Analysis of genomic region(s) and gene(s) associated with cranial cruciate ligament rupture in the dog

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Analysis of genomic region(s) and gene(s) associated with cranial cruciate ligament rupture in the dog

by

Vicki Lea Wilke

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Animal Breeding and Genetics (Molecular Genetics)

Program of Study Committee:
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Iowa State University
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2006

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For the Major Program
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ABSTRACT

Rupture of the cranial cruciate ligament (CCLR) in the dog is the most common cause of hind limb lameness. When CCLR occurs it results in instability in the knee leading to progressive, debilitating arthritis and lameness. Particular breeds of dogs (e.g. Labrador retriever, Newfoundland) are predisposed to CCLR while other breeds (e.g. Greyhound) have dramatically reduced frequency of this disorder supporting a heritable basis for the CCLR trait. For this study, we first estimated the economic impact to veterinary clients for the medical and surgical management for CCLR and determined the prevalence rate for CCLR in Newfoundlands that were seen at the Iowa State University Veterinary Teaching Hospital (ISU-VTH). Two hundred fifty six diplomates of the American College of Veterinary Surgeons (ACVS) and 1,083 small animal veterinarians responded from a survey sent to 4500 individuals. The results revealed that the dog owning public spent an estimated $1.3 billion for CCLR medical and surgical management in the U.S. in 2003. Next, we examined medical records for a diagnosis of CCLR for all Newfoundlands that were presented to the ISU-VTH. One hundred sixty three Newfoundlands were evaluated from January 1, 1996, through December 31, 2002, and 22% were diagnosed with CCLR. In addition, a large-scale recruitment study was undertaken from the National Newfoundland Registry and local breeders to collect five-generation pedigrees from Newfoundlands. Newfoundlands identified for the recruitment study were examined by a veterinarian and classified as affected with CCLR based on typical signs of CCLR. These signs included pain on hyperextension of the knee, knee effusion, decreased range of motion, positive cranial drawer sign or cranial tibial thrust, radiographic evidence of knee arthritis, and/or surgical confirmation of a ruptured cranial cruciate ligament. Pedigrees were constructed using Pedigraph version 1.1. Inbreeding coefficients were calculated using SAS v 8.2. Segregation analysis was performed to predict mode of inheritance. The recruitment study included 411 Newfoundlands, of which 92 (22%; 53 females and 39 males) were affected with CCLR and 319 (182 females and 137 males) were unaffected. The average inbreeding coefficient for those animals that were inbred was 0.05 (range 0.004 – 0.17). Segregation analysis was performed iteratively, with updating of penetrance values from prevailing genotype probabilities. This analysis predicted a recessive pattern of inheritance. The frequency of the recessive allele was 0.60 with 51% penetrance. Biological candidate gene analysis yielded single nucleotide polymorphisms (SNPs) in COL9A1, COL9A2, COMP, and FBN. Association analyses using restriction fragment length polymorphisms designed from the SNPs were performed on 90 dogs selected from a population of Newfoundlands; 45 unaffected and 45 affected with CCLR. There was no statistically significant association with the SNPs and CCLR status, although COL9A1 revealed statistically close association (P = 0.10). Two
cumulative genome scans were performed, first with 97 microsatellites (MSATs) and then, in a
collaborative effort with UC-Davis, an additional 320 MSATs were used (MSAT interval
approximately every 6.7 cM). Initial results indicate CCLR status association to seven chromosomes
that had 4 or more markers with statistical significance; chromosomes 3, 5, 10, 14, 18, 23, and 27. A
partial list of potential positional candidate genes located on chromosomes 3, 10, and 23 includes
several related growth factor genes; fibroblast growth factor binding protein, fibroblast growth factor
receptor substrate 2, insulin like growth factor-1 receptor, transforming growth factor alpha,
transforming growth factor beta type II receptor, and the related bone morphogenetic protein 10
precursor; as well as several proteoglycan related genes (versican, proteoglycan link protein 2
precursor, aggrecan core protein precursor, and chondroitin synthase 1) and interleukin receptors
(interleukin-1 receptor type I and II, and interleukin-2 receptor beta). In summary, CCLR is a
debilitating disease due to its resultant progressive arthritis. Surgery is presently recommended as the
treatment of choice in the majority of dogs, costing the public over $1.3 billion dollars each year. The
incidence of CCLR in the ISU-VTH population of Newfoundlands was excessive (22%). Analysis
revealed a heritability of 0.27 and potential recessive mode of inheritance with 51% penetrance. This
implies that only 51% of the dogs with the recessive genotype will exhibit clinical signs of CCLR
making diagnosis harder. A microsatellite based genome scan in a population of Newfoundlands
determined several chromosomal regions to be associated with CCLR status. Fine mapping these
chromosomal regions should further narrow the list of potential CCLR candidate genes.
CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Rupture of the cranial cruciate ligament (CCLR) is considered one of the most common causes of hind limb lameness in dogs, representing nearly 20% of dogs that present to university hospitals for lameness (Johnson et al. 1994). The CCLR causes stifle joint instability and inflammation. This leads to the development of lameness and, despite surgical intervention, osteoarthritis (OA) (Vasseur et al. 1992; Elkins et al. 1991; Chauvet et al. 1996). Although the severity of OA of the stifle joint does not correlate with the severity of lameness (Gordon et al. 2003) many patients do require treatment for OA and this treatment may be required for the duration of the patient’s life. Treatment options include surgical and nonsurgical management. Surgical management is generally recommended for dogs weighing over 35 pounds because without surgery lameness persists in greater than 80% of cases (Chauvet et al. 1996; Elkins et al. 1991; Vasseur et al. 1992). This contributes to the increased cost for CCLR management since the breeds of dogs that are most predisposed to CCLR are relatively large in size (Whitehair et al. 1993; Duval et al. 1999). The overall cost to the owner for CCLR treatment depends on several other factors, such as expertise of the surgeon, surgical technique performed, and region of the country. Non-steroidal anti-inflammatory drugs (NSAIDs) in combination with weight management, physical therapy, and disease modifying drugs are all nonsurgical treatments commonly used for OA in dogs. In addition, it is reported that up to 50% of patients initially present or develop bilateral CCLR and only 50% of cases that receive surgery have an excellent outcome (Doverspike et al. 1993; Smith et al. 1985). The magnitude of this problem is compounded by the cost of surgery which ranges from an average of $1,000 to $2,500 per knee at university teaching hospitals. The overall outcome is that veterinary clients, in the year 2003, spent $1.32 billion for CCLR treatment for dogs (Wilke et al. 2005).

In comparison, approximately 38,000 women sustain injury to the anterior cruciate ligament (ACL) each year in the U.S. (Toth et al. 2001). This estimate, however, is likely to grow as the number of women participating in athletics (either structured or unstructured) increases. The significance of this problem becomes more evident when one considers that athletically active females are 2 to 8 times more likely to injure their ACL than athletic males (Anderson et al. 2001; Arendt et al. 1995; Charlon et al. 2002; Fagenbaum et al. 2003; Gwinn et al. 2000; Lohmander et al. 2004; Sciore et al. 1998; White et al. 2003; Wojtys, Ashton-Miller et al. 2002; Wojtys, Huston et al. 2002). In addition, the injury in women (in contrast to men) is frequently reported to be via non contact mechanisms such as landing from a simple jump or changing direction when running (Hewett...
2000; Malinzak et al. 2001; Toth et al. 2001). The outcome for the majority of these athletes is a shortened sports career, debilitating arthritis, and resultant decreased quality of life, as demonstrated by a study done by Roos et. al. that assessed outcome 12 years after ACL injury in young female soccer players (Lohmander et al. 2004; Roos et al. 1995). In addition, young athletes and teenagers that sustain injuries to the knee have a three fold increase in the risk of OA development by the age of 65 (Gelber et al. 2000). One would thus expect this group to require total knee arthroplasty to address the knee OA at a much earlier age than expected, increasing their risk for aseptic loosening and revision (Harrysson et al. 2004; Knutson et al. 1994). The economic burden to the health care industry for surgical care alone per person ranges from $3,679 to $17,000. Based on the fact that each year approximately 250,000 people, or 1 in 3,000, experience ACL injury (Colby et al. 2000), the total cost for surgery per year approaches $2 billion (Novak et al. 1996; Kao et al. 1995; Boden et al. 2000). Rupture of the ACL most commonly occurs in people < 40 years old and development of OA is common, this economic burden would be further increased if the cost of rehabilitation and medical management were added.

