574	42305231	1	BICF2S23235509	47138591	13
BICF2P436181	42733742	1	BICF2S23349464	47321328	13
BICF2G630723627	42923396	1	BICF2S23356476	47463718	13
BICF2S23354710	43136958	1	BICF2S2334718	47520123	13
BICF2G630724151	43597873	1	BICF2P1327845	47746638	13
BICF2G630724281	43789044	1	BICF2P1278270	47831998	13
BICF2S23137417	43836428	1	BICF2P171920	48072175	13
BICF2S22957329	44308092	1	BICF2S23336942	48325733	13
BICF2P712400	44785193	1	BICF2S23344202	48331086	13
BICF2S23331432	44980629	1	BICF2P1468876	48489093	13
BICF2S23745911	45187887	1	BICF2S23059102	48504864	13
BICF2P859516	45351171	1	BICF2S23150657	48568622	13
BICF2S23335031	45502089	1	BICF2P1154531	48608683	13
BICF2S2446088	45816753	1	BICF2S23717994	48852330	13
BICF2S23342760	46216534	1	BICF2S2313737	48933758	13
BICF2S23648150	47130003	1	BICF2P53488	49052384	13
BICF2S23332289	47448293	1	BICF2S24318448	49104790	13
BICF2S23748662	47565955	1	BICF2S23753224	49369147	13
BICF2S23229162	48012013	1	BICF2S23040001	49395325	13
BICF2S23735372	48026187	1	BICF2P1346659	49603225	13
BICF2S2302578	48032056	1	BICF2P168493	49703633	13
BICF2S23339349	48046729	1	BICF2S23323053	49792447	13
BICF2S23334509	48250338	1	BICF2S23335532	49842712	13
BICF2S23330175	48983314	1	BICF2S2368441	50122175	13
BICF2P1037303	49314836	1	BICF2S22960785	50174905	13
BICF2S23116033	49818042	1	BICF2P1101427	50198870	13
BICF2P600785	50049732	1	BICF2S23762895	50260623	13
BICF2G630725420	50211077	1	BICF2S23421415	50375760	13
BICF2P1161563	50729000	1	BICF2S23024682	50589336	13
BICF2S23354438	51040310	1	BICF2S23759341	51060679	13
BICF2S2322487	51218321	1	BICF2S2451432	51324335	13
BICF2S23153369	51639510	1	BICF2S2319117	51428216	13
BICF2S23147389	52045088	1	BICF2S23010274	51797686	13
BICF2P1006091	52520864	1	BICF2S2443933	51825881	13
BICF2S23912610	52709786	1	BICF2S236858	51864288	13
BICF2S23322081	52919738	1	BICF2S23323096	52044262	13
BICF2S23321222	55042346	1	BICF2S2338586	52100454	13
BICF2S23056890	55231305	1	BICF2P1451812	52131463	13
BICF2S23449462	55455065	1	BICF2S23312899	52338079	13
BICF2S23346068	56479731	1	BICF2S2453064	52532013	13

BICF2S23613346	56762645	1	BICF2P316628	52570352	13
BICF2S22931416	57346192	1	BICF2S23327421	52687782	13
BICF2S23339718	57634964	1	BICF2S23429666	52772796	13
BICF2S23346766	57843847	1	BICF2S23332437	53253040	13
BICF2S23012	58613330	1	BICF2S23234199	53310666	13
BICF2S2331932	58872076	1	BICF2S23319247	53442018	13
BICF2P932423	59199025	1	BICF2S23612091	53547518	13
BICF2S23553705	59382749	1	BICF2S2412848	53728424	13
BICF2S23139587	59553653	1	BICF2S23131503	53848255	13
BICF2S23035172	59648285	1	BICF2S232859	53920426	13
BICF2S2338393	60243597	1	BICF2S23355267	54051703	13
BICF2P181563	60571533	1	BICF2S23326163	54301235	13
BICF2S23757532	60741577	1	BICF2S2363383	54434550	13
BICF2S230392	60816938	1	BICF2P319458	54513420	13
BICF2P1146321	61525462	1	BICF2S22912758	54617670	13
BICF2P1255021	61700778	1	BICF2P456999	54908411	13
BICF2P786832	62109037	1	BICF2S2331875	55059378	13
BICF2S23425890	62365635	1	BICF2P233034	55196084	13
BICF2S23015531	62376782	1	BICF2S23414519	55215567	13
BICF2S22921751	62726637	1	BICF2S23041300	55397987	13
BICF2S23323362	62969838	1	BICF2P978439	55412902	13
BICF2S2331647	63617638	1	BICF2S23349760	55506895	13
BICF2P260198	64370298	1	BICF2S23326706	55518793	13
BICF2S23610603	64523268	1	BICF2S23317487	55608235	13
BICF2S23553384	64833875	1	BICF2S23724993	55938294	13
BICF2P657118	65262614	1	BICF2S23341702	56078590	13
BICF2S23259729	65406895	1	BICF2P1322907	56160631	13
BICF2P1019872	65568448	1	BICF2S234298	56422600	13
BICF2S23356349	65778631	1	BICF2P1277338	56861990	13
BICF2S2339364	66396802	1	BICF2S23312697	57107455	13
BICF2S23329564	66664582	1	BICF2S23764906	57156965	13
BICF2P339384	66809310	1	BICF2P694929	57379292	13
BICF2S24310925	67173228	1	BICF2S23330456	57501988	13
BICF2S23146106	67695038	1	BICF2P1362720	57664713	13
BICF2P169546	67932392	1	BICF2S2333762	27466828	34
BICF2S23334210	68136988	1	BICF2P4895	28016932	34
BICF2P1350924	68611372	1	BICF2P401724	28488489	34
BICF2P208850	68615624	1	BICF2P452210	28683065	34
BICF2S2454604	69188060	1	BICF2P449899	28946317	34
BICF2S2315618	69338261	1	BICF2P377171	29065256	34
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BICF2P965815	34147435	28

Part II

Studying of other skeletal dysplasia in other breeds

Chapter 5

Polymorphic microsatellite markers for genetic analysis of collagen genes in suspected collagenopathies in dogs

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Abstract

Defects in collagen proteins cause a variety of disorders in man. It can be expected that collagen gene mutations are involved in collagenopathies in dogs. The collagen genes COL3A1, COL5A1, COL5A2, COL6A1, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, and COL11A1 were identified on the canine genome based on the homology with the human genes. Simple sequence repeats (microsatellites) were found in the chromosomal regions of these genes and investigated for polymorphism in Labrador retrievers, Bernese Mountain dogs, Boxer dogs and German shepherd dogs by PCR and subsequent detection of the DNA products. Nine informative microsatellite markers were identified. The markers closely situated to COL9A1, COL9A2 and COL9A3 were used to investigate the involvement of the genes in cranial cruciate ligament rupture in Boxer dogs. It was found that these genes are probably not involved in this abnormality. The markers described here will be useful for a candidate gene approach of suspected collagenopathies specific to dog breeds.

Introduction

Collagen is the collective term of the filamentous protein component of connective tissue. Defects in collagen are at the basis of a large number of diseases in humans, and these disorders are often caused by inherited mutations in genes encoding collagen proteins (Mundlos and Olsen, 1997; Myllyharju and Kivirikko, 2001; Eyre, 2002; Reginato and Olsen, 2002). A number of suspected collagenopathies occur in dogs, for example chondrodysplasia and dwarfism in Alaskan Malamute (Fetch et al., 1973), miniature poodles (Riser et al., 1980), Labrador retriever (Carrig and Sponenberg, 1988), Scottish deerhounds (Breur et al., 1989), Great Pyrenees (Bingel and Sande, 1994) and Samoyed (Aroch et al., 1996). The pathological findings of multiple epiphyseal dysplasia in Beagles were shown to be similar to multiple epiphyseal dysplasia in man by morphological and histochemical features (Rasmussen, 1975). Ehlers-Danlos like syndrome (also called cutaneous asthenia or collagen dysplasia) has been seen in dogs with clinical signs of skin hyperextensibility and fragility. In addition, this entity has been described in dogs with vessel fragility, subcutaneous hematomas and joint laxity (Minors et al., 1983; Paciello et al., 2003; Barrera et al., 2004). To analyze collagen genes as candidates for involvement in these disorders in dogs, closely situated polymorphic DNA markers form a useful tool.

Microsatellites are tandem repeats of di-, tri-, or tetranucleotides which are being found dispersed along genomes of vertebrates (Ellegren, 2004). A natural variation in repeat number occurs frequently at a microsatellite locus, resulting in variation of DNA sequence length and polymorphism. These length variants or alleles are stably transmitted to offspring and the markers can be used to study genetic linkage or association with phenotypes such as an inherited disorder. A disease which has been linked to a microsatellite marker is, at least in part, caused by DNA mutations in the proximity of that marker.

We identified microsatellite markers in the canine genome which are associated with ten collagen genes, including the gene coding for collagen type III alpha 1 (COL3A1), COL5A1, COL5A2, COL6A1, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1 and COL11A1. The genes COL9A1, COL9A2 and COL9A3 are candidate genes for involvement in rupture of the cranial cruciate ligament (CCLR) of the stifle joint in dogs (Wilke et al., 2005). In most dogs, this condition is associated with development of stifle arthritis and it is one of the most common causes of hind limb lameness in certain breeds as Rotweilers and Newfoundland dogs (Whitehair et al, 1993). A high incidence of CCLR in a birth cohort of Boxer dogs studied at our Department was observed. We genotyped the markers closely situated to COL9A1, COL9A2 and COL9A3 genes in Boxer dogs from the cohort with CCLR to explore the involvement of collagen IX in the disorder.

Material and methods

Animals

DNA samples of 12 Boxer dogs, 12 Bernese mountain dogs, 12 German shepherd dogs and 12 Labrador retrievers who did not share parents were selected. All Labrador retrievers were from the breeding program of the Royal Dutch Foundation for Guide Dogs for the Blind. The dogs were purebred Labrador retrievers, except for one dog who was a 1:1 mix of Labrador and Golden retriever. The dogs from the other three breeds were also purebred and registered by the Dutch Kennel Club. Apart from these, 32 Boxer dogs from 12 families with CCLR were selected for molecular genetic studies. These families were chosen from the birth cohort of the Dutch Boxer dog population which has been extensively studied (van Hagen et al., 2005). CCLR was diagnosed by physical finding of stifle problems including cranial drawal sign, positive cranial tibia thrust and confirmed by radiograph or arthrotomy during corrective operation. Genomic DNA was isolated from EDTA blood using the salt extraction method (Miller et al., 1988), and frozen at -20°C until use.

Identification of collagen genes and microsatellites

The cDNAs of the collagen genes of interest were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and compared with the assembled canine genome sequence, build 2.1, using BLASTN software (http://www.ncbi.nlm.nih.gov/genome/seq/CfaBlast). The Genbank accession numbers were: NM_000090 (COL3A1), NM_000093 (COL5A1), NM_000393 (COL5A2), NM_001848 (COL6A1), NM_004369 (COL6A3), NM_078485 (COL9A1), NM_001852 (COL9A2), NM_001853 (COL9A3), NM_000493 (COL10A1) and NM_001854 (COL11A1). The resulting hits displayed high similarity to the cDNAs and could be identified as the coding regions of the corresponding collagen genes. The coding DNA sequences of the human and dog genes were approximately 90% identical and the dog genes were in each case the only genes that displayed this high level of similarity. The localization of these reconstructed genes on the canine genome is shown in Table 1. COL3A1 was localized closely to COL5A2 on canine chromosome 36 and the same polymorphic marker can be used for these two genes. The genomic regions of the genes were screened for LINE and SINE elements by RepeatMasker (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) and microsatellite DNA sequences were selected outside these elements by Tandem Repeat Finder software (Benson, 1999). For most genes, repeats of the dinucleotide sequence CA were chosen which were not interrupted by other DNA sequences. The exceptions were COL10A1 for which a TC repeat was selected and the COL6A1 gene region were a GAAA repeat was chosen. The lowest number of repeats was 14.

Table 1. The chromosomal location of canine collagen genes with known phenotypes in man.

Gene	CFA[a]	Mb[b]	Human phenotype of gene mutations
COL3A1	36	33.7	
COL5A1	9	43.5	Skin hyperextensibility, skin and vessel fragility, joint laxity
COL5A2	36	33.7	
COL6A1	31	41.6	Patellar luxation, hyperextension syndrome
COL6A3	25	51	
COL9A1	12	36	
COL9A2	15	5	Multiple epiphyseal dysplasia
COL9A3	24	49.4	
COL10A1	12	74.5	
COL11A1	6	48.9	Legg-Perthes disease

[a] CFA = Canis familiaris chromosome number
[b] Position in Mega base pairs of chromosomal DNA sequence

Genotyping of microsatellite markers

Oligonucleotides (primers) for PCR amplification of DNA fragments containing the repeat were designed with Primer3 software (Rozen and Skaletsky, 2000). A phage M13 based tag (GTTTTCCCAGTCACGAC) was added at the 5'end of forward primers. PCR amplification was carried out in a reaction volume of 15 µl containing 25 ng of genomic DNA, 1 µM M13 tagged forward primer, 10 µM reverse primer, 10 µM M13 based tag primer labeled at the 5'end with 6-FAM (Eurogentec, Maastricht, The Netherlands), 1X PCR gold buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.3 U Amplitaq Gold (Applied Biosystems, Foster

City, CA, USA). Thermal cycling was performed in a GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA) with the following program: 5 minutes at 95°C, followed by 10 cycles of 30 seconds at 95°C, 15 seconds at the annealing temperature (Table 2), 30 seconds at 72°C, then another 25 cycles of 30 seconds at 92°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C. The program was completed with 10 minutes at 72°C.

The PCR reactions were diluted 10-30 fold with H_2O and 2 µl of the dilution was mixed with 10µl formamide and 0.2 µl GS500 LIZ or TAMRA size standard (Applied Biosystems, Foster City, CA, USA). The products were analyzed after capillary electrophoresis and automatically detected using the Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA, USA). The DNA products were classified by size with Genescan Analysis version 3.7 software (Applied Biosystems, Foster City, CA, USA) and alleles were assigned.

Results

Identification of collagen genes and polymorphic microsatellites

Location specific microsatellite repeats were readily detected after masking of LINE and SINE elements in genomic DNA sequences of the dog. Mainly dinucleotide repeats were chosen because these tend to be more stably inherited than tetranucleotide repeats. The lowest number of repeats was 14 for the COL6A1 and COL5A1 markers. The polymorphic nature of these microsatellites was investigated by genotyping 12 dogs of four different breeds. Each of the markers displayed three or more alleles. More importantly, a majority of markers displayed a high level of at least 50% heterozygosity (Table 2). Only the COL5A1 marker showed low levels of heterozygosity in each of the investigated breeds. The COL6A1 microsatellite, like COL5A1 with a low number of repeats in the genome sequence, showed a high level of heterozygosity in all breeds with the highest percentage of 92 % in Labrador retrievers. The markers for COL9A1, COL9A2 and COL9A3, candidate genes for CCLR, showed a high level, up to 100% of heterozygosity in Bernese Mountain dogs breeds.