Rupture of the CCL in the dog has been reported to be associated with trauma, immune-mediated mechanisms (Galloway et al. 1995; Lawrence et al. 1998; Niebauer et al. 1987), age related degeneration of the CCL (Vasseur et al. 1985), obesity (Whitehair et al. 1993), and conformational abnormalities such as a patella luxation (Aiken et al. 1995) and a narrowed femoral intercondylar notch (Aiken et al. 1995; Shelbourne et al. 1998). In addition, a number of publications have suggested that the slope of the tibial plateau, or tibial plateau angle (TPA), may predispose dogs to CCLR. Many dogs develop CCLR from a single high-load traumatic event however it is also common for dogs with CCLR to have a history of only mild trauma (the effect of daily mechanical wear) (Moore et al. 1996). Statistical analyses would suggest that CCLR is an exceedingly prevalent disease, particularly in some breeds of dogs (Wilke et al. 2006). Whitehair et al. was the first to report that the Newfoundland, along with the Rottweiler and Staffordshire terrier, had an increased prevalence of CCLR (Whitehair et al. 1993). This finding was later supported when Duval et al. concluded that the Newfoundland breed had an increased risk for CCLR with an odds ratio of 6.65 (Duval et al. 1999). In contrast, the odds ratio was decreased for the Golden Retriever (0.48), Doberman Pinscher (0.33), and German Shepherd (0.25). At the ISU-VTH, 22% of all Newfoundlands that presented for care in a six year period were diagnosed with CCLR (Wilke et al. 2006). Recently, CCLR has been diagnosed at an earlier age than expected within these same breeds with no known history of trauma or other possible cause for the CCLR (Duval et al. 1999). These findings strongly support that CCLR, in some dog breeds, has a heritable component.
RESEARCH OBJECTIVES

Cranial cruciate ligament rupture is a very prevalent, costly disorder in the dog and has a similar clinical course in young, female athletes. The ability to identify an underlying genetic basis for CCLR in the dog would allow huge advances in understanding the etiopathogenesis of the disorder in humans as well as provide a large animal species to study therapeutic and preventative treatment options before their use in humans. The goal of chapter two was to establish the overall economic impact of medical and surgical treatment for CCLR in the dog in order that veterinary and consumer agencies will prioritize funding to seek a better understanding of the injury. Chapter three establishes the underlying genetic basis for CCLR in a population of Newfoundland dogs and proposes a potential mode of inheritance for the trait. Information gained from this chapter allowed us to classify CCLR as having a simple pattern of inheritance and led us to initiate our work using the candidate gene approach. Chapter four focused on the biological candidate gene approach and presents association analysis results of CCLR status with SNPs identified in \textit{COL9A1}, \textit{COL9A2}, \textit{FBN1}, and \textit{COMP}. Although there were no statistical associations between the identified SNPs and CCLR status, the PCR-RFLP tests described may be useful for association studies involving dogs that are affected with other musculoskeletal diseases, such as hip or elbow dysplasia. Chapter five uses the microsatellite based genome scan to identify CCLR associated chromosomal region(s). The results of this chapter will be explored further through fine mapping methods using positional candidate genes. All of the projects listed in the thesis are a systematic approach to attempt to identify an associated or causal mutation for CCLR in the dog, which would then be applied to attaining the long range goal of this project of identifying the cause of the increased incidence of ACL injury in athletic women.

THESIS ORGANIZATION

The remainder of this chapter is the literature review of the clinical course of the disorder in the dog with appropriate comparisons to young, female athletes and an explanation of the methodology used. The remainder of the thesis is organized into chapters based on research papers that have been either accepted for publication or will be submitted once final data is analyzed. Chapter two is the paper titled “Estimate of the annual economic impact of cranial cruciate ligament injury in dogs in the United States” which is available in the Journal of the American Veterinary Medical Association vol. 227(10), pp. 1604-1607 (2005). This work was conducted out of the Iowa State University Veterinary Orthopaedic Research Laboratory under the direction of Professor Michael G. Conzemius, who also made significant contributions to the paper. Dr. Vicki Wilke was involved in data analysis, Dr. Duane Robinson was responsible for data collection and organization.
and Professor Richard Evans was involved in mentoring in study design and statistical analyses. Professor Max Rothschild contributed suggestions and corrections to the paper.

Chapter three consists of the paper “Inheritance of rupture of the cranial cruciate ligament in Newfoundlands” which is available in the Journal of the American Veterinary Medical Association vol. 228(1), pp. 61-64 (2006). This work was conducted by Dr. Vicki Wilke under the direction of Professors Max Rothschild and Michael Conzemius. Max Rothschild was involved in numerous discussions concerning the progress of this work and both Max Rothschild and Michael Conzemius made significant contributions to the paper. Drs. Paula Macrossan and Brian Kinghorn performed the segregation analyses that identified the potential mode of inheritance for CCLR. Weigo Cai is a graduate student in the Department of Animal Science and was involved in the computer programming for the heritability determination using MTDFREML.

Chapter four consists of the paper “SNP detection and association analyses of candidate genes for rupture of the cranial cruciate ligament in the dog” published by the journal Animal Genetics vol. 36, pp. 519-521 (2005). This work was conducted by Vicki Wilke, while Max Rothschild provided research guidance and both Max Rothschild and Michael Conzemius contributed suggestions and corrections to the paper.

Chapter five consists of the paper “Chromosomal associations with cranial cruciate ligament injury in the Newfoundland dog.” This paper will be submitted to Mammalian Genome once genotyping results and a final analysis of the genome scan have been completed. Genotyping was completed by two different facilities, GeneSeek, Inc., Lincoln, NE and Veterinary Genetics Laboratory, Davis, CA. Alison Ruhe oversaw the laboratory work at the Veterinary Genetics Laboratory. The work was conducted by Vicki Wilke. Professor Richard Evans was involved in the computational programming for the statistical analyses. Max Rothschild was involved in numerous discussions concerning the progress of this work and made significant contributions to the paper. Michael Conzemius contributed suggestions and corrections to the paper.

Chapter six summarizes the general conclusions from each of the projects described in chapters two through five. It discusses the systematic methodology followed to identify the economic impact, prevalence, and underlying genetic basis to CCLR in the Newfoundland dog. Suggestions for future research based on the findings of this study are provided.
LITERATURE REVIEW

Anatomy

The intra-articular CCL originates on the caudomedial aspect of the lateral femoral condyle and inserts on the cranial intercondylar area of the tibial plateau (Evans et al. 1979), see Figure 1. The CCL has two main bands; the cranio-medial band which is taut in flexion and extension, and the caudolateral band which is taut only in extension (Moore et al. 1996, Part I). Thus, the CCL is primarily responsible for preventing hyperextension of the stifle because both bands are taut in extension. The cranio-medial band, because it is taut in both flexion and extension, is important for stabilizing against excessive cranial displacement of the tibia on the femur, which is known as cranial drawer movement. Blood supply to the CCL is from synovial tissue vessels that arose from branches of the genicular artery. Additional blood supply comes from longitudinal endoligamentous vessels that anastomose with the synovial vasculature. Unfortunately, the core of the midsection of the CCL, the most common site of rupture (Paatsama 1952), has a poorer blood supply than the proximal or distal portions of the CCL (Arnockzy et al. 1979).

The cranial cruciate ligament is composed of mostly collagen, water, proteoglycans, and elastin (less than 5 % of the dry weight). Collagen, which is the most abundant protein in the human body, makes up approximately 70-80% of the CCL. Type I collagen composes 88-91% of the CCL; Type III composes 9-12% of the CCL (Horton et al. 1996). Type I collagen is found in fibrous supporting tissue, such as the dermis, tendons, ligaments and bone. Major mutations in collagen genes are found in the Ehlers Danlos syndrome, a syndrome characterized by skin hyperextensibility, joint laxity, and tissue friability (Girotto et al. 2000). It is unlikely that CCLR is due to a major mutation in a collagen family gene. It is more likely that subtle changes in collagen structure, undetectable in puppies, may be important in common diseases, such as osteoarthritis, when dogs are mature. The majority of collagen stores are developed when the animals are young and adolescent and must withstand the lifetime of the animal. Thus, a minimal change in the amino acid sequence could impair the ability of the connective tissues to appropriately withstand normal daily mechanical loading. Mechanical load is concentrated at joint surfaces. This would lead to earlier than expected degeneration of tissues composed of collagen, more specifically rupture of the cranial cruciate ligament.

Proposed treatments and treatment outcomes

Surgical techniques to stabilize the stifle were introduced by Paatsama five decades ago (Paatsama 1952). The majority of research addressing CCLR in the dog since that time has focused on the development of new surgical techniques or adapting surgical techniques used in people for use
in the dog. The different techniques used in the dog consist of: 1) restoration of joint stability through periarticular tissue thickening (extracapsular repair), 2) transposing the lateral collateral ligament (fibular head transposition), 3) enhancement of the forces of the flexor muscles (tibial plateau leveling osteotomy) and 4) replacement of the ligament with autogenous grafts, allografts, xenografts, or synthetic material (intraarticular techniques) (Moore et al. 1996, Part II). Although many patients benefit from surgery it is well established that regardless of the surgical technique performed osteoarthritis progresses (Lazar et al. 2005). The expected outcome of surgical treatment is clinical improvement in 85% to 90% of dogs; unfortunately, less than 50% of dogs will achieve complete soundness (Doverspike et al. 1993; Smith et al. 1985). In summary, force platform gait analysis, an objective measure of limb function, has found that surgical technique has no influence on outcome (Conzemius et al. 2005).

**Determining causative factors**

**Tibial plateau angle.** In a normal standing animal, the proximal tibial plateau is compressed against the femoral condyles due to the forces of weight bearing and contraction of the gastrocnemius muscles. The CCL passively controls the cranial advancement of the tibia on the femur, hence when the CCL is ruptured, cranial translation of the tibia with respect to the femur can occur. Read and Robins were the first to describe the proximal tibial plateau angle (TPA) and report its association with CCLR in five dogs (Read et al. 1982). They theorized that a steep TPA predisposed the dogs to CCLR. Slocum reported the slope to be 22.6° in a population of dogs and suggested that this angle placed excessive strain on the CCL and predisposed dogs to tear of the CCL (Slocum et al. 1983). This data, however, was derived from a study population that did not define the dog's age, weight, breed or CCL status. Morris et al. reported that the slope of the TPA in dogs with CCLR was 23.76° while it was 18.1° in dogs without cranial cruciate ligament injuries (Morris et al. 2001). Dogs were considered without CCLR if they had no cranial drawer sign, no evidence of a medial buttress and no radiographic abnormalities of the stifle. In contrast, Caylor et al. reported the mean slope of the TPA in dogs without CCLR to be 23.5° and Reif et al. reported it to be 25.0° (Caylor et al. 2001; Reif et al. 2001). Neither of these two studies considered a breed effect even though it is reported that some breeds (e.g. Labrador Retriever, Newfoundland, Rottweiler) are predisposed to CCLR, and some breeds (e.g. Greyhound) are protected from CCLR (Duval et al. 1999). Since that time, three additional reports have found no clinically relevant difference in the TPA between dogs with and dogs without CCLR, refuting the TPA as a cause for rupture of the cranial cruciate ligament (Rooney et al. 2002; Reif et al. 2003; Wilke et al. 2002).
Patella luxation and postural abnormalities. The stifle joint is constrained by soft tissue instead of osseous structures; it is dependent on ligaments for stability (Arnoczky et al. 1977). Hence, abnormal stifle biomechanics are involved in the initiation of injury to the CCL and osteoarthritis development. The quadriceps mechanism consists of the quadriceps muscles, the patella, the patella tendon and its insertion on the tibial tuberosity. A luxating patella in an immature animal creates an abnormal force on the patella tendon and affects the development of the femur and tibia. Medial patella luxation is associated with internal tibial rotation and depending on the severity of the luxation, coxa vara; lateral patella luxation may be associated with external rotation of the foot and coxa valgum (Vasseur et al. 2003). The incidence of CCL associated with a luxating patella has not been specifically studied. In one study that evaluated the clinical outcome of 34 dogs that had surgery for a medial patella luxation, only two had been diagnosed with CCLR (Willauer et al. 1987).

Femoral intercondylar notch width. The femoral intercondylar notch (ICN) in the normal dog consists of the bell-shaped cranial outlet (the narrowest part of the femoral ICN), the intercondylar shelf, the caudal arch, and the caudal outlet and is almost completely filled by the cranial and caudal cruciate ligaments and fat (Fitch et al. 1995). The intercondylar shelf contacts the CCL between approximately 0° to 30° of stifle flexion (or near full extension) and this point of contact is the most common site for the CCL to rupture (Fitch et al. 1995). Therefore, a narrowed femoral ICN may predispose the CCL or a replacement intraarticular graft to failure. Narrowing of the CCL may be congenital or, more commonly, acquired. In humans, acquired femoral ICN narrowing is usually due to osteophyte formation secondary to degenerative joint disease caused by rupture of the ACL (Houseworth et al. 1987; Souryal et al. 1988; Souryal et al. 1993). Few reports have studied the femoral ICN in dogs. Aiken et al. evaluated femoral ICN in dogs that sustained a CCL injury and found a direct correlation between ICN width and CCL injury (Aiken et al. 1995). It is unknown whether the narrowed intercondylar notch in dogs is congenital, developmental, or secondary to OA formation. Women are reported to have a femoral ICN width that is narrower than the ICN width of men and this narrowed notch may explain the predisposition to ACL injury (Charlton et al. 2002; Shelbourne et al. 1998). A similar report suggested that an athlete with stenosis of the intercondylar notch is 26 times more likely of injuring their ACL in a non-contact sport than are athletes with a normal intercondylar notch width (Souryal et al. 1993). However, the most apparent criticism of this hypothesis is if the narrowing predisposes to ACL injury or is the OA change as its result (Hill et al. 2005; Quasnichka et al. 2005). In addition, one study reported that notch width was not statistically different between sex groups (Anderson et al. 2001). That and another study suggested that the volume of the ACL is less in female athletes when compared to men.
(Anderson et al. 2001; Charlton et al. 2002). They concluded that this decreased ligamentous volume may not be able to withstand the mechanical demand applied by athletic women. Unfortunately, these findings and conclusions are limited by a lack of correlative body size, body weight and mechanical strength data.

**Degeneration.** The CCL has been found to have decreased tensile stiffness and increased degeneration associated with an increase in age and body weight (Vasseur et al. 1985); this correlates to similar findings in clinical studies that have noted an increase in frequency of CCLR in older dogs and dogs weighing over 15 kg (Duval et al. 1999; Whitehair et al. 1993). Microscopic degenerative signs include loss of ligamentocytes, metaplasia of surviving ligamentocytes to chondrocytes, and failure to maintain collagen fibers and primary collagen bundles (Vasseur et al. 1985). These lesions tend to be concentrated in the central core of the CCL, the site that has been noted to have less vascularity than the rest of the CCL (Arnoczky et al. 1979; Paatsama 1952). This is also the site that the two functional bands of the CCL (the craniomedial and the caudolateral bands) twist upon each other. One study hypothesized that these microscopic degenerative lesions were due to ischemia from acquired loss of vascularity to the midportion of the CCL (Vasseur et al. 1985). The ligamentocytes that survived the ischemia were thought to do so by undergoing metaplasia to chondrocytes that were then able to use anaerobic metabolic pathways. The loss of collagen fibers and primary collagen bundles was considered a secondary effect of the ischemia on the ligamentocytes. These microscopic degenerative lesions of the CCL, specifically chondroid metaplasia and loss of collagen fibers, have been confirmed in other studies (Muir et al. 2002; Narama et al. 1996).