Table 2. Polymorphic microsatellite markers close to collagen genes of the dog.

gene	O[a]	primer (5'-3')	T[b]	range (bp)	#[c]	Het[d]			
						LR	Box	BMD	GSD
COL3A1	F	ttgatacataccattggacc	60	494-503	5	59	75	8	33
COL5A2	R	tatgtgtataggcattggct							
COL5A1	F	ctgtaagcctggaactctga	58.5	355-362	3	13	0	0	17
	R	ctgtgattctcataggccag							
COL6A1	F	aactgcttactcattgaagg	60	204-216	4	92	83	58	67
	R	acttgcaaccatcacattac							
COL6A3	F	gtcacttggcaccaggttag	60	330-335	4	36	38	58	50
	R	gtcaccagatgcagctcaga							
COL9A1	F	gattaactccaggcagaatc	60	306-312	4	75	38	100	33
	R	cctaggagtgactctctgct							
COL9A2	F	ctaccagcaggcaactccaa	60	353-357	3	75	25	50	50
	R	caggatgccaacgagaacaa							
COL9A3	F	gctccagagttcaggctcag	55	213-223	4	25	50	50	50
	R	aggaggtcggagatggagat							
COL10A1	F	gaagaagagagacaggtgtg	60	310-318	4	80	0	0	67
	R	cttgtctacgtggcttagtc							
COL11A1	F	catcacaatctctaaggcgt	60	389-414	3	37	83	42	0
	R	gcatgatgagttcggttagg							

[a] Orientation of oligonucleotides; F= forward primer, R= reverse primer
[b] Annealing temperature in ^{o}C
[c] Number of alleles
[d] Percentage of heterozygous Labrador retrievers (LR), Boxer dogs (Box), Bernese Mountain dogs (BMD) and German shepherd dogs (GSD)

Genetic analysis of CCLR in Boxer dogs

The incidence of CCLR in a birth cohort of Boxers in the Netherlands was 22% in dogs upto the age of 9 years (Data not shown). To investigate the involvement of the genes COL9A1, COL9A2 and COL9A3 in CCLR in this breed, the closely situated microsatellite markers were analyzed in 32 affected dogs from 12 pedigrees (Fig. 1). Because there is no single allele that is shared by all the affected dogs, the results indicated that COL9A1, COL9A2 and COL9A3 probably do not play an important role in the etiology of CCLR. For instance, for the COL9A1 marker we found that dog 2 of pedigree B does not share an allele with dogs 3, 4, 6 and 7 of the same pedigree (Fig. 1). Similarily, COL9A2 can be excluded because dog 1 of pedigree I does not have a marker allele in common with dog 2 of pedigree J and COL9A3 can be excluded because dogs 1 and 2 of pedigree C does not share an allele of the gene marker with dog 3 of the same pedigree.

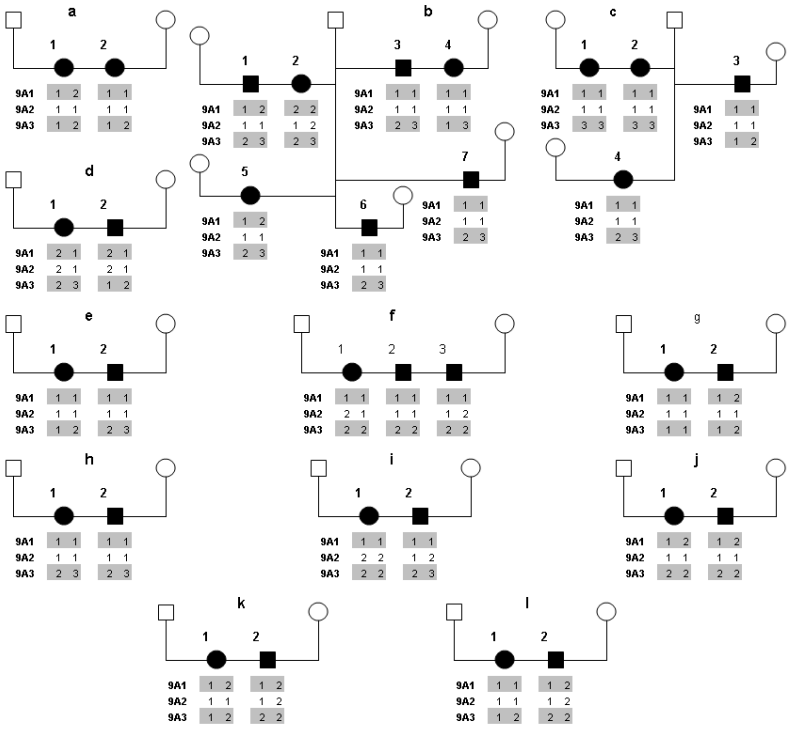

Figure 1. Pedigrees of Boxer dogs, including 32 dogs affected by cranial cruciate ligament rupture (CCLR).

Genotypes for COL9A1, COL9A2 and COL9A3 are shown by allele numbers below individual dogs. Circles and squares represent female and male dogs, respectively. Filled symbols indicate CCLR affected dogs; the phenotype of the other dogs is unknown.

Discussion

With the advent of the PCR technique and the identification of polymorphic microsatellite markers, the number of identified human disease genes has surged. Currently, the complete genome sequence of the dog is available, so that genes can be identified based on the similarity to human gene sequences and microsatellites can be found by scanning the chromosomal regions of these genes. In this study, we identified informative markers that are useful for the evaluation of a variety of collagen genes in suspected collagenopathies in dogs.

CCLR is one of the most common causes of lameness of the hind limb in certain breeds of dogs (Whitehair et al., 1993). Wilke et al. (2005) suggested a recessive mode of inheritance with reduced penetrance for this trait, and excluded the genes coding for fibrillin 1 (FBN1) and cartilage oligomeric matrix protein (COMP), and the genes COL9A1 and COL9A2 as candidates for the disorder in Newfoundland dogs because no significant linkage was observed. Markers for COL9A1, COL9A2 and COL9A3 were developed and tested for association to CCLR in a cohort of Boxer dogs with a high incidence of the condition. The breed predisposition to CCLR suggests that a common genetic factor is involved in the etiology of the disorder. Because each of the developed markers is located very close to the corresponding collagen gene, the chance of recombination between the marker and the gene is small. Therefore, it can be expected that affected dogs are homozygous for the same allele if the gene is involved in CCLR by a recessive allele. In the case of a dominant effect it is expected that affected dogs share one allele from a common ancestor. For each of the genes under investigation, we identified affected Boxer dogs which do not share a marker allele with other affected Boxer dogs (Fig.1). In the case of COL9A1 and COL9A3, affected dogs were found from the same pedigree which did not share an allele of the closely situated marker. In the case of COL9A2 we identified affected dogs from different families which did not share marker alleles. We therefore conclude that the collagen IX genes do probably not play a role in CCLR in Boxer dogs. This analysis demonstrates the usefulness of the markers described here.

It is known that mutations of type VI collagen genes can cause Ullrich congenital muscular dystrophy and Bethlem myopathy (Lampe, 2005). In dog puppies which start to walk, a similar disorder (hyperextension syndrome) has been observed (Rudy, 1965). Clinical features of muscular contracture in patella luxation that resemble some features of Ullrich congenital muscular dystrophy also warrant further investigation of collagen type VI genes in dogs with this disorder. The markers for COL6A1 and COL6A3 described here can be used for such an investigation. It should be noted that the genes COL6A1 and COL6A2 co-localize in the genomes of man, mouse and chicken and that in genome build 1.1 of the dog these genes were also closely situated on an assembly of contiguous DNA sequences of unknown

chromosomal origin. In build 2.1, COL6A1 is localized on CFA31, while the COL6A2 localization is still not known. Possibly, the dog genome is ambiguous in the COL6A1 region and the polymorphic marker described here for COL6A1 may prove to be also useful for the evaluation of COL6A2.

Genetic studies of collagenopathies in dogs with the markers described here should reveal the possible involvement of the corresponding candidate genes.

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Chapter 6

Evaluation of candidate genes as cause of chondrodysplasia in Labrador retrievers

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Abstract

Chondrodysplasia (CD) is a disabling, hereditary disease in Labradors with short long-bones, warranting genetic screening in families at risk. The familial relationship has been studied in 8 CD-affected Labradors as well as the involvement of 8 candidate collagen genes (i.e., COL9A1, COL9A2, COL9A3, COMP, MATN3, COL2A1, COL11A1 and COL11A2) and of a sulphate transporter glycoprotein (SLC26A2) gene in these dogs and in 14 control Labradors. CD seems to segregate as an autosomal recessive trait. The candidate genes could not be implicated in CD.

Introduction

Eight Labradors with chondrodysplasia (CD), referred in a 4 years period, revealed characteristic features due to short frontlegs with radius curvus (Figure 1). All affected dogs and some unaffected littermates were radiographed, revealing disturbed development of long bones. Biopsies from two affected dogs taken at 12-weeks from the 9th rib at the physeal area were stained. Microscopy revealed disorganized chondrocyte columns and irregularities in endochondral ossification. Retina dysplasia, as reported in Labradors with ocular skeletal dysplasia (OSD) (Du et al., 2000) was not present in these cases. The Labrador CD described here has strong similarities with multiple epiphyseal dysplasia (MED), spondyloepiphyseal dysplasia congenita (SEDC), and with Stickler syndrome in man (Lachman, 1998). In man, MED coincides with mutations in genes encoding collagen COL9A1, COL9A2, COL9A3, COMP, matrilin 3 (MATN3), and SLC26A2 (Briggs and Chapman, 2002). Mutations in the gene encoding collagen COL2A1 have been associated with SEDC (Lee et al., 1989). Stickler syndrome is caused by mutations in genes encoding COL11A1, COL11A2, and COL2A1 (Sirko-Osadsa et al., 1998). Collagen type 2, 9 and 11 are important extracellular matrix proteins which are produced by chondrocytes in the growth plates of the long bones (Kivirikko, 1993).

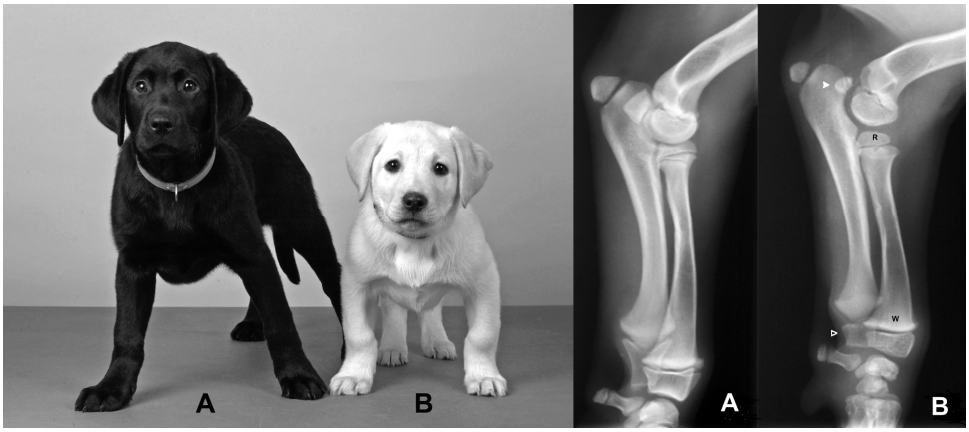

Figure 1. Comparison of normal and chondrodysplatic Labrador retrievers.

Mediolateral radiographs of the antebrachium of a normal male (A) and a chondrodysplastic female Labrador of 12 weeks of age (B) with absence of the anconeal process (closed arrow head), cubital configuration of the ulnar styloid process (open arrow head), widened metaphyseal areas (“W”), underdevelopment of the radial head (“R”), and absence of a pronounced medial humeral.

Materials and methods

Marker selection and genotyping

The matrilin 3 gene was identified on the canine genome based on comparison with the human MATN3 cDNA sequence (NM_002381). Repetitive DNA sequences in proximity of the gene were selected using Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html). A repeat of the dinucleotide sequence CA was selected from intron 1 of the gene. The target DNA sequences for the PCR of this repeat were 5'-cacacgtgctcgttttcatc-3' and 5'-ctttatgcccaagcaccagt-3'. Table 1 describes the origin of the other markers used in this study.

A phage M13 based sequence tag (5'-gttttcccagtcacgac-3') was added at the 5' end of the forward primers of COL9A1, COL9A2, COL9A3, MATN3, and COL11A1. The tail allowed fluorescent labeling of the products with a universal third primer.

The amplification was carried out in a reaction volume of 15 µl containing 25 ng of genomic DNA, 0.03 µM forward primer, 0.3 µM reverse primer, 0.3 µM M13 tag sequence primer labeled at the 5' end with 6-FAM, 10 X PCR Gold buffer, 2.5 mM $MgCl_2$, 0.2mM dNTP's and 0.06 µl AmpliTaq Gold (Applied Biosystems). The PCR was initiated with 95°C for 5 minutes followed by 10 cycles of 95°C for 30 seconds, 15 seconds at 55°C and 30 seconds at 72°C, followed by 25 cycles at 92°C for 30 seconds, 55°C for 15 seconds, and 30 seconds at 72°C.

Table 1. Microsatellite markers associated with candidate genes for MED and SEDC

Gene	Chromosome	Marker	Distance to gene	Reference
COL9A1	12	NA	195 kb	Salg [a]
COL9A2	15	NA	650 kb	Salg [a]
COL9A3	24	NA	300 kb	Salg [a]
COMP	20	FH3771	117 kb	Guyon [b]
MATN3	17	NA	intragenic	Present study
COL2A1	27	FH4001	70.5 kb	Guyon [b]
COL11A1	6	NA	1.6 Mb	Salg [a]
COL11A2	12	FH2200	1.6 Mb	Guyon [b]

[a] Salg, et al., 2006. Am. J. Vet. Res. 67, 1713-1718.

[b] Guyon, et al., 2003. Proc. Natl. Acad. Sci. U.S.A. 100**,** 5296-5301.

The products were analyzed by capillary electrophoresis and automated detection using the 3100 Genetic Analyzer (Applied Biosystems). The alleles were assigned with GeneScan Analysis version 3.7 software (Applied Biosystems).

DNA Sequence analysis

The cDNA of the human SLC26A2 (NM_000112) gene was selected and compared with the canine genome using BLASTN software. Primer sets for the SLC26A2 gene were developed using Primer3 http://fokker.wi.mit.edu/primer3/input.htm (Table 2).

For each PCR reaction, 25 ng of canine genomic DNA was amplified with 5 µl 5x Gibco buffer, 0.5 µM of each F and R primer, 1.25 Unit of Recombinant Taq (Invitrogen), and 1.5 mM $MgCl_2$ in a final volume of 25 µl. The PCR program consisted of a denaturation step of 10 min at 95°C, followed by 35 cycles of 30 sec 95°C, 30 sec at 55°C, 30 sec 72°C and a final extension at 72°C for 10 minutes.

Phage M13 based tags were added to the 5' end of the forward (tag: 5'-gttttcccagtcacgac-3') and reverse (tag: 5'-caggaaacagctatgac-3') primers except for SLC26A2 exon 1 primers F2 and R2, which were used for the PCR and, separately, for the DNA sequencing reactions (Table 2).

The PCR products were processed for DNA sequence analysis with Big Dye chemistry version 3.1 as recommended by the manufacturer (Applied Biosystems). The sequencing products were analyzed using the 3100 Genetic Analyzer (Applied Biosystems).

Table 2. Oligonucleotides for DNA sequence analysis of SLC26A2

Fragment	Orientation	M13-tail	Sequence 5'-3'
Exon 1-1	F1	+	ttggttttgttttcccatca
	R1	+	tcccttgcagacctgagatt
Exon 1-2	F2	-	cagaagagttgccagtgcag
	R2	-	ccaaaaatgcccacagaaat
Exon 2-1	F1	+	gacattttgcgatgctctga
	R1	+	gccaatggcatacatttcct
Exon 2-2	F2	+	tgctgggtctattcccacag
	R2	+	gaggggctacaaaacggaat
Exon 2-3	F3	+	taccatgctgtcctctgcac
	R3	+	ggccatcaacttcaaaggaa

Results

Pedigree analysis showed that the patients shared one common ancestor. Three litters with patients displayed inbreeding or marriage loops within 4 generations from the patients. This finding suggested that this type of CD is inherited recessively (Figure 2.).

The possible involvement of the candidate genes COL9A1, COL9A2, COL9A3, COMP, MATN3, COL2A1, COL11A1, and COL11A2 was examined by using polymorphic microsatellite markers and of SLC26A2 by DNA sequence analysis. Blood samples were collected from 8 affected Labradors and 14 unaffected related Labradors and DNA was isolated. Unaffected littermates of 4 litters and 3 of 13 parents could be traced and sampled.

The affected Labradors are expected to be homozygous for a polymorphic marker in close proximity of the responsible gene. The analysis of each of the DNA markers revealed that at least one affected Labrador was heterozygous for the microsatellite markers situated close to COL9A1, COL9A2, COL9A3, COMP, COL2A1, COL11A1, and COL11A2. The affected dog of family G was homozygous for another allele of the MATN3 marker than the other affected dogs. These results suggest that there is no association between the studied genes and the CD phenotype, presented by this group of Labradors.

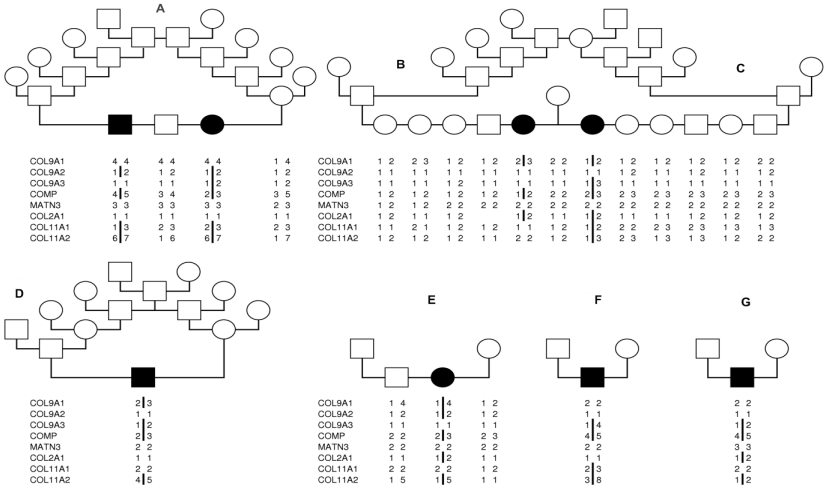

Figure 2. Genotypes of DNA markers close to candidate genes for chondrodyplasia in Labradors.

The pedigrees of E, F, and G were not known. The genotypes are displayed below the dogs of which DNA was available. The vertical bars indicate heterozygous genotypes of affected dogs, suggesting the corresponding genes are not involved in chondrodysplasia.

The DNA sequence analysis of the candidate SLC26A2 did not reveal a mutation when compared to the gene sequence from the canine genomic assembly or to the gene sequence of healthy siblings. Information about the markers and the genotyping protocol is supplied in the supplementary material.

None of the known genes causative for MED and SEDC in humans can be implicated in the development of MED in Labradors. Another important collagen of the extracellular matrix in physeal cartilage, collagen type 11, is also not involved in the development of MED in Labradors.

Collagen type 2 and collagen type 11 were excluded as causative genes in this study. Both collagen types are synthesized as pro-collagens that undergo extensive post-translational processing before they are secreted into the extracellular matrix (Kivirikko, 1993). Dysfunctional post-translational processes due to mutations in genes encoding the enzymes involved, could lead to disruption of the construction of the extracellular matrix with skeletal dysplasia as a result. This is likely in Labradors with OSD. Although expression of type 2 collagen was decreased in these Labradors, no mutation in COL2A1 was found (Du et al., 2000). The extracellular matrix of physeal cartilage is comprised of many different proteins that undergo post-translational processes and interact in numerous ways (Kivirikko, 1993). Consequently, numerous candidate genes exist and genome wide linkage analysis is warranted to localize the responsible gene.

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Chapter 7

Radiographic and genetic study of hereditary subluxation of the radial head in Bouviers des Flandres

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Submitted

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Abstract

Objective- To study radiographic and genetic aspects of hereditary radial head subluxation in Bouvier des Flandres.

Procedure- Twenty six related Bouvier des Flandreses affected with bilateral subluxation of the radial head and ten unaffected related dogs were radiographically studied. The DNA of these dogs and of 29 unrelated Bouviers, diagnosed with other, non-skeletal, diseases were analyzed with a genome wide screen of 1536 Single Nucleotide Polymorphisms (SNPs). In addition, an unaffected dam and her affected offspring were karyotyped.

Results- Both front legs of affected Bouviers were disproportionately short with caudolateral (sub-)luxation of the radial head. Angulation of the radial axis at the mid-diaphysis ranged from 9.3^{o}-30.3^{o} (mean $14.9 \pm 6.1°$), with an analyzed age of onset estimated between 0 and 4 months. Poorly defined medial coronoid processes and osteoarthritis of the elbow joint, cranial bowing of the olecranon, disturbed growth in length of the ulna with sharply demarcated spurs were noticed on radiographs of affected dogs. Genealogical analysis indicated that most affected Bouviers were closely related, but the mode of inheritance is not clear. The DNA analysis found that 205 SNPs were monomorphic in the affected Bouviers. Conventional chromosome staining revealed no numerical chromosomal aberration.

Conclusion and clinical relevance- Hereditary radial head (sub-)luxation in these Bouviers is characterized by angulation of the radial axis leading to caudolateral subluxation of the radial head and by insufficient growth of the distal ulna together with cranial bowing of the olecranon. Heredity based on genomic instability is a possible but yet unproven explanation.

Keywords; Bouvier des Flandres, elbow subluxation, hereditary radial head subluxation

Introduction

Non-traumatic elbow luxation has been described as congenital or hereditary elbow luxation in different breeds, including Miniature Poodle, Pekingese, Pug and Pomeranian (Bingel and Riser 1977, Lau 1977, Milton and others 1979). The use of the term "congenital" or "hereditary" suggests that the abnormality is present at birth and that familial involvement has been demonstrated, but most publications describe a single case (Bingel and Riser 1977, Lau 1977, Milton and others 1979). According to Kene and colleagues (1982), three forms of non-traumatic elbow luxation can be distinguished in the dog: type I is characterized by a caudolateral dislocation of the radial head with little or no displacement of the ulna; type II is characterized by a marked rotational dislocation of the ulna and a lesser degree of displacement of the radial head; and type III is marked by dislocation of both the radial head and ulna. Although no longitudinal studies have been published, the disorder is thought to develop postnatally. The subluxation of the radial head is suggested to be related to abnormal growth of the distal physis of the ulna (Lau 1977, Gurevitch and Hohn 1980). Although movement of the elbow joint is not painful, the range of motion of the joint may become limited. Several surgical interventions have been described, such as corrective osteotomies, transposition of the radial head and temporary transarticular fixation, and radial head resection (Newton 1974, 1985, Milton and others 1979, Gurevitch and Hohn 1980, Morgan and Griffiths 1981), but severe degenerative joint disease due to the malformation of the elbow joint makes the prognosis guarded.

In the present study, we report on an extended family of Bouvier des Flandres with subluxation of the radial head. In addition to clinical and radiographic findings, we also present genealogical, cytological and molecular genetic findings, together with a hypothesis regarding the pathophysiology of this trait.

Materials and methods

Animals

Two 10-month-old Bouviers des Flandres with clinical signs of bilateral front leg lameness and bilateral deformity were presented at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University. In collaboration with the Dutch Bouvier breeders club, a group of 43 Bouviers, consisting of 24 affected Bouviers and 19 non-affected relatives (34 dogs from the Netherlands and 9 dogs from Sweden), was investigated. The dogs were 2 months to 11 years old and all underwent complete orthopedic examination (Hazewinkel and others 2009). The family relationship between affected Bouviers (Figure 1) was investigated and a pedigree diagram was drawn (Figure 2). The investigated pedigree comprised 31 affected Bouviers, of which 24 were clinically ascertained. In addition, the DNA of 29

Bouviers, unrelated to the aforementioned group and diagnosed with other, non-skeletal, diseases were used as control group for molecular genetic investigation.

Figure 1. Bouviers des Flandres with hereditary radial head subluxation
The abnormal front legs of affected dogs including valgus deviation of the front feet, and disproportionately short front legs can be noticed.

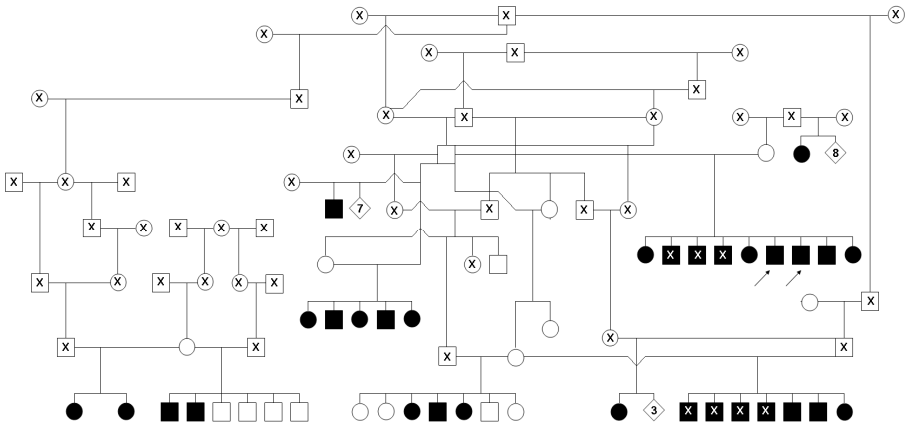

Figure 2. Pedigree diagram of Bouviers des Flandes with subluxation of the radial head
Square symbols are males. Circle symbols are females. Filled symbol and opened symbols are affected and healthy Bouviers, respectively. The number in diamond depicts the number of reamaining litter-mates (with know gender but unknown status). The first two probands are indicated by an arrow. Dogs with X have not been examined; DNA was not available but the elbow status became available by history taking. Two litters on the left bottom were Bouviers bred in Sweden but related to the common founder of the Bouviers in the Netherlands.

Radiographic study

Standard craniocaudal (CrCd) and mediolateral (ML) radiographs of the right antebrachium and a ventrodorsal (VD) view of the pelvis with the hind legs extended were obtained from 26 (16 affected and 10 unaffected dogs) of the 43 Bouviers des Flandres. These radiographs were examined with regard to configuration, alignment, congruity and subluxation of the elbow joint, evidence of sclerosis of the incisura trochlearis, deviation of the radial head, degree of curvature of the radius, relative length of the ulna, configuration of distal ulnar and radial metaphyses, and presence of a core of cartilage in the distal radial and ulnar metaphyses (Figure 3).

The axis of the distal and the proximal parts of the radius was drawn on each CrCd view, according to Newton (1974), and the angle between these axes was measured (in degrees) with a goniometer. Measurements (mean ± SD) were obtained in all 26 dogs. In addition, the distance between the point of angulation and the proximal metaphysis (a) and between the point of angulation and the distal metaphysis of the radius (b) was measured, and a/b and a/(a+b), the latter expressed as percentages (±SD), were calculated. The statistical significance of differences in the angle between the axes and a/(a+b) in affected and unaffected Bouviers was tested with Student's t-test (SPSS V.15).

On the ML view of the antebrachium, a straight line was drawn between the top of the olecranon process and the tip of ulnar styloid process; the configuration of

the ulna and olecranon process was evaluated in terms of the proportion of this line lying outside the ulnar projection (Figure 4).

Genotyping

Blood samples were taken from all affected Bouviers and the available unaffected relatives, and DNA was isolated according to Miller and colleagues (1988). The set of 1536 single nucleotide polymorphisms (SNPs) was used, which were evenly spread along the dog genome, as has been described earlier (Leegwater and others 2007). The genotyping of the SNP set in the DNA from 24 affected and 48 unaffected dogs was performed with the GoldenGate assay (Illumina, San Diego, CA, USA). Alleles were clustered and assigned by the Illumina beadstudio program (Illumina, San Diego, CA, USA). Genotype frequencies of related and unrelated Bouviers, and of affected and unaffected Bouviers were compared.

Cytogenetic study

For chromosome analyses or karyotyping, blood was obtained from an unaffected bitch and one of her affected female offspring of a litter with two probands (Figure 2). Metaphase chromosomes were obtained from blood lymphocytes stimulated with 25µg/ml concanavalin A (Sigma-Aldirch BV, Zwijndrecht, The Netherlands) following standard procedures. In short, cells were cultured for 72 hours and treated with 0.4 µg/ml colchicine (Sandoz, Bazel, Switzerland) for 60 minutes at the end of culture. Cells were swollen in 0.075M KCl (15 minutes, 37°C) and fixed in a mixture of methanol and glacial acetic acid (3:1). Chromosome preparations were made and slides were conventionally stained with Giemsa (Sigma-Aldrich BV, Zwijndrecht, The Netherlands). In well-spread metaphases the total number of chromosomes and the presence of the X chromosome were determined, using a Leica DMRA microscope equipped with the GENUS software of Applied Imaging (San Jose, CA, USA).

Results

Physical features

None of the breeders noted abnormal alignment of the front legs when the pups were born. In affected dogs, both front legs revealed valgus deviation of the feet at the age of investigation (Figure 1). The elbow joints of affected Bouviers were wider at the level of the radial head than those of unaffected dogs. In most affected dogs, the configuration of the elbow joint was abnormal, with a decreased range of motion, as was the antebrachium, and the front limbs were relatively shorter than the hind limbs. Affected Bouviers suffered from lameness, difficulty of movement, and limited function of the elbow joint. For these reasons, seven of the affected dogs were euthanized before the age of 12 months preceding this study; no samples were

available for pathological investigation. With the exception of these abnormalities, the dogs had a normal appearance without deformity of the face or skull. No evidence of ocular disease or other abnormalities was found in any of the affected Bouviers.

Radiographic study

Moderate to advanced elbow osteoarthritis with periarticular new bone formation at the medial aspect of the humeral condyle, anconeal process, and radial head was seen in 12 affected dogs. Moderate to severe sclerosis of the incisura trochlearis of the ulna was detected in 14 affected dogs and in 1 unaffected dog; the latter was not associated with luxation of the radial head or angulation of the radius. In addition, the cranial margin of the medial coronoid process was blunted and had a poorly defined contour in 15 affected dogs. Six affected dogs younger than 9 months had a retained cartilage core in the distal ulnar metaphysis, but this was not seen in mature affected dogs. A sharply demarcated "spur like" formation of new bone arising from the mid-caudal portion of the ulna and extending in a caudoproximal direction was seen in 5 affected dogs, but in none of the unaffected dogs (Figure 3). The radiographic features are summarized in Table 1.