**Trauma.** Approximately 20% of all cases of CCLR are considered to be due to trauma (Moore et al. 1996). However, there is speculation that the ligaments may have been weakened by another process, such as degeneration or primary arthritis, that actually allowed the ligament to traumatally rupture. These latter animals would be considered to be predisposed to CCLR. The CCL is vital in maintaining joint stability by preventing cranial drawer motion, limiting internal rotation of the tibia on the femur, and preventing hyperextension of the stifle. True traumatic events that exceed the breaking strength of the CCL (considered to be approximately 4 times the body weight of a dog (Gupta et al. 1969)) can occur through three main mechanisms: hyperextension of the stifle, excessive internal rotation while the stifle is in partial flexion, or jumping. An example of hyperextension would be a dog that stepped into a hole while running; excessive internal rotation could occur by a pivotal shift while the limb is in a weight-bearing stance. Jumping is said to lead to CCLR through excessive cranial tibial thrust exceeding the breaking strength of the CCL. Cranial
tibial thrust is the cranially directed force of the tibia on the femur, the basis of the tibial compression mechanism (Slocum et al. 1983).

**Primary vs. secondary osteoarthritis.** Conflicting results have been reported concerning whether the osteoarthritis (OA) associated with CCLR is the inciting cause or secondary to joint instability and whether the OA is inflammatory or non-inflammatory (Doverspike et al. 1993; Galloway et al. 1995; Lawrence et al. 1998; Lemburg et al. 2003; Moore et al. 1996 Part I; Quasnichka et al. 2005). A study that characterized synovial fluid changes in dogs with partial and complete CCLR supports that CCLR in the majority of cases is non-inflammatory; however cases with partial CCLR did have inflammatory changes present in the synovial fluid (Griffin et al. 1992). Another study determined the presence of anticollagen antibodies in the synovial fluid of dogs with CCLR, suggesting an immune mediated mechanism as a cause of CCLR (Niebauer et al. 1987). They also determined that the presence of antibodies was more common in dogs with acute CCLR. Other studies have suggested that the presence of the antibodies may have been secondary to the damaged ligament and cartilage produced by the process of CCLR (Lemburg et al. 2003) and anticollagen antibodies have been found to be elevated in osteoarthritis associated with conditions other than CCLR (de Rooster et al. 2000). Numerous investigations of the role of inflammatory mediators (e.g. interleukins, TNF, metalloproteinases) in CCLR have improved insight into the disease process (Hegemann et al. 2002; Chu et al. 2002; Johnson et al. 2002). This information, however, is generally geared toward treatment of osteoarthritis, not the prevention of CCLR. It is known that the CCL is essential for stability of the stifle joint. Instability in a joint leads to inflammation and arthritis formation. The quandary, therefore, is whether these mediators caused the instability or are presented as part of the inflammatory process. Since these mediators are present in all forms of arthritis and to date, no one has shown any relationship between these mediators and the predisposed breeds, most researchers agree that although they contribute to the progression of CCLR they do not contribute to the cause of the condition.

**Obesity.** Obesity is considered a risk factor for CCLR through the placement of excessive strain on the ligament during normal weight bearing (Moore et al. 1996 Part II; Vasseur et al. 1993). Most studies support that female dogs have a higher incidence of CCLR (Whitehair et al. 1993) which is hypothesized to be due to the increased incidence of obesity in spayed female dogs (Edney et al. 1986). In rats, the effect of ovariectomy on the hip joint capsule was to decrease elastin content and the diameter of collagen fibers (Vasseur et al. 1993). It is unknown whether a similar effect may occur in dogs.
Neuromuscular differences. Studies addressing neuromuscular differences between men and women have yielded conflicting reports. Specifically, women were found to have similar hamstring muscle activation to men and had greater stifle flexion angles than men, both are known as protective mechanisms for the ACL (Fagenbaum et al. 2003). In contrast, Hollman et al. reported less hamstring activity in females than males (Hollman et al. 2003) and Malinzak et al. found women to have less flexion angles, greater quadriceps activation, and less hamstring activation than men (Malinzak et al. 2001). Other studies have found significant muscular differences between sexes that may contribute to the increased risk in females, such as imbalances in strength and coordination, timing activation, and lower extremity muscle pattern recruitment (Henry et al. 2001). It has also been suggested that women have inadequate muscular protection against anterior tibial translation (Anderson et al. 2001; Charlton et al. 2002). These findings were supported in work that revealed the quadriceps mechanism, which has been shown to increase anterior tibial translation when contracted, had increased coactivation in females compared to males, placing the ACL at a higher risk for injury (Shultz et al. 2001; White et al. 2003). Neuromuscular training has also been hypothesized to have an effect on the incidence of ACL injury in female athletes, supported by evidence that female athletes with additional neuromuscular training had a decreased incidence of ACL injury (Henry et al. 2001; Mykebust et al. 2003). When Hewett studied neuromuscular training, trained high school female athletes had no statistically significant differences in incidence of ACL injury when compared to untrained high school male athletes, and untrained female athletes had a statistically significant increase in incidence of ACL injury compared to the trained female athlete (Hewett 2000). While these neuromuscular-related hypotheses are interesting their direct link to an increase in ACL injury in women has not been shown.

Hormonal changes. Many studies have focused on hormonal causes for an increased incidence of ACL injury, such as relaxin, estrogen, and progesterone (Slauterbeck et al. 2001). These hormones are present at various levels during the menstrual cycle and are hypothesized to increase joint laxity while decreasing neuromuscular control (Dragoo et al. 2003; Hewett 2000). Relaxin has collagenolytic effects and a correlation exists between relaxin protein levels and interstitial collagenase production (Bryant-Greenwood et al. 1995). In addition, estrogen surges, which occur near the time of ovulation, have been shown to increase target organs response to relaxin, with relaxin levels also highest near ovulation (Winn et al. 1994). This could affect the integrity of the ligament and more ACL injuries have in fact been reported during the ovulation phase of the menstrual cycle (Wojtys, Ashton-Miller et al. 2002; Wojtys, Huston et al. 2002). Estrogen, progesterone and relaxin receptors have been localized in ACL cells, supporting their potential role in affecting the integrity of
the ACL (Dragoo et al. 2003; Liu et al. 1996). In addition, measurement of serum estradiol concentration revealed a negative correlation with ACL stiffness during the menstrual cycle near ovulation (Romani et al. 2003). This data might suggest that differences in genes affecting the receptors or the production of hormones could be involved in the incidence of ACL injury. Hormonal differences between sexes are obvious and are simple to measure, unfortunately like other propositions in etiology contradictions exist. Measurement of serum relaxin levels during the course of the menstrual cycle have failed to demonstrate a correlation with hormone levels and joint laxity (Arnold et al. 2002) and the increased incidence of ACL injury continues to occur in eumenorrheic women and women taking oral contraceptives.

**Genetics.** One of the greatest limitations to measuring phenotype is that environmental influences can change the variable measured. It is possible that the increased frequency in dogs is due to a variation in genotype. This suggestion is not totally unique as there is strong evidence that the susceptibility of OA is of a genetic nature. Osteoarthritis (OA) has an incidence rate that is higher in post-menopausal women than in men, suggesting that sex hormones may play a role (Fytiti et al. 2005). Predisposition to OA and ACL injury may have a similar basis since it has been shown that complete ACL rupture is more common in patients that have clinical signs of OA than in those without (Hill et al. 2005). Several sex hormone receptors have been identified that increased the risk of OA, such as estrogen receptor β and androgen receptor (Fytiti et al. 2005; Spector et al. 1989), and regions on human chromosomes 2, 4 and 16 were found to be associated with an increase susceptibility to OA based on genetic linkage (Loughlin 2001). One study by Fytiti et al. that reported specific mutations in the estrogen and androgen receptors to be associated with OA of the knee found that women with a specific microsatellite long allele genotype (LL) had an increased risk for OA development (Fytiti et al. 2005). A study by Uitterlinden et al. reports an association with a Vitamin D receptor genotype and radiographic knee osteoarthritis (Uitterlinden et al. 1997).

However, limited molecular work has been performed and, to the best of our knowledge, no research addressing the genetics of ACL injury has been published. Foos et al. determined expression of RNA proteins of all known matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) by reverse transcription polymerase chain reaction (RT-PCR) (Foos et al. 2001). They reported that numerous genes encoding tissue remodeling effector proteins are expressed in the human ACL. Sciore et al. reported that estrogen and progesterone receptor transcripts are expressed in ACL tissue of male and female rabbits and humans and that alterations in receptor expression occur in ligaments during pregnancy (Sciore et al. 1998). Female vs. male
differences were either not addressed or not found in either study (Foos et al. 2001; Sciore et al. 1998).

**Candidate Gene Approach**

There are several different approaches for identifying genes that are responsible for genetic disorders (Rothschild et al. 1997). The comparative candidate gene approach is dependent on the existence of a known mutation for a specific disorder in a particular species and the same disorder’s occurrence in the species of interest. No known comparative candidate gene exists for injury of the ACL. However, this is a method that could be applied to compare information gained in this study as compared to the human genome. The biological candidate gene approach relies on presumed knowledge of gene functions and their potential role in the disease etiology. This approach requires that gene sequence and function be known. However, this approach is limited by the fact that there are numerous genes that may have a potential role in a disorder, and more than one gene may be responsible for the disorder.

The positional candidate gene approach uses first the identification of a chromosomal region that is associated with a disorder, and then identification of all genes located in that region. The human genome map is typically used to identify these positional candidate genes due to its near completeness. The genes are then organized and selected according to different roles based on gene ontology, such as cellular component, molecular function, or physiologic function. This allows a more specific narrowing of the list of potential genes involved in a disorder, rather than choosing one that may be related. Since the dog genome has been initially sequenced one can use that information, in consultation with the human genome and the comparative map, to choose the best positional candidates.

A SNP is a one base pair difference in a gene’s sequence, either in coding or non-coding sequence. Other sequence differences that may be associated with a disease include an insertion or deletion of multiple base pairs (an INDEL), or alternative splice sites (splice sites are gene locations where introns, or non-coding sequences, are cut out of the gene sequence after the gene has been transcribed). One base pair changes in the coding region of the gene are the most common type of mutation noted in the Human Gene Mutation Database, 2003. This consists of missense mutations, a one base pair change that changes the amino acid it codes for, and nonsense mutations, a one base pair change that changes the amino acid to a stop codon (Sargan et al. 2001). Deletions cause approximately 22% of all noted mutations, insertion/duplications 7%, whereas regulatory-type mutations account for only 0.01%. Most commonly, sequences are compared or pooled into groups between animals known to have the disease and animals known to be free from the disease.
Primer Design: Selected genes have to be sequenced in order to identify the SNPs. A brief description of the process follows. We begin by using the web site for the National Center for Biotechnology Information (NCBI, Bethesda, Maryland) to identify the sequence for the gene. This step will either produce canine sequence directly, if known, or more commonly, we will use the human sequence to determine the dog sequence using the BLAST algorithm against the canine whole genome shotgun sequence. Once sequence is known, it will be aligned using human exonic/intronic boundaries as determined by Ensembl (European Bioinformatics Institute and Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK). Primers are then designed using Oligo 6.0 software (Molecular Biology Insights, Cascade, CO) or Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA). Primers will be designed on any sequence information that is known for the gene, which may include but is not limited to 5' or 3' non-coding sequence, exons and introns.

PCR-RFLP Design: DNA is amplified based on the primers designed for a specific region for different pools of animals. Each pool is sequenced separately, and the sequence is viewed to search for SNPs using Sequencher software (Gene Codes Corporation, Ann Arbor, MI). Typically it is rare to easily identify a one base pair mutation that is responsible for a disease, known as a causative mutation. More commonly, an associated, linked mutation(s) is identified. This is typically a collection of SNPs or mutations (called haplotype) that are identified as occurring in affected animals and not in normal animals. Thus an affected animal will have a haplotype containing specific SNPs or mutations that are found to have a closer association with the disease status than an unaffected animal. This process can begin by preparing an easily performed PCR reaction that contains the SNP. Then, a restriction enzyme (RE) would be used that would only cut a particular DNA sequence, such as a SNP that is associated with the disease, leaving some animal’s DNA sequence uncut. The PCR-RFLP is thus digesting the PCR product (the amplified DNA sequence from an individual animal) with the RE, and running the product on a gel. Larger fragments will migrate slower and hence normal (or uncut DNA sequences) will migrate less than an affected animal (or cut DNA sequence). Having multiple PCR-RFLP tests will increase the probability of correctly identifying an affected animal. Although this latter method does not determine the actual genetic cause for the disorder, it does identify an affected or carrier individual. This information can then potentially be used to identify predisposed individuals, modify treatment modalities, or predict response to therapies.

Fine Mapping: Fine mapping is based on the same premise as the genome scan but instead of scanning the entire canine genome containing 38 autosomes and the sex chromosomes for disease associated chromosomal regions, scanning is only performed between markers in a narrower range.
This can narrow the potential positional candidate gene list even more, allowing the search for the disease causing mutation to proceed more quickly. This approach will be the quickest and most efficient method for narrowing the gene search for CCLR loci and has been used successfully in other disease studies (Lowe et al. 2003; van de Sluis et al. 2002).

Analysis: Initial analysis of each marker includes number and frequency of alleles and genotypes, cumulated from individuals, followed by a chi square analysis performed on each marker. Such analyses compared the allele and genotypic frequencies for each marker for each group of dogs; those assumed to be homozygous unaffected and those assumed to be homozygous affected. Some of the contingency tables (CCLR status by allele and CCLR status by genotype) have cell counts (observations) of less than five, a violation of an assumption of classical chi square tests. Therefore, randomization chi square tests were used for all the markers. Briefly, the null hypothesis of the randomization test is that CCLR status is independent of allele or genotype, so that the chi square statistic from the data should be consistent with chi square statistics generated after randomly shuffling the CCLR status relative to the markers. If the original chi square statistic is an outlier (infrequent) relative to the distribution of randomly generated chi square statistics (less than 5% are greater than the original statistic, i.e.; P<0.05), then the statistical test is considered nominally statistically significant. For each marker, 5000 random chi square statistics give a stable distribution to compare with the original chi square statistic. All markers may be tested further using homozygosity mapping. Homozygosity mapping is based upon a specific homozygous genotype occurring more frequently in the affected animals than in the unaffected animals, as in the case of a simple recessive disorder (Lander et al. 1987). This technique has been used to localize chromosomal regions for both mucopolysaccharidosis IIIC in humans and copper toxicosis in Bedlington terriers (Ausseil et al. 2004; van de Sluis et al. 2000).