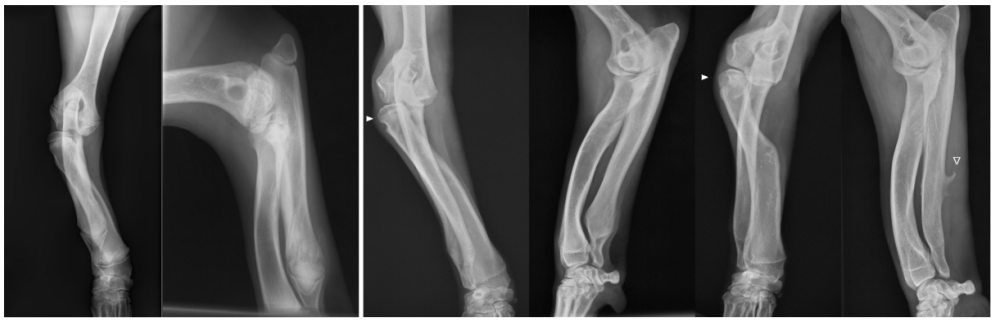

Figure 3A. **Figure 3B.**

Figure 3. Radiographic appearance of hereditary radial head subluxation in Bouviers des Flandres.

The radiographs in Figure 3A are of an affected dog at 4 months of age, with a flared ulnar and retained cartilage in the ulnar metaphysis, and marked lateral deviation of the radial head. The radiographs in Figure 3B are of two affected Bouviers at adult age. An abnormal elbow configuration, and incongruity, and various degrees of subluxation of the radial head can be seen (filled arrow). The cranial margin of the coronoid process was not normal. Moderate to severe sclerosis of the trochlear notch can be seen. A spur-like lesion can be seen on the mediolateral projection at the caudal aspect of the ulna (opened arrow) in one the dogs.

The angulation of the radius at mid-diaphysis was greater and the radial head showed a marked lateral deviation in affected dogs compared with unaffected dogs (Figure 4). The degree of radius angulation had a mean of mean 14.9± 6.1^{o} with a range in between 9.3^{o} and 30.3^{o} in affected dogs (n= 16) and from 0^{o} to 6.6^{o} (mean 4±1.8^{o}) in

Table 1. Radiographic features of hereditary radial head subluxaton in affected Bouviers and in non-affected related Bouviers.

Radiographic findings	Number of affected dogs (n= 16)	Number of unaffected dogs (n= 10)
Malalignment, incongruity, or abnormal configuration of the elbow joint	16	none
Osteophyte formation in the elbow joint	12	none
Ill-defined contour or blunted cranial margin of the medial coronoid process	14	1
Moderate to severe sclerosis of the incisura trochlearis	14	1
Cranial bending of the olecranon	8	0
Shortened ulna	14	0
Spur-like appearance at mid-caudal ulna	5	0
Flared distal metaphysis of the radius and ulna	16	0
Cartilage core at distal metaphysis of the ulna	5	0

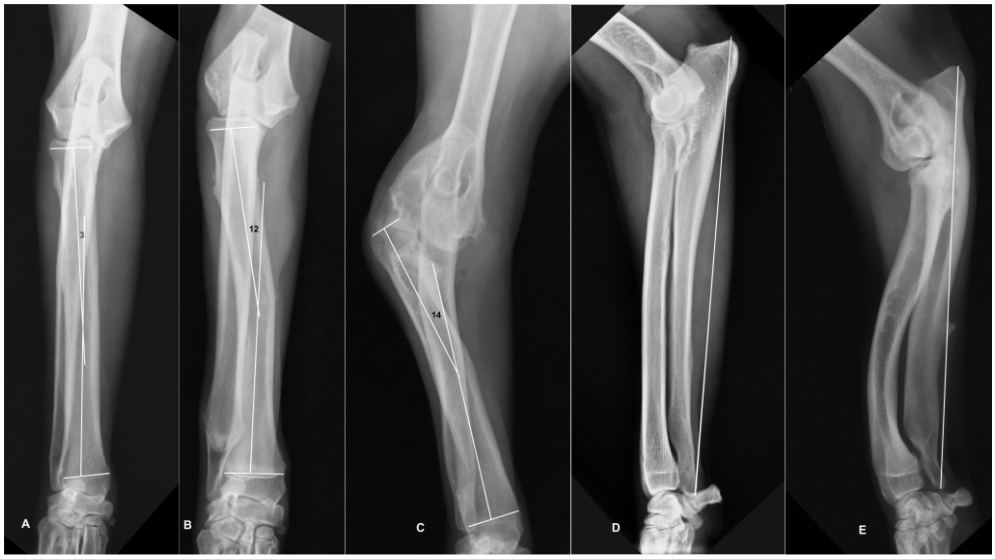

Figure 4. Line indicating radial angulation and ulnar curvature
A line drawn in the axis of the proximal (a) and distal (b) part of the radius will be straight in unaffected dogs (A), but indicates a pivot point (B & C) in which the angle of the lines can be determined, indicating the angulation of the radius. The length of line a can be expressed as percentage of the whole length (a+b) of the radius. Line drawn from the olecranon till the ulnar styloid process helps to objectivate the curvature of the ulnar shaft in normal (D) and affected (E) dogs.

unaffected dogs (n= 10), and the difference between 2 groups was statistical significant ($p < 0.05$). The ratio of a/b was 1/0.99± 0.29 and 1/1.54± 0.48 in affected and unaffected Bouviers, respectively (Table 2) ($p = 0.009$). The percentage of the

proximal part of the radius of the overall length of the radius, a/(a+b), was mean 51.7 ± 9.7% (range 42.2– 85.5%) in affected Bouviers whereas it was mean 39.8±6.5% (range 31.4– 46%) in healthy Bouviers ($p = 0.002$).

Cranial bending of the olecranon was marked in 8 affected Bouviers, as indicated by the line between the top of olecranon process to the distal end of the ulna styloid process (Figure 4). In these dogs at least 50% of the line was within the boundary of the projection of the ulna, whereas 100% of the line was outside the boundary in all normal Bouviers (Table 3). The pelvis and hip joints were normal in all affected dogs, except for one case of mild osteoarthritis of both hip joints.

Table 2. Angulation and configuration of the radius in 16 Bouviers with radial subluxation and 10 non-affected Bouviers

Radiographic findings	Affected dogs	Unaffected dogs
Degree of angulation of the radius, range (mean± SD)	9.3°-30.3° (14.9± 6.1)	0°-6.6° (4±1.8)
Ratio of a/b (mean ± SD)	1/0.99±0.29	1/1.54± 0.48
Percentage of a/(a+b), range (mean± SD)	45.2-85.5% (51.7± 9.7)	31.4-46.3% (39.8± 6.5)

Genetic studies

A pedigree diagram was constructed for 31 affected Bouviers (13 males and 18 females) (Figure 2). Twenty one affected dogs were born in three litters in which no normal dogs were born (Figure 2). One sire fathered two of these litters and was the grandfather of the third litter. He also fathered another litter with an affected dog. Another sire fathered two litters, one with exclusively affected dogs, and the same can be said for one of the dams. Segregation analysis was not possible because not all the litters with probands were completely ascertained.

We hypothesized that loci with alleles contributing to the phenotype were fixed or nearly fixed in the breeding line concerned. To explore the genetic divergence of the breeding line from the general population, we compared the allele frequencies of a set of 1536 SNPs, evenly located along the canine chromosomes, in the related and unrelated Bouviers. Of the set, 1319 SNPs passed the quality control and were not monomorphic in all Bouviers. A total of 205 SNPs were homozygous in the affected Bouviers and 163 of these were homozygous in the relatives of these dogs as well. Only 3 SNPs were homozygous in the group of unrelated control Bouviers. The average level of heterozygosity was 29%, in affected dogs and 35% in unrelated control dogs.

We examined whether the deformity could be the result of a gross chromosomal rearrangement or instability. Analyses of ten Giemsa stained metaphases of an unaffected bitch and of one of her affected pups revealed that both dogs had 76

telocentric autosomes and two submetacentric X chromosomes (78,XX). Besides the X chromosomes, no other bi-armed chromosomes were observed.

Discussion

Reports on hereditary subluxation of the radial head in the Bouvier des Flandres were not found in the literature. We investigated the physical and radiographic features and the genealogy of this type of skeletal disorder in a group of Bouviers. The disease was characterized by moderate to severe angulation of the radius, shortening of the ulna, laterocaudal displacement and subluxation of the radial head, and elbow osteoarthritis of varying severity.

Owing to the angulation of the radius, neither the location of the pivot point nor the angulation of the axis measured on standard radiographs should be considered as precise measurements but were suitable for analysis. In the unaffected Bouviers, a pivot point with angulation between the proximal and distal axis of < 6° was seen, located at 39.8 ±6.5% of the total length of the radius. Since the proximal growth plate of the radius contributes 28-38% to the growth in length of the radius (Olson and others 1979, Newton 1985, Conzemius and others 1994), it is likely that this pivot point was originally localized in the midpoint of the primary ossification center of the radius in these unaffected newborn dogs. Since this pivot point was located more distally in the affected dogs with a reduced total length of the radius, we conclude that the distal radial growth plate contributes less to the growth in length of the radius in affected dogs. The growth in length of the ulna, which is almost entirely dependent on the growth of the distal ulnar growth plate, was also diminished, as reflected by the observation that the ulnar styloid process did not reach the accessory carpal bone. Five affected Bouviers had evidence of a retained cartilage cone of less than 2 cm; this did not necessarily hamper growth (Voorhout and others 1986) because 11 dogs with radial head subluxation without a cartilage cone also showed diminished growth. A cartilage core in the distal ulnar metaphysis was seen in 5 young affected dogs (4 dogs aged 4 months and 1 dog aged 9 months at radiography) but not in older (≥12 months) affected dogs. A cartilage core of 1 cm or less is not considered to be clinically relevant (Voorhout and others 1994). Thus this abnormality is not a radius curvus syndrome due to ulnar growth plate trauma (Theyse and others 2005), osteochondrosis (Olsson 1980), or excessive intake of calcium (Voorhout and Hazewinkel 1987a) or vitamin D (Tryfonidou and others 2003). Therefore we conclude that this developmental abnormality originates in the mid-diaphysis of the radius at birth, with decreased growth in the length of the distal radius. The angulation is located at the insertion site of the interosseous ligament, which firmly connects the radius and ulna.

Table 3. Measurement of angulation and configuration of the radius and ulna

Animal	Affected status (2=affected, 1= unaffected)	Angulation (degree)	a/b	a/(a+b) %	Line between olecranon and ulnar styloid process
1	2	14	1/1.4	46.6	in
2	2	9.3	1/1.19	45.6	in
3	2	17.5	1/1.28	43.75	in
4	2	30.3	1/0.87	53.6	in
5	2	20.3	1/1.4	46.6	in
6	2	11	1/1.02	48.4	in
7	2	17.6	1/0.94	51.5	in
8	2	26.3	1/1.04	49	out
9	2	15.6	1/1.21	45.2	in
10	2	10.6	1/1.3	47	out
11	2	11	1/1.08	48	out
12	2	11	1/0.17	85.5	out
13	2	12.6	1/0.76	56.6	ND
14	2	10	1/0.82	54.8	out
15	2	10.3	1/0.79	52.5	out
16	2	11.3	1/0.88	53	out
17	1	4.2	1/1.87	45.7	out
18	1	5	1/1.22	45	out
19	1	4.3	1/2.18	31.4	out
20	1	6.6	1/1.17	47.2	out
21	1	4	1/1.75	36.4	out
22	1	0	ND	ND	out
23	1	4.6	1/1.16	46.3	out
24	1	4.6	1/2.12	32	out
25	1	2.3	1/2	33.3	out
26	1	5.6	1/1.43	41.2	out

a: Distance between the point of angulation and the proximal radial metaphysis
b: Distance between the point of angulation and the distal radial metaphysis
a and b are given in centimeters, a/(a+b) is given as percentage of total length of the radius.
Line between the olecranon and ulnar styloid process is given as “in” when at least 50% of the line was inside the ML projection of the ulna.

The growth in length of the proximal part of the radius did not seem to be reduced in affected dogs. However, due to the angulation at the pivot point, the radial head passes the humerus and the humeral condyle rotates around the anconeal process. The radiographic signs of osteoarthritis can be explained by the abnormal architecture of the elbow joint or the occurrence of fragmentation of the coronoid process (Voorhout and Hazewinkel 1987b), although a common cause of chondrodysplasia and osteoarthritis cannot be ruled out (Kannu and others 2009).

Several authors have suggested that soft tissue is involved in this disorder (Bingel and Riser 1977, Milton and others 1979). Underdevelopment of the collateral ligament could be considered a consequence of abnormal growth in the length of the radius and ulna. Subluxation of the radial head of the elbow and bending of the olecranon have not been reported in other cases of asynchronous growth of the radius and ulna, including radius curvus syndrome or short radius syndrome. Thus we speculate that, apart from the angulation of the radius, abnormal forces from the supinatus muscle may also play a role in the luxation of the radial head, and that forces from the triceps brachii muscle may play a role in the cranial bending of the olecranon. These forces probably act in conjunction with the laxity of the lateral collateral ligament and annular ligament, which allows the radial head to move away from the ulna (Bingel and Riser 1977, Milton and others 1979) while the mid-radius is strongly held by the interosseous ligament.

The spur-like lesion occurring close to the point of insertion of the deep digital flexor muscle in the mid-caudal ulna, seen in 5 affected Bouviers, has not been reported in other cases of hereditary elbow subluxation or in normal Bouviers. The cause of this lesion is not known.

The radial head subluxation arose at the distal growth plate of the radius and affected the function and configuration of the elbow joint. In the present study, all affected dogs also had malfunctioning elbow joints. This type of subluxation of the elbow joint is incidentally found in chondrodystrophic breeds, and isolated cases have been described in other breeds including the Akita (Piermattei and others 2006). Our findings are similar to those described in congenital elbow luxation type I, where the radial head moves away from the humeral condyle (Kene and others 1982). However, the clinical findings were also similar to those of chondrodysplasia described in isolated cases of the Saint Bernard, Newfoundland, Lapphund, and German Shepherd (Fjeld 1990). In these breeds no hereditary aspects have been demonstrated. The valgus deformity is the consequence of asynchronous growth of the radius and ulna, resulting in abnormal development of the carpal and elbow joints (Carrig 1983). This finding has also been described in dogs with traumatic radius curvus syndrome where often only one limb is affected with the direct consequence of loss of normal joint configuration (Theyse and others 2005). Therefore we conclude that valgus deformation in these Bouviers is not per se a consequence of hereditary luxation of the radial head.

All affected dogs were closely related over five generations (Figure 2) and had at least one founder in common. The disease was seen both in male and female dogs (ratio 1:1.4). Since the phenotype of radial head subluxation seems to have accumulated in this group of dogs with a high level of inbreeding, and the parents did not display the phenotype, recessive inheritance would be the most likely mode of inheritance. However, in recessive disorders it is uncommon that all siblings are affected, as was seen in three litters. This observation could suggest that environmental factors play a role in combination with a genetic effect. Although traumatic or nutritional factors cannot be ruled out at this stage, the fact that the affected dogs came from different breeders makes it unlikely that they all made similar errors in husbandry or preparation of the puppy diet. Severe growth plate abnormalities can also occur as a result of trauma (Theyse and others 2005), with valgus deviation or short radius syndrome, and as a result of oversupplementation (Voorhout & Hazewinkel 1987a, Voorhout and others 1994, Schoenmakers and others 2000, Tryfonidou and others 2003); however, these dietary induced abnormalities never coincide with radius angulation and elbow subluxation, but instead occur with radius curvus syndrome.