Animal models – Induced

To date, the animal models used to investigate the cause of the increased incidence of women to ACL injury have focused on the role of hormonal fluctuations. In 2003, Strickland et al. studied estrogen levels and its correlation to knee joint laxity in sheep and found a lack of hormonal influences on mechanical properties of sheep knee ligaments between the sexes (Strickland et al. 2003). Sciore et al. measured ligament protein expression of estrogen and progesterone receptors and found no difference in the levels present in male vs. female or human vs. rabbit ligamentous tissue (Sciore et al. 1998). Both of these models are limited by the facts that they are an induced situation, and although ACL injury occurs via mechanical overload in humans, the studies were performed in species that rarely get naturally occurring ACL injury (rabbit and sheep) (Sciore et al. 1998;
Strickland et al. 2003). The dog has been used for decades as an induced animal model (via transection of ACL) to investigate osteoarthritis, meniscal tears, and various treatment alternatives in people (Brandt et al. 1991). No models of an animal that experiences naturally occurring ACL injury are available to date that could be used to study the epidemiology or genetics behind the increased incidence of ACL injury reported in women.

**Similarities between ACL Injury in the Dog and Women**

Naturally occurring CCLR in the purebred dog is similar to the injury in athletic females in that increased incidence is: 1) usually found in specific breeds of dogs more commonly than other breeds (i.e. predisposed population), 2) found in female and neutered (female and male) dogs more commonly than in sexually intact male dogs, 3) occurs at a young age in the predisposed populations and, 4) usually linked to an activity that places only a modest mechanical demand on the CCL that shouldn’t lead to failure (e.g. force is not more than four times the body weight). Whitehair et al. was the first to report that the Newfoundland, along with the Rottweiler and Staffordshire Terrier, had the highest prevalence of ACL injury (Whitehair et al. 1993). This finding was later supported when Duval et al. concluded that the Newfoundland breed had an increased risk for ACL injury with an odds ratio of 6.65 (Duval et al. 1999). In contrast, the odds ratio was decreased for the Golden Retriever (0.48), Doberman pinscher (0.33), and German shepherd (0.25). This finding, that specific breeds are either predisposed or protected from developing ACL injuries, strongly supports that injury of the ACL in some dog breeds has a heritable component. This parallels the ACL incidence in people, a population that is predisposed (woman) or protected (man).

These epidemiological studies have also demonstrated an increased incidence of ACL injury in female dogs (Griffin et al. 1992; Whitehair et al. 1993); again, this compares favorably to the condition in female athletes. It is also interesting to note that neutered dogs (both males and females) have an increased incidence (3x) as compared to sexually intact dogs (Duval et al. 1999). Both of these findings suggest a role for sex hormones in the incidence of ACL injury in the dog. In addition, these studies report that within these predisposed breeds (all of which are large breed dogs weighing over 15 kg) ACL injuries occurred at a younger age (Newfoundlands commonly present under 24 months of age with bilateral ACL injuries and a history of only minimal trauma; e.g. jumping off a porch) (Duval et al. 1999; Harasen 1995; Moore et al. 1996; Whitehair et al. 1993). The highest incidence of ACL injury has been noted in athletic girls less than 19 years of age (Baker et al. 1998).

Additional similarities have been reported between phenotypic conditions in the dog and in women. Specifically, Aiken et al. evaluated femoral intercondylar notch in dogs that sustained an
ACL injury and found a direct correlation between notch width and ACL injury (Aiken et al. 1995). The narrowed intercondylar notch in humans is theorized to impinge on the ACL and lead to premature rupture. It is unknown whether the narrowed intercondylar notch in dogs is congenital, developmental, or secondary to OA formation. Arcand et al. reported that mechanoreceptors were present in canine ACL, with an increased proportion found in the proximal third compared to the rest of the ligament (Arcand et al. 2000). This simulates mechanoreceptor work suggested as a potential etiology for ACL injury in women. Wingfield et al. determined that Rottweilers, a predisposed breed, require less load per body mass than the greyhound, a protected breed, to rupture the ACL and reported differences in ACL volume between breeds (Wingfield et al. 2000, 2000). Again, this parallels work suggesting differences in ACL volume between men and women to explain the increased incidence of ACL injury in women.

**Comparative genomics**

The dog serves as an excellent model for studying human genetic diseases. First, there are over 350 reported inherited canine diseases, of which greater than 50% are also experienced by humans (Nicholas et al. 2001; Nicholas et al. 2003; Ostrander et al. 2000). In addition, humans and dogs share high sequence homology, much higher than humans share with the mouse (Kirkness et al. 2003). This combined with the sequence information gained from completion of sequencing of the canine genome opens many doors into comparative genomic studies. Domestication and selection of the dog into several breeds has led to high inbreeding within each breed, increasing the frequency of recessive diseases that are experienced within that breed, similar to an isolated human population, such as a particular ethnicity or race. The linkage disequilibrium experienced by dogs is much higher than humans (about 50 times higher), therefore a whole genome association study can be performed with as little as 10,000 single nucleotide polymorphisms (SNPs) for the dog versus as many as 500,000 SNPs needed for similar studies in humans (Parker et al. 2004). This implies that a comparatively much smaller number of SNPs would be needed in the dog to identify the associated chromosomal region(s) than if performing the same study in humans. Therefore a disease with similar pathology in humans and dogs can be found much more quickly and efficiently in the dog model, speeding up the disease mutation identification process.

In summary, CCLR in the dog has many possible associations including trauma, immune-mediated mechanisms, age related degeneration of the CCL, obesity, and conformational abnormalities. Many dogs develop CCLR from a single high-load traumatic event however it is also common for dogs with CCLR to have a history of only mild trauma (the effect of daily mechanical wear). Statistical analyses would suggest that CCLR is an exceedingly prevalent disease, particularly
in some breeds of dogs such as the Newfoundland. Recently, CCLR has been diagnosed at an earlier age than expected within the predisposed breeds with no known history of trauma or other possible cause for the CCLR. These findings strongly support that CCLR, in some dog breeds, has a heritable component.
Figure 1: Intra-articular view of stifle. Anterior cruciate ligament is synonymous with cranial cruciate ligament and posterior cruciate ligament is synonymous with caudal cruciate ligament.
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CHAPTER 2. ESTIMATE OF THE ANNUAL ECONOMIC IMPACT OF CRANIAL CRUCIATE LIGAMENT INJURY IN DOGS IN THE UNITED STATES

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ABSTRACT

Objective—To estimate the economic impact to veterinary clients for the medical and surgical management for rupture of the cranial cruciate ligament (CCLR) in the dog for the year 2003.

Design—Economic impact survey.

Sample population—501 diplomates of the American College of Veterinary Surgeons (ACVS) indicating that their area of surgical emphasis was small animal orthopedic surgery or small animal general and orthopedic surgery, and 4,000 veterinarians indicating to the AVMA that their professional area was small animal practice exclusive or mixed animal practice (at least 80% small animal).

Procedure—Veterinarians were surveyed concerning the cost for medical and surgical management for CCLR for 2003. The economic impact was calculated by multiplying the number of CCLR surgeries performed by the mean cost of surgery. This was added to the number of CCLR cases managed medically multiplied by the mean cost of medical management. This estimate for survey responders was extrapolated to the total number of veterinarians in the study population for the ACVS or AVMA, respectively.

Results—Estimates for the total cost of surgery was $171,730,134.72 and $1,020,167,907 for veterinarians in the ACVS and AVMA populations, respectively. The cost of medical management was $2,885,687.86 and $126,558,155.16 for veterinarians in the ACVS and AVMA populations, respectively. After combining the ACVS and AVMA populations, we estimated that owners spent $1.32 billion for the treatment of CCLR in the United States in 2003.