Since none of the parents of the affected litters had elbow subluxation, it is also unlikely that hereditary elbow luxation in Bouviers is a dominant trait. The lack of radial head subluxation in the parents suggests incomplete penetrance of the genotype at risk. The observation that all pups of three litters were affected and none of the parents was affected makes dominant inheritance in combination with reduced penetrance unlikely. A novel phenotype can arise if a new mutation occurs abruptly at some point in the breeding line, if there is chromosome rearrangement or replication, or if there is accumulation of the mutation in the breeding line. Chromosome rearrangement might cause a particular chromosome region to be duplicated, lost, or fused to a chromosome region that disrupts its regulation, thereby contributing to the phenotype. Although conventional chromosome staining revealed that the two dogs investigated had a total number of chromosomes that reflects the normal female karyotype (78, XX) (Selden and others 1975). Due to the relatively low number of metaphases and the poor quality of the metaphase spreads, GTG-banding was not carried out in the present study. More detailed chromosome analysis, for instance by comparative genome hybridization, is needed to investigate the involvement of structural abnormalities in the phenotype.

We consider genomic instability in the form of gradual expansion of a DNA repeat over generations in this breeding line to be an attractive explanation for our data. This repeat could disrupt the expression of an associated gene, and disease severity could be associated with the length of the repeat. Zlotogora (1994) postulated a similar model of repeat expansion with an autosomal dominant effect and reduced penetrance for split-hand/foot malformation with long bone deficiency in humans, even though the disorder was observed in a number of consanguineous families. Naveed and colleagues

(2007) showed that this disorder was inherited in dominant digenic mode with reduced penetrance in a large consanguineous family. SNP genotyping of the Bouviers with radial head subluxation revealed a high level of homozygosity in the affected pedigree. Finemapping and evaluation of a larger cohort is required to limit the number of positional candidate regions. High-throughput DNA sequencing of these regions may be required to confirm the presence of an expanding repeat and to understand the developmental process that underlies radial head luxation in Bouviers.

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Chapter 8

General discussion

In veterinary orthopedics, fragmented coronoid process (FCP) is regarded as one of the most important diseases in dogs and a major component of elbow dysplasia. The defect is widespread in the canine population despite breeding regulations, because not all affected dogs display the disease phenotype and thus go undiagnosed. Indeed, several studies have shown that the sensitivity of routine radiology does not exceed 80% (Carpenter et al., 1993). Elucidation of the genetic basis of this defect could explain the disease biology of FCP, the pathogenesis of which is not currently known. The studies described in this thesis also included other diseases, such as cruciate ligament rupture (CCLR) in Boxers, chondrodysplasia in Labrador Retrievers, and hereditary subluxation of the radial head in Bouviers des Flandres. While our findings have answered some questions, others remain unanswered, necessitating further research. Various aspects of the different studies are discussed below.

FCP gene mapping

Little is known about the etiology and pathogenesis of FCP. There are two main theories regarding its pathogenesis, namely, a disturbance of endochondral ossification or biomechanical changes. The former was suggested when FCP was first described in veterinary orthopedics (Tirgari, 1974 and Olsson, 1977), whereas several research teams have hypothesized changes in biomechanical function in affected animals. Thus different genetic and environmental factors may play a role in the phenotypic expression of the genetic abnormality. The incidence of FCP in the Labrador Retriever population is 14.7%, which makes it a common disorder. The fact that FCP is a common disorder suggests that it is probably a complex disease because otherwise breeders would have bred the disease out, preventing its spread. Studying the genetic basis of FCP in combination with family data could improve our understanding of the disease etiology in the families studied and, by extrapolation, in the entire breed. FCP is not recognized as a skeletal disease in other species. As a consequence, there is little information available about candidate genes in the literature, which meant that we needed to identify promising candidates.

Collagen genes are the most promising candidates to investigate with regard to FCP etiology because of their involvement in the extracellular matrix of cartilage and bone, and especially during skeletal development (Mundlos and Olsen, 1997; Eyre, 2001). The availability of family data for Labrador Retrievers of the Royal Dutch Guide Dog Foundation, obtained by means of a screening protocol for elbow dysplasia, made linkage analysis the most suitable method for analysis. Linkage analysis compares inherited information contained in DNA with the inherited phenotype and can be applied either selectively to preselected candidate genes or aselectively to the whole genome. Mutated genes carried by affected offspring were expected to be inherited from their parents, on the ground of their inheritance pattern. With an

uncertain mode of inheritance as in FCP, mutated causative genes were expected to be frequently shared by affected sib-pairs. For this reason, we selected a number of collagen genes and developed markers to assess the involvement of these genes in FCP. The collagen markers investigated in the studies of this thesis were closely situated to collagen genes and were shown to be polymorphic. Several collagen genes, including COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1, were found not to be responsible for FCP in the investigated Labrador Retrievers.

We initially chose the candidate gene approach because the analysis can be completed in a short period of time. A limited number of markers can be selected to cover the candidate genes rather than coverage of a vast region, as with a whole genome approach. However, linkage analysis with collagen genes as candidate genes was not a successful approach in our studies. We knew too little about potentially relevant candidate genes because the disease, or a similar disease, does not occur in other species, including humans, which would have provided strong candidate genes. Moreover, more than one gene, and one pathway, may be involved in the pathogenesis of FCP, given the complexity of the disease.

Because the collagen genes we investigated were found not to be involved in FCP, we carried out genome-wide linkage analysis. We expected that with the sib-pair analysis large regions would be covered by the markers used. Finemapping of potentially relevant regions would limit the number of positional candidate genes. We used microsatellite markers in the genome-wide linkage analysis because these are highly polymorphic. However, genotyping of microsatellite markers was laborious and time-consuming despite the use of a pipetting robot, and we restricted the analysis to affected sibling pairs and their parents because we were uncertain about the status of dogs without FCP. Information on single nucleotide polymorphisms (SNPs), which became available when the whole canine genome sequence was completed, and the availability of high-throughput genotyping techniques enabled us to perform a genome-wide linkage analysis of FCP with a SNP set in parallel.

After genotyping with two separate markers sets, linkage analyses revealed possible FCP loci on CFA01, CFA06, CFA13, CFA28, and CFA34. We refined these loci by using densely spaced SNPs, and the responsible loci were ultimately limited to a region on CFA01 and a less significant region on CFA13. Although the FCP loci were identified, the actual genes responsible for FCP were not. The 23-Mb region of the highest significance linkage on CFA01 was the most promising, but there are up to100 genes in the region. We were not able to identify a smaller common haplotype shared by FCP-affected dogs across the families.

Analysis of SNPs selected for finemapping of the FCP locus on CFA01 revealed that 3 SNPs showed an allele distribution with a significant association with FCP in the cohort of litters with multiple cases (data not shown). However, these SNPs

were not significantly associated with FCP when analyzed in FCP-affected Labradors Retrievers and healthy Labradors Retrievers not related to the familial cases. It is possible that the inability to distinguish between positives and true negatives affected our association test. Moreover, the density of SNPs used for finemapping may have been too low to detect common haplotypes. The average distance between adjacent SNPs was 320 kb, while it has been shown that the linkage disequilibrium (D') drops below 0.5 at distances over 100 kb in Labrador Retrievers from the USA (Lindblad-Toh et al., 2005). In other words, the SNPs on the finemapping array we used in this study were not sufficiently correlated to allow complete comparison between dogs from different families. Likewise, the SNPs did not represent neighboring genes unless they were located close to or within the genes. Future studies should start with further finemapping of the CFA01 locus with SNPs that are located 50 kb apart.

It is worth noting that simple association analysis could be an alternative to sibling-pair analysis in the case of a complex disease such as FCP. In such an analysis, the allele frequencies of closely spaced DNA markers are compared in groups of unrelated affected dogs and unaffected dogs of the same breed. At the start of this project, the group of unrelated affected dogs was too small and high-throughput technology was not yet available to conduct an association study. Large-scale association analysis is now warranted, not only of the FCP loci identified here but also of the entire canine genome. Genes with moderate effect are difficult to detect with linkage analysis in terms of the power of the analysis; however, the power of association analysis can be substantial if the groups of affected and control animals are large enough (Risch and Merikangas, 1996).

Collagen marker analysis and other skeletal diseases

Chondrodysplasia in Labrador Retrievers

Collagen genes are strong candidate genes for osteochondrodysplasia in dogs because the disorder in humans is often caused by mutations of these genes. We detected polymorphism of the DNA markers for collagen genes in Labrador Retrievers and dogs of other breeds (Chapter 5). These collagen gene markers should be informative for assessing the involvement of collagen in potentially collagenopathic diseases, such as chondrodysplasia in Labrador Retrievers, and cruciate ligament rupture in Boxer dogs. Hereditary elbow luxation is seen in different breeds (Bingel and Riser, 1977; Lau, 1977; Milton et al., 1979), and we described, for the first time, this disorder in Bouvier des Flandres. It can be considered an expression of chondrodysplasia with possible involvement of collagen genes. Although we could not demonstrate the relationship between hereditary elbow luxation and chondrodysplasia, due to a lack of specimens for pathological investigation, the similarity of chondrodysplasia and hereditary elbow luxation in Bouviers can be assumed on the basis of findings for Labradors (these

studies) and St Bernards, Lapphounds, Newfoundland, and German shepherd dogs (Fjeld, 1990).

We investigated the presence of chondrodysplasia in Labrador Retrievers by evaluating DNA marker alleles carried by affected dogs. We carried out pedigree analysis and genotyping on the assumption of a recessive mode of inheritance. However, none of the collagen genes analyzed were involved in chondrodysplasia in Labrador Retrievers. The thesis provides the first description of a discrete and unique form of chondrodysplasia that affects long bones and vertebrae exclusively, and not ocular or other organs as previously reported in Labrador Retrievers and other breeds. The disease appeared to have severe and mild forms. In the severe form, the long bones were abnormally short and there was malformation of the hip joint, and in the mild form the long bones were abnormally short but the hip joint was normal (Brocks and Hazewinkel, 2004). In this respect, the chondrodysplasia seen in these dogs is similar to human multiple epiphyseal dysplasia, which occurs in the severe Fairbank type or the mild Ribbing type (OMIM #132400). We observed both severe and mild chondrodysplasia in the same pedigree, although it was not present in the same litter. The chondrodysplasia in Labrador Retrievers described here also bore similarities to spondyloepiphyseal dysplasia in humans, which can be indistinct from multiple epiphyseal dysplasia. Disease heterogeneity was seen in our chondrodysplastic Labrador Retrievers. This might have affected the genetic analysis, which we carried out on the basis of a simple Mendelian disease. We took into account that the assumed recessive mode of inheritance might not be completely correct and tested linkage with various degrees of penetrance of the phenotype without significant results. Thus on the basis of our results, we cannot conclude that chondrodysplasia in Labrador Retrievers has genetic factors in common with FCP.

Further study of chondrodysplasia in Labrador Retrievers should focus on genome-wide linkage analysis. However, first it should be established whether the phenotype is heterogeneous and could be caused by different independent genes.

Cranial cruciate ligament rupture (CCLR) in Boxer dogs

Although CCLR is known to have a traumatic origin, its frequent diagnosis in particular breeds, such as Boxers (van Hagen, 2004; Temwichitr et al., 2007), Rottweilers, and Newfoundland dogs (Whitehair et al., 1993), is an indication that this abnormality has a genetic background (Wilke et al., 2005). We excluded the possible contribution of the collagen genes COL9A1, COL9A2 and COL9A3 to CCLR in Boxers. There are a few studies of CCLR in dogs that support a genetic cause of CCLR (Muir, et al., 2005; Wilke et al., 2005). Further genetic study of CCLR should start with segregation analysis to establish the mode of inheritance and the complexity of the disease.

Radial subluxation in Bouviers des Flandres

We described radial head subluxation, with malconfiguration of the elbow joint, and curvature of the proximal part of the radius in a cohort of Bouviers des Flandres. Although the abnormality in these dogs has not been proven to be chondrodysplasia, the phenotype is very similar to that of chondrodysplasia in St Bernards, Lapphounds, Newfoundland and German shepherd dogs (Fjeld, 1990). A hereditary cause of radial head subluxation subsequent to premature closure of the ulnar physis has been suggested (Lau, 1977; Gurevitch, 1980) but we did not find premature closure to be a predisposing factor in Bouviers. In the Bouviers investigated, we found the radial head subluxation to be caused by angulation of the radial bone (cause unknown) and possible involvement of the supinatus and triceps brachii muscles and the collateral and annular ligaments. The forces most likely to affect the medial coronoid process in FCP originate from the annular ligament (Wolschrijn and Weijs, 2004) and/or from joint incongruity of the radius, ulna, and humeral condyle. The subluxation of the radial head in Bouviers affects the humerus and forces the medial humeral condyle to rotate around the anconeal process and ultimately forces it toward the medial coronoid process. This latter mechanobiology is somewhat similar to the possible role of the long radius in FCP, as reviewed in this thesis (Chapter 2), and could explain the observations that the contour of the medial coronoid process in the affected Bouviers was abnormal and that the ulna showed signs of increased sclerosis, both findings typical for FCP.

The angulation of the radius in radial head luxation appeared to occur between 0 to 4 months of age. Angulation takes place at the midshaft, where the inter-osseous ligament is strongly attached between radius and ulna. The different length of the proximal and distal parts of the radius from the pivot point in affected and unaffected Bouviers suggested that the growth rate of the distal physis of the radius was less in the affected than in unaffected Bouviers, indicating a decreased growth in length of the distal radial physis. Also the growth in length of the ulna was less than normal. This finding explains the shortening of the front legs but not the radial subluxation in the affected Bouviers, because neither traumatic growth arrest of the radius nor traumatic growth arrest of the ulna give rise to radial subluxation.

Further study of this disease in Bouviers should focus on the development process with special attention to the occurrence of radial angulation. Histopathologic studies to specify the development of this defect are warranted but depend on the cooperation of owners of affected dogs. This Bouvier pedigree should be studied further to determine the inheritance pattern, which cannot be explained as yet. The finding that complete litters were suddenly affected will possibly lead to novel modes of inheritance.

In conclusion

We studied the occurrence of FCP in Labrador Retrievers by sibling-pair analysis, with collagen genes as candidate genes and could exclude a number of collagen genes as candidate genes for FCP. We then performed genome-wide linkage analysis with two sets of markers, microsatellite markers and SNPs. Finemapping of the loci enabled us to localize the linkage loci to CFA01 and to a lesser extent to CFA13. The linkage regions on CFA01 and CFA13 were shared by affected sibling pairs more often than by chance, which suggests that these regions contain the genes responsible for FCP, although these have yet to be identified.

FCP is a complex disease and the model-free linkage analysis described here did not allow us to unambiguously identify a genetic component. In fact, only a few complex diseases have been successfully resolved by linkage analysis (Risch and Merikangas, 1996). Chondrodysplasia in Labrador Retrievers seemed to be less complex than FCP and there are similar diseases in humans and other species. The failure to confirm the involvement of collagen genes in chondrodysplasia in our study group of Labrador Retrievers does not disqualify the linkage analysis or candidate gene approach. Genome-wide linkage analysis should be performed for chondrodysplasia in Labrador Retrievers because genotyping is easier to perform nowadays.