Conclusions and Clinical Relevance—Rupture of the cranial cruciate ligament is a prevalent, costly injury. Results may motivate veterinary and consumer agencies to prioritize funding for a better understanding of the injury.
INTRODUCTION

Rupture of the cranial cruciate ligament (CCLR) is the most common cause of lameness in dogs. Rupture of the cranial cruciate ligament causes stifle joint instability, joint inflammation, and lameness. Although treatment options include surgical and nonsurgical management, surgery is generally recommended for dogs > 15 kg (33 lb) because it provides for an improved prognosis. This contributes to the increased cost for CCLR management because the breeds of dogs that are most predisposed to CCLR are comparatively large in size. Veterinarians have dozens of surgical techniques to choose from to treat CCLR and the cost to the owner depends on several factors. Expertise of the surgeon, surgical technique performed, and region of the country would also be expected to factor heavily into cost comparisons. Unfortunately, even with surgical management, osteoarthritis develops in most patients. Although the severity of osteoarthritis of the stifle joint does not correlate with the severity of lameness many patients do require treatment for osteoarthritis, and this treatment may be required for the duration of the patient’s life. Nonsteroidal anti-inflammatory drugs (NSAIDs) in combination with weight management, physical therapy, and disease modifying drugs are all nonsurgical treatments commonly used for osteoarthritis in dogs.

Although CCLR is an exceedingly prevalent disease, particularly in some breeds of dogs, only a limited amount of research funding is available for investigators to explore the cause of CCLR and establish the best treatment options for patients. This limitation in funding may be attributable to, in part, to the fact that the economic impact of CCLR is considered not substantial and thus funding agencies prioritize research in this field comparatively low, or that the economic impact of CCLR is high and agencies are not aware of the economic impact. Therefore, we hypothesized that CCLR treatment is costly. The purpose of the study reported here was to estimate the economic impact to veterinary clients for the medical and surgical management for CCLR for the year 2003.
MATERIALS AND METHODS

Survey

Two groups of veterinarians were surveyed. First, all veterinarians that were diplomates of the American College of Veterinary Surgeons (ACVS) and indicated that their area of surgical emphasis was small animal orthopedic surgery or small animal general and orthopedic surgery (n = 501) were surveyed. This mailing list was purchased from the ACVS after they had approved both the cover letter and survey. The cover letter and survey were mailed directly by the authors. Second, 4,000 veterinarians that had indicated to the AVMA that their professional area was small animal practice exclusive or mixed practice (at least 80% small animal) were randomly chosen. This mailing list was purchased from the AVMA, through the list manager for the AVMA, after the AVMA approved both the cover letter and survey. The list manager mailed the letter and survey.

Survey design

Similar surveys were designed for each group with a goal to ascertain the combined cost of surgical and medical treatment for CCLR for each veterinarian surveyed. Survey questions were specific for the year 2003 and cases of CCLR treated at the veterinarian’s hospital by that veterinarian. Estimates of costs for cases referred for treatment at a different hospital were specifically addressed in the survey and not included in the overall economic estimate. Questions included the average number of CCLR cases evaluated each month, the number of cases managed surgically and medically, cost range for surgical and medical management, and mean cost of surgical and medical management. We did not ask what type of surgical or medical treatment was offered. To determine normality of responses between responders and nonresponders, 25 nonresponders from the ACVS study population were randomly chosen and called for their survey response.

Calculation of economic impact

A 1-way ANOVA was performed to compare responses that were mailed with responses obtained via telephone. If no significant difference was detected between these response groups, data were then pooled. An economic calculation was performed for each group of veterinarians and their data were added to estimate the overall economic impact of CCLR. To calculate the economic impact from responders, the number of CCLR surgeries performed was multiplied by mean cost of surgery. This was added to the number of CCLR cases managed medically, multiplied by the mean cost of medical management. This estimate for responders was then extrapolated to the total number of veterinarians reported in the study population by the ACVS (n = 501) or AVMA (n = 4,000). The equation used for the ACVS group was:

\[ \text{Total Cost} = (\text{Number of CCLR Surgical Cases} \times \text{Mean Cost}) + (\text{Number of CCLR Medical Cases} \times \text{Mean Cost}) \]
\[(\text{number of CCLR medical cases}) \times \text{(mean cost)} = \text{ACVS}^{T1}\]

\[\text{ACVS}^{T1} \times \text{ACVS study population} = \text{ACVS}^{T2}\]

\[
\text{number of responders}
\]

where $\text{ACVS}^{T1}$ was the total cost of management reported from responders, and $\text{ACVS}^{T2}$ was the total cost of management estimated from all potential responders.

In addition to economic data, the reported year of graduation from veterinary school, gender, and state in which a veterinarian practiced was collected from each veterinarian in the AVMA population. These data were examined for associations with the mean cost for surgery and evaluated by use of a Pearson product moment correlation. When appropriate, data is expressed as mean ± SEM with $P < 0.05$ considered significant.

**RESULTS**

Of 501 surveys mailed to the ACVS study population, 231 responded. In addition, 25 (9.3%) nonresponders from the ACVS study population were contacted via telephone for data collection. No significant difference was detected between these 2 groups for any survey response and these data were pooled. In effect, data were collected from 256 of 501 (51.1%) members of the ACVS study population. Included in this group were 9 (3.5%) responders that did not treat CCLR cases. Of the 38,959 veterinarians listed by the AVMA as practicing small animal practice exclusively or mixed practice (at least 80% small animal), 4,000 surveys were mailed and 1,083 (27.1%) responses were returned. Included in this group were 111 (10.3%) responders that did not treat CCLR cases.

In the ACVS response group, the mean number of CCLR cases managed surgically per year was 186.24 (range, 0 to 720; median, 156) and the mean number of cases managed medically per year was 23.88 (range, 0 to 120; median, 12.0). In the AVMA response group, the mean number of CCLR cases managed surgically per year was 29.16 (range, 0 to 480; median, 24.0) and the mean number of cases managed medically per year was 12.24 (range, 0 to 180; median, 12.0).

The mean cost for a single surgery reported by veterinarians in the ACVS response group was $1,840.50 (range, $0 to 5,000; median, $1,900.00) and the mean cost for medical management was $241.20 (range, $0 to 1,000; median, $225.00). The mean cost for surgery reported by veterinarians in the AMVA response group was $898.00 (range, $0 to 5,000; median, $875.00) and the mean cost for medical management was $265.40 (range, $0 to 1,000; median, $225.00).
The estimate for the total cost of surgery (by use of the mean) reported by the ACVS response group was $87,750,328.32. When multiplied by the total number of veterinarians that fit the ACVS inclusion requirements (indicated that their area of surgical emphasis was small animal orthopedic surgery or small animal general and orthopedic surgery, n = 501), the estimate was $171,730,134.72. The cost of medical management reported by the ACVS response group was $1,474,523.14. When multiplied by the total number of veterinarians that fit the ACVS inclusion population, the estimate was $2,885,687.86. Similarly, the estimate for the cost of surgery (by use of the mean) reported by the AVMA response group was $28,359,091.44. When multiplied by the total number of veterinarians that fit the AVMA inclusion population (indicated to the AVMA that their professional area was small animal practice exclusive or mixed animal practice with at least 80% small animal, n = 4,000), the estimate was $1,020,167,907. The cost of medical management reported by the AVMA response group was $3,518,121.17. When multiplied by the total number of veterinarians that fit the AVMA inclusion population, the estimate was $126,558,155.16. After combining the ACVS and AVMA populations, we estimated that owners spent $1.32 billion for the treatment of CCLR in the United States in the year 2003. Similarly, calculation of the economic estimate using the median reported for surgical and medical management would have resulted in a total > $1.28 billion.