To date, linkage analysis to identify the genes that cause complex diseases has not been a success (Risch and Merikangas, 1996). This was reflected in this study of FCP. With hindsight, we can conclude that association analysis of FCP would have been more appropriate. However, our cohort mainly consisted of related affected dogs. The biomechanical pathogenesis of FCP, which is important and not yet clarified, should be investigated further. Identification of both genetic and biomechanical causes of FCP could lead to better diagnostic tools and effective preventive measures.

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Chapter 9

Summary

Fragmented medial coronoid process (FCP) is the main component of elbow dysplasia (ED), which includes osteochondrosis of the medial humeral condyle (OCD), elbow incongruity (INC), and ununited anconeal process (UAP). FCP is recognized as a hereditary disease in many breeds and is a major concern in working dog breeds such as the Labrador Retriever, which is the main breed used as guide dogs for the blind and assistance dogs for the disabled. FCP manifests as front leg lameness but is can only be diagnosed by effective imaging techniques or at arthroscopic or exploratory surgery.

In **Chapter 2**, different aspects of FCP were described, such as the development of the elbow joint, possible pathogeneses of FCP, and genetic studies to identify the gene for FCP. The elbow joint develops from the mesenchymal cells of the limb bud. Both the primary and secondary ossification centers of the bone develop from a cartilaginous template by the process of endochondral ossification, as does the medial coronoid. During the growth and development of the medial coronoid process, several different processes can lead to its fragmentation, such as disruption of endochondral ossification and/or imbalance of biomechanical forces from the annular ligament, incongruity of the radio-ulnar joint or of the humeral-ulnar joint, and asynchronous growth between radius and ulna. A hypothesis for the heterogeneous pathogenesis of FCP is presented in chapter 2, which also describes different types of genetic investigations that could be carried out to identify the gene causing FCP. Knowledge of the genetics of this orthopedic disorder can be summarized as follows: the disease is common in the Dutch Labrador Retriever population (14.7% of the population is affected), FCP is more common in male dogs, and it is thought to have a recessive mode of inheritance, but this is not proven. Two different genetic markers were considered appropriate for the molecular genetic study of FCP, namely, microsatellite markers and single nucleotide polymorphisms (SNP). The former are short nucleotide repeats that can vary between homologous chromosomes, and the latter are changes within a single nucleotide at the same chromosomal location. A SNP can be varied up to two alleles while a microsatellite marker can have a high number of alleles. Both marker types are stably inherited by offspring and can be used to represent nearby genes.

Two techniques were used to try to identify the FCP gene, namely, linkage analysis and association analysis. Linkage study makes use of genetic markers and the familial relationship between affected individuals to determine whether the markers are inherited in a manner consistent with a specific mode of inheritance. Association analysis compares the frequency of marker alleles in affected and unaffected animals, regardless of their familial relationship. The availability of information about the familial relationship between the Labrador Retrievers investigated made linkage analysis a promising approach. Sibling-pair analysis should enable the identification of marker alleles that are frequently inherited together with FCP or that are significantly shared by animals with the disorder. Sibling-pair analysis, known as model-free or

non-parametric linkage, does not require the mode of inheritance to be known, unlike traditional linkage or parametric linkage analysis. If the linkage is significant, then the marker is closely situated to the gene responsible for FCP.

A candidate gene approach was initially used to try to identify the FCP gene **(Chapter 3).** The choice of candidate gene to use is often based on a comparison of the same disease in different species, including humans, or on genes possibly involved in the FCP pathogenic pathway. However, FCP does not occur in other species, and so this way of selecting a candidate gene for FCP was not possible. Collagen protein is the major and most plentiful component of the extracellular matrix of cartilage and bone, and the coronoid process develops from a cartilage template into bone during skeletal maturation. Because mal-development of bone and cartilage could cause FCP, we speculated that collagen genes could play an important role in the pathogenesis of FCP. Microsatellite markers were developed that were closely situated to different genes encoding various collagen proteins, namely, COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1. In general, siblings randomly share 50% of alleles, and we expected that disease marker alleles would be significantly shared by affected sibling pairs in the same litter. The candidate-gene study was carried out with 13 litters originating from 10 pedigrees of Labrador Retrievers; however, we found that none of the collagen genes investigated was shared to a significant extent by FCP-affected sibling pairs. We failed to identify marker alleles that we would expect to be shared by affected Labrador Retrievers and therefore concluded that the collagen genes investigated were unlikely to play an important role in the pathogenesis of FCP. We also excluded the vitamin D receptor gene (VDR), which is located close (80 kb) to COL2A1, as candidate gene for FCP.

Completion of the second-generation genomic map of the domestic dog in 2004 led to the location of microsatellite markers from other genetic maps, including a radiation hybrid map and a linkage map, comparable to the physical map of the reference canine genome. The reference canine genome also provided extensive information about SNPs across the genome. This knowledge made it possible to use both microsatellite markers and SNPs as marker sets in a genome-wide linkage analysis instead of studying a particular region of chromosome, as in linkage analysis with candidate genes. The results of our study to identify the FCP gene using genome-wide, model-free linkage analysis with 320 microsatellite markers are described in **Chapter 4**. Samples from affected sibling pairs from 10 pedigrees were analyzed. At the same time, we performed a genome-wide analysis using 1536 SNP markers, which are equally spread along the genome. This study was made possible by the advent of high-throughput technology. Comparison of the results of the two genome-linkage analyses revealed 5 loci on different chromosomes that could be involved in FCP, namely, CFA01, 06, 13, 28, and 34. These potential loci were analyzed further by

finemapping with a customized 384 SNP array. Extended markers, 384 SNPs, and samples from 23 litters from 17 pedigrees were included in the finemapping study. Results identified CFA01 and CFA13 to be potential linkage loci but the gene for FCP was not identified (Chapter 4). Research into possible candidate genes at these loci is currently in progress.

Although we did not find collagen genes to be major causative genes of FCP, these genes have been shown to be polymorphic. We assessed the polymorphisms of these genes in different breeds, including Boxers, Bernese Mountain dogs, German shepherds and Labrador Retrievers. We found they mostly exhibited high levels of heterozygosity in those breeds (**Chapter 5**). We also investigated the involvement of these collagen genes in cruciate ligament rupture in Boxers, a breed considered to be at high risk of this disorder. Unfortunately, we could not show that collagen genes were involved in the pathogenesis of cruciate ligament rupture in Boxers.

The short stature, with disproportionately short long bones, of dogs affected by chondrodysplasia was accompanied by elbow incongruity and irregular configuration of the elbow joint. We investigated chondrodysplasia in eight Labrador Retrievers and their relatives (**Chapter 6**), using the candidate gene approach with linkage analysis on the assumption of a recessive mode of inheritance, as determined by analysis of the pedigrees. Knowledge of the gene causing chondrodysplasia would further our understanding of this disorder in dogs, and possibly by extrapolation our knowledge of other possibly related diseases such as FCP. Since the chondrodysplasia in the Labradors Retrievers studied was similar to multiple epiphyseal dysplasia and spondyloepiphyseal dysplasia seen in humans, we evaluated the involvement of the genes encoding cartilage oligomeric matrix protein (COMP), COL9A1, COL9A2, and COL9A3, Matrilin-3, and solute carrier family 26 member 2 (SLC26A2) in chondrodysplasia in these dogs. We expected affected Labrador Retrievers to have homozygous marker alleles if these represented the gene responsible for chondrodysplasia. However, we did not find any marker allele to be exclusively homozygous in the Labrador Retrievers with chondrodysplasia, even though these genes are responsible for chondrodysplasia in humans.

We documented hereditary radial subluxation in Bouvier des Flandres for the first time (**Chapter 7**). This abnormality manifests as disproportionately short front legs with valgus deformity and affects the configuration of the elbow, leading to various degrees of elbow luxation and malformation. These features are similar to those seen in congenital elbow luxation in Miniature Poodles, Skye terriers, and other breeds, and are also similar to those of chondrodysplasia in St Bernards, Lapphounds, Newfoundland, and German Shepherd dogs. The radial subluxation in Bouviers was much more severe than seen in the Labradors with chondrodysplasia as described in Chapter 6. The disproportionate stature of the Bouviers was limited to the front legs whereas it affected all long bones in the Labrador Retrievers. We hypothesized that the

radial head subluxation in these Bouviers was the consequence of angulation of the radial bone and was aggravated by forces exerted by the supinatus muscle and by laxity of the annular ligament. The cranial bending of the olecranon could be caused by force originating from the triceps brachii muscle. Angulation of the radius could occur at birth or soon thereafter and was seen at the mid-diaphysis of the radius, at the insertion of interosseous ligament.

Our findings suggested that the most likely mode of inheritance was dominant with a reduced penetrance, by which affected Boviers do not always demonstrate disease phenotypes. We carried out a genetic study based on the hypothesis that the radial head subluxation was caused by instability of a chromosomal region including a repeat expansion or loss of part of a chromosome over a generation. However, the number of chromosomes was normal and we did not detect chromosomal abnormalities, findings that do not support our hypothesis. However, the high level of homozygosity observed in a group of affected Bouviers warrants further genetic investigation.

The studies described in this thesis were performed to identify the basic defect of FCP. Our findings suggested possible responsible loci and ruled out a number of candidate genes. However, it should be borne in mind that disease is not always the consequence of genetic factors, and that environmental factors may play an important role in the manifestation of an abnormality. The genetic study of FCP described in this thesis could serve as a model for the investigation of other orthopedic diseases in veterinary medicine, such as chondrodysplasia and radial head subluxation.

Nederlandse samenvatting

Van de verschillende aandoeningen die vallen onder de aanduiding elleboog dysplasie, zoals osteochondritis dissecans, elleboog incongruentie, los processus anconeus en los processus coronoïdeus (LPC), is deze laatste het meest belangrijk. LPC wordt in vele hondenrassen herkend als erfelijke aandoening. Het vóórkomen van LPC is vooral een probleem bij werkhonden zoals de Labrador Retriever, dat het belangrijkste ras is dat wordt getraind voor blindengeleidehond en hulphond. Het LPC openbaart zich als een kreupelheid van de voorextremiteiten, maar kan uitsluitend met zekerheid worden gediagnosticeerd met behulp van gevoelige beeldvormende technieken of door middel van arthroscopie of –tomie.

In **hoofdstuk 2** worden verschillende aspecten van LPC behandeld, zoals de embryonale en postnatale ontwikkeling van het ellebooggewricht, de mogelijke pathogeneses van LPC en de verschillende benaderingen waarmee het gen zou kunnen worden geïdentificeerd dat een rol speelt bij het ontstaan van LPC. Het ellebooggewricht ontwikkelt zich uit de mesenchymale cellen van de pootaanleg. Zowel de primaire als secundaire ossificatiecentra van de botten ontwikkelen zich, net als het processus coronoïdeus, vanuit een kraakbenig voorstadium via het proces van endochondrale ossificatie. Gedurende de groei en ontwikkeling van het mediale processus coronoïdeus, kunnen verschillende processen de fragmentatie ervan veroorzaken, zoals een stoornis in het proces van endochondrale ossificatie en/of een disbalans in de biomechanische krachten die ontstaan door het ligamentum annulare, de incongruentie van het radio-ulnair of humero-ulnair gewricht en de asynchrone groei van radius en ulna. Een hypothese van de heterogene pathogenese van het LPC wordt gegeven in hoofdstuk 2, waarin tevens verschillende strategieën van genetisch onderzoek worden beschreven die uitgevoerd zouden kunnen worden om het gen te identificeren dat bij het ontstaan van het LPC betrokken is. De genetische kennis met betrekking tot deze orthopedische aandoening kan als volgt worden samengevat: de aandoening komt veel voor bij de Nederlandse Labrador Retriever (waarvan 14.7% van de populatie positief is voor LPC), LPC komt vaker voor bij reuen dan bij teven en wordt verondersteld recessief te vererven, alhoewel dit laatste niet bewezen is.

Twee verschillende typen genetische markers werden verondersteld bruikbaar te zijn voor de moleculair-genetische studies van LPC, en wel microsateliet markers en Single Nucleotide Polymorfismen (SNP). De eerste zijn herhalingen van korte DNA sequenties waarbij het aantal herhalingen kan variëren tussen homologe chromosomen. Een SNP is een variatie van een enkele nucleotide van de DNA sequentie, zoals die voor enkele diersoorten voor elk chromosoom vastgesteld is. Een SNP heeft hooguit 2 allelen, terwijl een microsatelliet een groot aantal allelen kan hebben. De allelen van beide typen markers worden stabiel overgeërfd en kunnen gebruikt worden om nabij gelegen genen te vertegenwoordigen.

Twee analysetechnieken werden gebruikt om te proberen om het LPC gen op te sporen, namelijk koppelingsanalyse en associatie analyse. Bij koppelingsstudies

wordt gebruik gemaakt van genetische markers en de familierelatie tussen aangedane dieren om te bepalen of de markers vererfd worden op de wijze die past bij een bepaald vererving spatroon. Bij associatie analyse vergelijkt men de frequentie waarin marker allelen in aangedane en niet-aangedane dieren voorkomen, dit ónafhankelijk van de familierelatie tussen de dieren. De beschikbaarheid van informatie van familieverwantschap tussen de Labradors onderling, maakte de koppelingsanalyse veelbelovend. Bij nestgenoten-analyse zou het mogelijk moeten zijn de marker-allelen te identificeren die veelal tezamen vererven met LPC of die significant veelvuldig voorkomen bij dieren met de aandoening. Nestgenotenanalyse is non-parametrisch, dat wil zeggen dat de wijze van vererven niet op voorhand bekend hoeft te zijn, in tegenstelling tot het gebruikelijke parametrische koppelingsonderzoek. Als de koppeling statistisch significant is, is de marker dicht bij het gen gelegen dat verantwoordelijk is voor LPC.

Allereerst werd een kandidaatgen-benadering toegepast bij de poging het LPC gen te identificeren (**Hoofdstuk 3**). De keuze voor kandidaatgenen wordt vaak gebaseerd op de analogie met eenzelfde aandoening bij andere species, zoals de mens, of op genen die mogelijkerwijze betrokken zijn in de pathogenese van LPC. Collageeneiwit is de belangrijkste en meest voorkomende component van de extracellulaire matrix van kraakbeen en bot, met mogelijke betrokkenheid bij de vorming van het processus coronoïdeus vanuit het kraakbenig voorstadium in bot tijdens het proces van skeletontwikkeling.

Omdat een ontwikkelingsstoornis van bot en kraakbeen een LPC zou kunnen veroorzaken, veronderstelden we dat collegeen-genen een belangrijke rol zouden kunnen spelen in de pathogenese van LPC. Microsatellietmarkers werden ontwikkeld die gelegen zijn dichtbij verschillende genen die coderen voor de verschillende collageeneiwitten, namelijk COL1A1, COL1A2, COL2A1, COL3A1,COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2 en COL24A1. In het algemeen delen 2 nestgenoten een willekeurige 50% van de allelen. Het mag echter verwacht worden dat allelen van markers in de nabijheid van een oorzakelijk gen significant vaker dan deze 50% gedeeld worden door aangedane nestgenoten. De studie naar kandidaatgenen werd uitgevoerd met 13 nesten afkomstig van 10 verschillende afstammingen Labrador Retrievers. We vonden echter dat geen van de onderzochte collageengenen significant vaker dan 50% gedeeld werden door paren van nestgenoten die beide een LPC hadden. Met andere woorden slaagden we er niet in marker allelen te identificeren die relatief vaak gedeeld werden door Labrador Retrievers met een LPC, en daarom concluderen we dat het onwaarschijnlijk is dat de onderzochte collageengenen een belangrijke rol spelen bij het optreden van het LPC. We concludeerden ook dat de receptor van vitamine D (VDR), die op 80 kb van het COL2A1-gen gelegen is, en een kandidaatgen was voor LPC, geen rol speelt in deze aandoening.

Toen in 2004 de tweede versie van de assemblage van de DNA sequentie van het hondengenoom gereed kwam, werd de precieze locatie bekend van microsatelietmarkers afkomstig van andere kaarten zoals de zogenaamde *radiation hybrid* kaart en de genetische kaart. Dit bekende hondengenoom verschafte ook uitgebreide informatie over SNPs verdeeld over het gehele genoom. Dit stelde ons in staat met zowel microsatelietmarkers als SNPs het gehele genoom te bestuderen in plaats van een bepaald gebied van een chromosoom, zoals werd gedaan bij de koppelingsanalyse bij het onderzoek van kandidaatgenen. De resultaten van ons onderzoek van het gehele genoom om het LPC-gen te identificeren, door middel van modelvrije koppelingsanalyse met gebruikmaking van 320 microsatelietmarkers, is beschreven in **hoofdstuk 4**. Hiertoe werden DNA-monsters van aangedane nestgenoten uit 10 verschillende nesten paarsgewijs geanalyseerd. Tegelijkertijd verrichtten we een onderzoek van het gehele genoom met gebruikmaking van 1536 SNP-markers, die gelijkmatig verspreid liggen over het hondengenoom. Dit onderzoek werd mogelijk gemaakt door de beschikbaarheid van nieuwe laboratoriumtechnologie waarmee grote aantallen SNPs gelijktijdig geanalyseerd konden worden. Vergelijking van de resultaten van deze twee analyses van het genoom toonde 5 loci aan op verschillende chromosomen die bij LPC betrokken kunnen zijn, en wel op chromosoom 1 (CFA01), CFA06, CFA13, CFA28 en CFA34. Deze potentiële LPC loci werden nader geanalyseerd door middel van "*finemapping*" met een array van 384 hiervoor speciaal geselecteerde SNPs. De DNA-monsters van 23 nesten van 17 families werden gebruikt in het finemappingsonderzoek. Dit resulteerde in de identificatie van aan LPC gekoppelde loci op CFA01 en CFA13, maar het lukte niet het gen voor LPC nader te identificeren (Hoofdstuk 4). Verder onderzoek naar de mogelijke kandidaat-genen op deze loci vindt op het ogenblik plaats.

Alhoewel we geen collageengenen konden aanwijzen die in belangrijke mate betrokken zijn bij het ontstaan van LPC, bleken de microsatellietmarkers voor deze genen wel polymorf te zijn. We stelden het polymorfe karakter van deze markers vast bij verschillende hondenrassen zoals Boxer, Berner Sennenhond, Duitse Herder en Labrador Retriever. We vonden dat er een grote mate van heterozygositeit voor deze markers bestaat (**Hoofdstuk 5**) . We onderzochten ook of deze collageengenen betrokken zijn bij de ruptuur van de voorste kruisband bij Boxers, een ras waarvan de honden een groot risico lopen om deze aandoening te verkrijgen. Jammer genoeg konden we niet aantonen dat de collageengenen betrokken waren bij de pathogenese van de kruisbandruptuur bij Boxers.

Het gedrongen postuur met disproportioneel korte pijpbeenderen bij honden lijdend aan chondrodysplasie werd gezien in combinatie met incongruentie en onregelmatige belijningen van het ellebooggewricht. We onderzochten chondrodysplasie bij 8 Labradors en hun verwanten (**Hoofdstuk 6**), met gebruikmaking van de kandidaatgen benadering. Hierbij werd de koppelingsanalyse

uitgevoerd met de vooronderstelling, zoals bleek uit de analyse van de stamboom, dat de aandoening recessief vererft. Als het gen dat chondrodysplasie veroorzaakt bekend is, dan zou dit onze kennis van deze aandoening bij honden kunnen vergroten en mogelijk door extrapolatie van deze kennis van andere, mogelijk verwante, aandoeningen zoals het LPC. Omdat de bestudeerde chondrodysplasie bij Labradors gelijkenis vertoont met *multiple epiphyseal dysplasia* en *spondyloepiphyseal dysplasia* zoals dit bij de mens voorkomt, bestudeerden wij de betrokkenheid van de genen die coderen voor het cartilage oligomeric matrix collagen (COMP), COL9A1, COL9A2 en COL9A3, Matrilin-3, en voor SLC26A2 bij chondrodysplasie bij deze honden. We verwachtten dat Labrador Retrievers met chondrodysplasie homozygote markers zouden hebben van het gen als dat gen deze aandoening zou vertegenwoordigen. We vonden echter geen enkele marker die uitsluitend homozygoot bij de Labradors met chondrodysplasie aanwezig was, alhoewel deze genen verantwoordelijk zijn voor chondrodysplasie bij de mens.

We beschreven als eersten de erfelijke radius-luxatie bij de Bouvier (**Hoofdstuk 7**). Deze afwijking manifesteert zich door de disproportionele korte voorextremiteiten met valgusstand en veranderingen in het ellebooggewricht die leiden tot verschillende graderingen van ellebooglуxatie en -misvorming. Deze verschijnselen vertonen overeenkomst met aangeboren ellebooglуxatie bij Dwergpoedels, Skye Terriers en andere rassen, en die ook beschreven zijn in geval van chondrodysplasie bij St Bernards, Finse Lappenhonden, Newfoundlanders en Duitse Herders. De subluxatie van de radiuskop bij Bouviers was veel ernstiger dan werd gezien bij de Labrador Retrievers met chondrodysplasie, zoals beschreven in hoofdstuk 6. De disproportionele lichaamsbouw van de Bouviers met chondrodysplasie was beperkt tot de voorextremiteiten, terwijl bij de Labrador Retrievers alle vier extremiteiten betrokken waren. Wij poneerden de hypothese dat de subluxatie van het radiuskopje bij deze Bouviers het gevolg was van een hoek in de as van de radius en dat de verplaatsing van het radiuskopje verergerde door de krachten uitgeoefend door de musculus supinator en de losheid van het ligametum annulare. De buiging naar voren toe van het olecranon zou veroorzaakt kunnen worden door de trekkracht die de musculus triceps brachii hierop uitoefent. De hoek in de as van de radius zou kunnen ontstaan in de periode vlak na de geboorte of kort daarna en werd gezien in het pijpbeengedeelte van de radius, ter hoogte van de insertie van het ligamentum interosseus. Onze bevindingen wijzen er op dat de meest waarschijnlijke wijze van vererving dominantie met een verminderde penetrantie zou kunnen zijn, zodat aangedane Bouviers niet altijd de ziekteverschijnselen zullen vertonen. We voerden een genetische onderzoek uit die gebaseerd was op de hypothese dat de radiuskop-subluxatie werd veroorzaakt door een genetische instabiliteit dat door het toepassen van zogenaamde lijnteelt homozygoot in de aangedane honden aanwezig zou zijn en waarvan de instabiliteit door de generaties heen zou toenemen. Echter het aantal chromosomen bleek normaal en we konden geen

abnormaliteiten aan het karyotype ontdekken, zodat onze hypothese nog niet aangenomen kon worden. De grote mate van homozygotie, die in de groep van aangedane Bouviers werd aangetoond, vereist echter nader genetisch onderzoek.
Het onderzoek dat in dit proefschrift is beschreven, werd uitgevoerd om het onderliggende defect van het LPC aan te tonen. Door onze bevindingen zijn mogelijk verantwoordelijke loci gelokaliseerd en zijn enkele kandidaatgenen uitgesloten. Er moet echter worden bedacht dat een ziekte niet altijd het gevolg is van genetische factoren alleen en dat omgevingsfactoren een belangrijke rol kunnen spelen in het optreden van een afwijking. Het genetische onderzoek van het LPC dat in dit proefschrift is beschreven, kan als voorbeeld dienen voor het onderzoeken van andere aandoeningen in de veterinaire orthopedie, zoals chondrodysplasie en radiuskopluxatie.

บทสรุปภาษาไทย

โรคชิ้นกระดูก coronoid แตก (FCP) เป็นโรคหลักที่สำคัญของกลุ่มอาการโรคข้อเสื่อมในสุนัข นอกเหนือจาก FCP แล้ว โรคข้อเสื่อมสุนัขจะประกอบไปด้วย โรค osteochondrosis ของกระดูก medial humeral condyle, โรคกระดูกข้อศอกไม่สัมพันธ์กัน และโรค anconeal process ไม่เชื่อมต่อ เป็นที่ตระหนักว่าโรคชิ้นกระดูก coronoid แตกเป็นโรคที่ถ่ายทอดทางพันธุกรรม ซึ่งสามารถพบในสุนัขหลายสายพันธุ์ แต่ที่พบได้บ่อยคือสุนัขสายพันธุ์ใช้งานดังเช่น ลาบาร์ดอร์รีทีฟเวอร์เป็นต้น สุนัขสายพันธุ์นี้จะถูกใช้งานเป็นสุนัขนำทางคนตาบอดและผู้พิการ โรค FCP จะแสดงออกให้เห็นอาการคือ ขากระแผลก หากแต่การวินิจฉัยจำต้องใช้การถ่ายภาพรังสีที่มีประสิทธิภาพ การส่องตรวจด้วยกล้องหรือจากการผ่าตัดเท่านั้น

ในบทที่ 2 ได้อธิบายแง่มุมต่างๆของ FCP ดังเช่นการพัฒนาการของข้อต่อข้อศอก พยาธิกำเนิดที่เป็นไปได้ของ FCP และการศึกษาทางพันธุศาสตร์เพื่อตรวจหายีนที่ก่อโรค ข้อต่อข้อศอกเจริญพัฒนามาจากเซลล์ mesenchymal ของหน่อระยางค์ ศูนย์กลางการพัฒนาการของกระดูกทั้งส่วนแรกและส่วนรองจะพัฒนามาจากกระดูกอ่อนต้นแบบ ซึ่งก็เป็นเช่นเดียวกันในกรณีของกระดูก coronoid ในขณะที่มีการพัฒนาของชิ้นกระดูก coronoid นั้นมีหลายกระบวนการมาเกี่ยวข้อง ซึ่งก็เป็นไปได้ว่าความผิดปกติบางอย่างอาจนำไปสู่การแตกหักของชิ้นกระดูกได้ ดังเช่น การขัดขวางการพัฒนากระดูกอ่อนไปเป็นกระดูก และ/หรือ ความไม่สมดุลทางชีวกลศาสตร์ของ annular ligament, การไม่สัมพันธ์กันดีของข้อต่อระหว่าง radio-ulna และ humeral-ulna และการเจริญไม่สำพันธ์กันระหว่างกระดูก radius และ ulna นอกจากนี้บทที่ 2 ของวิทยานิพนธ์นี้ได้กล่าวถึงสมมุติฐานของพยาธิกำเนิดของ FCP ในแง่มุมต่างๆ รวมทั้งได้กล่าวถึงการศึกษาทางพันธุศาสตร์ที่จะนำไปสู่การค้นหายีนที่ก่อโรคนี้ ข้อเท็จจริงเกี่ยวกับพันธุกรรมของโรค FCP ดังเช่น พบใน 14.7% ของประชากรสุนัขลาบาร์ดอร์ในเนเธอร์แลนด์ พบในสุนัขเพศผู้ได้มากกว่าสุนัขเพศเมีย ลักษณะการถ่ายทอดทางพันธุกรรมของ FCP ในลักษณะยีนด้อยตามข้อคิดเห็นของหลายฝ่าย แต่ยังเป็นที่สรุปไม่ได้ รวมทั้งเรื่อง marker ทางพันธุศาสตร์ 2 ชนิดที่เหมาะสมกับการศึกษาทางด้านชีวะโมเลกุลทางพันธุศาสตร์ของโรค FCP คือ microsatellite marker และsingle nucleotide polymorphism (SNPs) โดยที่ marker แรกคือนิวคลีโอไทด์ช่วงสั้นๆ ที่ซ้ำติด ๆ กัน และ marker หลังเป็น marker ที่ความแตกต่างกันใน marker เองเพียงแค่ระหว่างนิวคลีโอไทด์เดียว นั้นก็หมายความว่าแต่ละ SNPs จะมีความหลากหลายในแต่ละตำแหน่ง marker อย่างมากเพียง 2 อย่าง (อัลลีล) ระหว่างคู่โครโมโซม ขณะที่ microsatellite จะมีได้มากกว่า marker ทั้ง 2 ชนิดจะสามารถถ่ายทอดสู่รุ่นลูกหลายอย่างคงที่ สามารถติดตามได้และสามารถใช้เป็นตัวแทนยีนที่อยู่ระยะใกล้เคียงได้เพื่อใช้ในการศึกษาทางพันธุศาสตร์

มี 2 วิธีที่จะนำไปใช้สำรวจยีนก่อโรค FCP ที่นำเสนอในบทที่ 2 นั้นคือ วิธีวิเคราะห์ linkage และวิธีวิเคราะห์ association โดยที่วิธี linkage จะใช้ marker ทางพันธุศาสตร์และข้อมูลระหว่างความสัมพันธ์ พ่อ แม่ ลูก ในการวิเคราะห์ว่า marker ได้ถูกถ่ายทอดไปสู่รุ่นลูกหลานในลักษณะเดียวกันกับที่เราคาดไว้หรือไม่ ในขณะที่วิธีวิเคราะห์ association จะทำการเปรียบเทียบความถี่ของ marker อัลลีล ระหว่างสัตว์กลุ่มตัวอย่าง (สัตว์ป่วย) และกลุ่มควบคุม โดยไม่ได้เอาข้อมูลความสัมพันธุ์ทางสายเลือดมาร่วมวิเคราะห์

กลุ่มสุนัขลาบาร์ดอร์ที่เราศึกษามีความสัมพันธ์ทางสายเลือดที่ชัดเจน ถือว่าเป็นข้อมูลที่เป็นประโยชน์อย่างมากโดยเฉพาะในการวิเคราะห์ linkage และหนึ่งในวิธี linkageที่ใช้ในการศึกษาคือการวิเคราะห์ linkage ด้วยวิธี sibling-pair โดยหลักการจะเป็นการหา marker อัลลีลซึ่งพบว่าถ่ายทอดไปสู่สุนัขที่เป็น FCP ได้มากกว่าปกติ หรืออีกนัยหนึ่งคือ อัลลีลนั้นจะถูกพบได้บ่อยมากในระหว่างคู่สุนัขที่เป็นโรคในคลอกเดียวกัน วิธีวิเคราะห์ linkage กับคู่

sibling ถือว่าเป็นวิธีที่ไม่ได้อาศัยลักษณะการถ่ายทอดทางพันธุกรรมของโรค FCP มาใช้ประกอบการวิเคราะห์ ซึ่งต่างจากกรณีของการวิเคราะห์ linkage โดยทั่วไป ซึ่งถ้าผลกรศึกษาพบว่ามีความ linkage อย่างมีนัยยะสำคัญทางสถิติก็หมายความว่า marker นั้นอยู่ใกล้กับยีนก่อโรคนั้นๆ

ในบทที่ 3 ได้กล่าวถึงวิธีการวิเคราะห์โดยอาศัยยีนคู่แข่งที่เหมาะสมมาศึกษาวิเคราะห์ว่ายีนนั้นเป็นยีนก่อโรค FCP หรือไม่ สำหรับการเลือกยีนคู่แข่งที่เหมาะสมในการวิเคราะห์นั้นขึ้นอยู่กับการเปรียบเทียบกับโรคเดียวกันในต่างสปีชี่ ซึ่งทั้งนี้รวมถึงโรคที่คล้ายคลึงกับมนุษย์ด้วย หรืออาจจะเลือกยีนคู่แข่งที่อาจเป็นไปได้กับการมีส่วนเกี่ยวข้องกับพยาธิกำเนิดและกลไกลการเกิดโรค FCP เป็นที่ทราบกันว่าไม่พบว่ามีโรค FCP ในมนุษย์และสัตว์สปีชี่อื่นๆ ดังนั้นการเปรียบเทียบหายีนคู่แข่งในลักษณะนี้จึงไม่สามารถทำได้

คลอลาเจนโปรตีนถือเป็นโปรตีนส่วนประกอบหลักและพบได้มากในบริเวณนอกเซลล์ของเซลล์กระดูกและเซลล์กระดูกอ่อน กระดูก coronoid เจริญมาจากกระดูกอ่อนจนไปเป็นกระดูก ซึ่งก็มีความเป็นไปได้ว่าร่างการเจริญผิดปกติอาจเกิดขึ้นได้ระหว่างนั้น ซึ่งอาจนำไปสู่โรค FCP ได้ จึงตั้งสมมุติฐานว่าคอลลาเจนยีนอาจมีส่วนเกี่ยวข้องอย่างมากกับการเกิดโรค FCP ดังนั้นการศึกษาวิจัยในบทที่ 3 จึงได้สำรวจและพัฒนา microsatellite marker ที่อยู่ใกล้กับคอลลาเจนโปรตีนต่างๆซึ่งประกอบด้วย COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2 และ COL24A1 และใช้ marker เหล่านั้นเป็น marker ของยีนคู่แข่งในการวิเคราะห์ linkage ของโรค FCP โดยหลักการพบว่าสุนัขครอกเดียวกันคู่ใดก็ตาม จะถือว่ามีอัลลีลที่เหมือนกันอยู่โดยเฉลี่ย 50% ของอัลลีลที่ได้รับการถ่ายทอดมาจากพ่อแม่เดียวกัน และเราสามารถเป็นที่คาดหมายได้ว่าอัลลีลของ marker ของยีนก่อโรคในคู่สุนัขที่เป็นโรคในครอกเดียวกันควรจะเหมือนกันอย่างมาก และมากกว่า 50% อย่างมีนัยทางสถิติ ดังนั้นการศึกษายีนคู่แข่งจึงได้ทำการศึกษาในสุนัขพันธุ์ลาบาร์ดอร์จำนวน 13 ตลอก จาก 10 เพดดีกรีด้วยวิธีวิเคราะห์ linkage กับคู่สุนัขที่เป็นโรค FCP และอยู่ในครอกเดียวกัน จากผลการศึกษาสรุปได้ว่า คลอลาเจนยีนดังกล่าวข้างต้น ดูเหมือนว่าจะไม่ได้มีส่วนสำคัญในการก่อโรค FCP ในสุนัขพันธุ์ลาบาร์ดอร์รีทีฟเวอร์ นอกจากนี้ยีน Vitamin D receptor (VDR) ซึ่งพบอยู่ใกล้กับ COL2A1 เพียง 80 kb ก็อาจไม่ได้มีส่วนในการก่อโรค FCP เช่นเดียวกับ COL2A1

หลังจากที่แผนที่จีโนมรุ่นที่สองของสุนัขเสร็จสมบูรณ์ในปีพ.ศ.2548 ได้นำไปสู่การจัดลำดับและวางตำแหน่งของ microsatellite masker ซึ่งแต่เดิมจากแผนที่จีโนมในลักษณะแตกต่างกันเช่น แผนที่ radiation hybrid และแผนที่ linkage ให้สามารถจัดนำมาแสดงบนแผนที่จีโนมรุ่นที่ 2 นี้ และได้ถือว่าเป็นแผนที่อ้างอิงในสุนัขให้กับยีนและ marker อื่นๆ ในโอกาสเดียวกันนี้แผนที่จีโนมอ้างอิงของสุนัขยังได้รวมถึง SNPs อย่างละเอียดและครอบคลุมตลอดจีโนมสุนัข ด้วยเหตุนี้จึงเป็นไปได้ที่จะใช้ทั้ง microsatellite marker และ SNPs ในการวิเคราะห์สำรวจหายีนในลักษณะไม่จำกัดบริเวณใดบริเวณหนึ่งดังเช่นการวิเคราะห์ยีนคู่แข่ง หากแต่เป็นการวิเคราะห์ที่คลอบคลุมตลอดจีโนมสุนัข จากผลการศึกษาในบทที่ 4 เป็นการศึกษาในลักษณะคลอบคลุมตลอดจีโนมสุนัขกับการวิเคราะห์ linkage มีการใช้ microsatellite marker 320 ตัว ในกลุ่มตัวอย่าง 10 เพดดีกรี ในขณะเดียวกันยังได้ศึกษาวิเคราะห์ linkage ด้วย SNPs 1536 ตัว ตลอดจีโนมไปพร้อมกันด้วย ผลของการศึกษาและทำการเปรียบเทียบระหว่าง 2 ชุดการศึกษา พบว่ามี 5 ตำแหน่งของโครโมโซม 1, 6, 13, 28 และ 34 มีส่วนเกี่ยวข้องกับการเกิดโรค FCP จากนั้นจึงได้ทำการศึกษาต่อเนื่องบนตำแหน่งดังกล่าวโดยมีการเพิ่มจำนวน SNPs เป็น 384 ตัว และขยายการศึกษาวิจัยครอบคลุมไปถึง 23

คลอก ใน 17 เพดดีกรี ในที่สุดพบว่าโครโมโซม 1 และ 13 มีความเป็นไปได้สูงมากที่จะมียีนที่เป็นต้นเหตุก่อโรค FCP ในลาบาร์ดอร์อยู่ ซึ่งการศึกษาวิจัยลงบนเฉพาะ 2 ตำแหน่งดังกล่าว ยังต้องดำเนินต่อไป

แม้ว่าการศึกษาไม่พบว่าคลอลาเจนยีนเป็นยีนที่ก่อโรค FCP ในสุนัขพันธุ์ลาบาร์ดอร์รีทีฟเวอร์ แต่ marker ของยีนเหล่านี้ได้แสดงออกถึงความหลากหลายซึ่งเป็นคุณลักษณะเฉพาะของ marker การศึกษาวิเคราะห์ตรวจสอบคุณลักษณะความหลากหลายของ marker ของยีนเหล่านี้ได้ถูกทำการทดสอบในสุนัขพันธุ์บ๊อกเซอร์, พันธุ์เบอร์นีสเมาเท่น, พันธุ์เยอรมันเชิฟเฟริด และพันธุ์ลาบาร์ดอร์รีทีฟเวอร์ ผลการศึกษาพบว่า marker ส่วนใหญ่มีคุณสมบัติของ heterozygosity สูงในทุกสายพันธุ์ ซึ่งได้กล่าวไว้ในบทที่ 5 นอกจากนี้ผลการศึกษายังพบว่าคอลลาเจน marker เช่น COL9A1, 9A2 และ 9A3 ไม่มีส่วนเกี่ยวข้องทางพันธุกรรมกับการเกิดโรคข้อเข่าฉีกในสุนัขพันธุ์บ๊อกเซอร์ในกลุ่มตัวอย่างที่ทำการศึกษา

ในบทที่ 6 ได้ศึกษาถึงโรค chondrodysplasia ในสุนัขพันธุ์ลาบาร์ดอร์รีทีฟเวอร์ ซึ่งโรคนี้แสดงออกโดยมีลักษณะของกระดูกระยางค์สั้น และไม่สมดุลกับโครงสร้างของร่างกาย นอกจากนี้ยังพบว่ามีโรคข้อศอกเสื่อม และกระดูกข้อศอกไม่สัมพันธ์กันซึ่งเป็นผลต่อเนื่องของโรคนี้ บทที่ 6 ได้ทำการศึกษายีนคู่แข่งกับโรค chondrodysplasia โดยใช้การวิเคราะห์วิจัยบนพื้นฐานของยีนด้อยเป็นยีนก่อโรคนี้ในสุนัขลาบาร์ดอร์รีทีฟเวอร์ในกลุ่มตัวอย่าง ด้วยคาดว่าผลจากการศึกษานี้จะทำให้เกิดความเข้าใจในโรค chondrodysplasia ในสุนัขมากขึ้นรวมถึงเป็นได้ที่จะเข้าใจถึงบางแง่มุมของพยาธิกำเนิดของโรคกระดูกและข้อต่อเช่น FCP

ด้วยเป็นเหตุที่ chondrodysplasia ในลาบาร์ดอร์รีทีฟเวอร์ ในกลุ่มศึกษานั้น คล้ายคลึงกับโรค multiple epiphyseal dysplasia และ spondyloepiphyseal dysplasia ในมนุษย์ การศึกษาจึงมุ่งไปวิเคราะห์ยีนคู่แข่งในการก่อโรคดังกล่าวในมนุษย์ ซึ่งประกอบด้วย cartilage oligomeric matrix protein (COMP), COL9A1, COL9A2, COL9A3, Matrilin 3 และ Solute carrier family 26 member 2 (SLC26A2) ในกลุ่มที่เป็นโรคนี้ โดยการศึกษาได้ดังสมมุติฐานว่าถ้ายีนเหล่านี้มีส่วนเกี่ยวข้องกับโรค chondrodysplasia ในสุนัขพันธุ์ลาบาร์ดอร์รีทีฟเวอร์ ก็เป็นไปได้ว่าจะพบ marker อัลลีลเหล่านี้ในลักษณะโฮโมไซกัสโดยเฉพาะในกลุ่มสุนัขที่เป็นโรคเท่านั้น ผลการศึกษาไม่พบว่า marker อัลลีลใดแสดงให้เห็นว่าเป็นโฮโมไซกัสเพียงเฉพาะสุนัขที่เป็นโรค chondrodysplasia

ในบทที่ 7 ได้ทำการศึกษาโรค radial subluxation ในสุนัขพันธุ์บูวิเอ เดส ฟลานเดส เป็นครั้งแรก โรคนี้แสดงออกโดยกระดูกระยางค์หน้า สั้นและไม่สมดุลกับร่างกาย และพบร่วมกับมีข้อศอกเสื่อม และข้อศอกหลุดในระดับต่างๆ ซึ่งลักษณะอาการเหล่านี้มีความคล้ายคลึงกับโรคข้อศอกหลุดที่ถ่ายทอดทางพันธุกรรมในสุนัขพันธุ์มินิเอเจอร์ พุดเดิ้ล สคายเทอเรีย และพันธุ์อื่นๆ นอกจากนี้ยังพบว่ามีส่วนคล้ายกันกับโรค chondrodysplasia ในสุนัขพันธุ์เซนท์เบอร์นาด, แลบสุลด์, นิวฟาล์วแลนด์ และเยอร์มันเชฟเฟริด โดยที่ radial luxation ในสุนัขพันธุ์บูวิเอ เดส ฟลานเดส จะมีความรุนแรงกว่าที่พบ (ในบางราย) ในโรค chondrodysplasia ในสุนัขพันธุ์ลาบาร์ดอร์ที่ได้กล่าวไว้ในบทที่ 6

ลักษณะโครงสร้างระยางค์สั้นที่พบสุนัขพันธุ์บูวิเอนั้นจะจำกัดให้เห็นเฉพาะขาหน้า ในขณะที่พบได้ทั้งขาหน้าและขาหลังในโรค chondrodysplasia ในพันธุ์ลาบาร์ดอร์ที่กล่าวถึงในบทที่ 6 ในบทที่ 7 นี้ได้ตั้งข้อสมมุติฐานว่า radial head subluxation ในสุนัขพันธุ์บูวิเอนั้นเป็นผลมาจากกระดูก radius โก่งงอ, ร่วมกับแรงจากกล้ามเนื้อ supinatus และการหย่อนของพังผืดรอบข้อต่อข้อศอก ในกรณีโก่งงอมาทางด้านหน้าของ olecranon ที่พบในโรคนี้อาจเป็นได้จากแรงกล้ามเนื้อ tricep brachii

จากการศึกษาพบว่าการโก่งงอของกระดูก radius เกิดขึ้นในช่วงระหว่างแรกเกิด และไม่นานหลังจากนั้น โดยพบว่าจุดของการโก่งงออยู่บริเวณจุดเกาะยึดของพังผืดกระหว่างกระดูก radius และ ulna

ผลการศึกษาในบทที่ 7 ได้แสดงถึงความเป็นไปได้ของลักษณะของการถ่ายทอดทางพันธุกรรมในลักษณะยีนเด่น และการแสดงออกของฟีโนไทป์ไม่สมบูรณ์ในกรณีมียีนก่อเป็นโรค ซึ่งสอดคล้องกับความเป็นไปได้ที่สัตว์ที่เป็นโรคไม่แสดงอาการให้พบเสมอไป นอกจากนี้การศึกษาได้ตั้งข้อสมมุติฐานว่าโรค radial head subluxation เป็นผลมาจากภาวะไม่คงตัวของบางตำแหน่งของโครโมโซม ซึ่งรวมถึงส่วนซ้ำของโครโมโซมบางส่วนได้ขยายบริเวณหรือสูญหายไปในแต่ละชั่วอายุ ผลการศึกษาพบว่าโครโมโซมของสัตว์ที่เป็นโรคมีจำนวนปกติ และลักษณะโครโมโซมเป็นปกติ ซึ่งไม่พบว่าสนับสนุนสมมุติฐานที่ตั้งไว้ อย่างไรก็ตามการพบภาวะโฮโมไซโกซิตี้อย่างมากในกลุ่มสุนัขที่เป็นโรค เป็นสิ่งที่ทำให้ต้องศึกษาเพิ่มเติมในแง่ของพันธุ์ศาสตร์ของโรค radial head subluxation ในสุนัขพันธุ์บูวิเอ เดส ฟลานเดส

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Curriculum vitae

The author was born in on 5 October 1969, Bangkok, Thailand. He attended secondary and high school at Wat Benjamaborpit school in Bangkok. He graduated from high school in 1988. He studied Veterinary Medicine in Faculty of Veterinary Medicine, Kasetsart Univerity, Bangkok, and graduated in 1993. After graduation from Veterinary school, he worked in Small Animal Teaching Hospital, Kasetsart University, Bangkein Campus, Bangkok, for 4 years. He was then assigned to be a staff member of the Department of Surgery (now is Clinical Sciences of Companion Animals after integration with section of Small Animal Medicine), Faculty of Veterinay Medicine, Kasetsart University in 1998. He married Pimjai Meethong in 2000. He was granted the scholarship from Commission of Higher education, Ministry of Education in 2003. He began PhD candidate in Department of Clinical Sciences of Companion Animal, Faculty of Veterinary Medicine, Utrecht University in 2004, under kindly supervised by Professor Dr. Herman A.W. Hazewinkel and Dr. Peter A.J. Leegwater.

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