Within the AMVA response group, responders included 739 males and 344 females. The year of graduation from veterinary school ranged from 1945 to 2002 and no significant correlation was detected when year of graduation was compared with cost for surgical management and no significant difference in cost for surgical management was detected between graduation dates between genders. However, when we compared the mean cost of surgery from veterinarians that reported that they performed CCLR surgery (ie, a zero dollar amount was not used for veterinarians that reported that they did not perform CCLR surgery), males reported a mean ± SE charge of $1,023.43 ± 18.54 and females reported a mean ± SE charge of $1,095.04 ± 33.86. This difference was significant (P < 0.05). At least 1 response was received from every state and large differences in cost to the owner for CCLR surgery were detected. For example, there was a significant (P < 0.001) difference in the mean cost for surgery between veterinarians practicing in Iowa ($769.20) and those practicing in California ($1,524.60).
DISCUSSION

Estimates provided by the ACVS population were expected because of the economic influence CCLR has at Iowa State University's Veterinary Teaching Hospital. The mean number of CCLR surgeries performed by the AVMA population (2.43 surgeries/month) was unforeseen because we had no experience to draw from. The economic impact of CCLR reported may be unexpected by some; however, it parallels the financial burden of the rupture of the anterior cruciate ligament (RACL) in humans. In the United States, it has been estimated that each year approximately 250,000 people, or 1 in 3,000, experience RACL. The economic burden to the health care industry for surgical care alone ranges from a mean of $3,679 to a mean of $17,000 with a total cost for surgery approaching $2 billion. Rupture of the ACL most commonly occurs in people < 40 years old and development of osteoarthritis is common, this economic burden would be further increased if the cost of rehabilitation and medical management were added. Unfortunately, we were not able to find comparative reports that included estimated costs of veterinary treatment for other injuries or diseases in small animals.

This survey was designed with guidance from the Center for Survey Statistics and Methodology at Iowa State University. Like many surveys, questions were designed to maximize response rate (eg, concise, nonintrusive questions) and maximize estimate accuracy (eg, question clarity and duplication). In our study, the response rate was higher than expected. A response rate of 20% is typical for surveys similar to those used in the study reported here. By completion of our study, > 46% of the ACVS and > 27% of the AVMA study populations had responded via mail. This encouraging response rate may have been in reaction to the importance of the problem as perceived by the veterinary groups studied. Regardless, the high response rate increases the accuracy of our estimate. Even if the reported mean cost is overestimated by 25%, the economic impact of CCLR would approach $1 billion. We would argue, however, that it is more likely that our estimate is below the actual financial burden. We surveyed only veterinarians that were active members of the AVMA and were classified by the AVMA as practicing exclusively small animal veterinary medicine or mixed animal practice (at least 80% small animal). We chose to study this group because the AVMA would be able to supply information necessary to perform the study and we believed that this group would have the greatest professional investment in the problem and thus would be more likely to participate. Many veterinarians who potentially treat dogs with CCLR did not participate because they either had a different classification from the AVMA and thus did not meet the study inclusion criteria or were not active members of the AVMA.

We recognize that there was a large range in the reported values for medical vs. surgical
management. One obvious difference in cost that we identified was explained by geographic location. The difference reported by veterinarians in the states of Iowa and California was expected because the authors are aware that similar differences exist between the mean costs of CCLR surgery performed by diplomates of the ACVS at our veterinary teaching hospital, compared with ACVS diplomates practicing in many metropolitan cities. Geographic location (and potential competition of veterinary services) would also affect the cost of nonsurgical management. However, the extent of nonsurgical management likely varies among veterinarians. Some veterinarians offer only traditional anti-inflammatory medications and patients of others routinely participate in rehabilitation programs immediately after surgery or for treatment of osteoarthritis. The 7% difference in mean cost between genders of practicing veterinarians would contribute to the variation in the range of costs. In contrast, the year of graduation from veterinary school did not influence mean cost of surgery, which was not expected. We had expected that recent graduates would charge less because that had less experience. However, it could be argued that with the high frequency of CCLR that even recent graduates gain rapid experience performing this type of surgery. Another possible contribution to variation would be the responder’s interpretation of the questions. For example, in the questionnaire we specifically asked for the total cost of surgery (such as anesthesia, equipment, hospitalization, and supplies) to the client. Some responders may have only reported the surgical cost as opposed to the total cost. Other factors that would have contributed to variation in cost of surgical or nonsurgical management of a CCLR would be the typical weight of the dog treated and type of treatment offered.

The objective of this survey was to provide an approximate calculation of the cost of CCLR to dog owners in the United States during 2003 thereby estimating the economic impact of this injury on the US economy. Rupture of the cranial cruciate ligament is a prevalent, costly injury and results of the study reported here may motivate veterinary and consumer agencies to prioritize funding for a better understanding of the injury.

ACKNOWLEDGEMENTS

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FOOTNOTES


REFERENCES

CHAPTER 3. INHERITANCE OF RUPTURE OF THE CRANIAL CRUCIATE LIGAMENT IN NEWFOUNDLANDS

A paper published in Journal of the American Veterinary Medical Association

V.L. Wilke, M.C. Conzemius, B.P. Kinghorn, P.E. Macrossan, W. Cai and M. F. Rothschild

ABSTRACT

Objective: To determine prevalence, level of inbreeding, heritability, and mode of inheritance for rupture of the cranial cruciate ligament (CCLR) in Newfoundlands.

Design: Retrospective and recruitment study.

Animals: 574 client-owned Newfoundlands.

Procedure: Medical records from January 1, 1996 to December 31, 2002 were evaluated for prevalence of CCLR. A pedigree was constructed by use of recruited Newfoundlands with CCLR status based on veterinary examination; level of inbreeding, heritability, and mode of inheritance were calculated.

Results: Hospital prevalence for CCLR was 22%; dogs in the pedigree from the recruitment study had a mean level of inbreeding of $1.19 \times 10^{-4}$, heritability of 0.27, and a possible recessive mode of inheritance with 51% penetrance for CCLR.

Conclusions and Clinical Relevance: Identification of a genetic basis for CCLR in Newfoundlands provides evidence that investigators can now focus on developing methods to identify carriers to reduce the prevalence of CCLR.

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INTRODUCTION

Rupture of the cranial cruciate ligament (CCLR) is the leading cause of lameness in dogs and represents nearly 20% of dogs evaluated at university hospitals for lameness. Dogs with CCLR develop stifle joint osteoarthritis and lameness from the instability and inflammation that occurs as a result of the CCL damage. Although many dogs develop CCLR from a single high-load traumatic event, it is also common for dogs with CCLR to have a history of only mild trauma.

In addition, statistical analysis suggests that some breeds of dogs are predisposed to CCLR where others appear protected. Whitehair et al. were the first to report that Newfoundlands, along with Rottweilers and Staffordshire Terriers, had the highest prevalence of CCLR. This finding was later supported when Duval et al. concluded that the Newfoundland breed had an increased risk for CCLR with an odds ratio of 6.65. In contrast, the odds ratio was decreased for Golden Retrievers (0.48), Doberman Pinschers (0.33), and German Shepherd Dogs (0.25).

These findings, in addition to a history of low-load trauma (the effect of daily mechanical wear) that causes CCLR, strongly support the notion that CCLR, in some dog breeds, has a heritable component. We hypothesized that there is a genetic basis for CCLR in dogs. The purpose of the study reported here was to determine the prevalence of CCLR in Newfoundlands evaluated at the Iowa State University College of Veterinary Medicine (ISU-CVM) and to determine the mean level of inbreeding, heritability, and mode of inheritance for naturally occurring CCLR in a sampled population of Newfoundlands.

MATERIALS AND METHODS

Data collection

Two populations of Newfoundlands were studied. First, to determine prevalence of CCLR in a hospital population, medical records for all Newfoundlands evaluated at the ISU-CVM from 1996 to 2002 were evaluated for diagnosis of CCLR. A diagnosis of CCLR was made only if CCLR was confirmed at the time of surgery. Second, to determine the level of inbreeding, heritability, and mode of inheritance for CCLR, a large-scale recruitment study was undertaken. The recruitment study targeted owners and breeders of Newfoundlands that were identified by the National Newfoundland Registry, web sites, and referred by other Newfoundland breeders and ISU-CVM clients. The study protocol was approved by the Iowa State University Committee on Animal Care and written consent was obtained by all owners of study participants. In addition, one of the study veterinarians (VLW) attended national Newfoundland shows to solicit study participants. All Newfoundland dogs were eligible for study enrollment if the owners were aware of its medical history, a five generation
pedigree was available for the dog, and if the dog was examined by a veterinarian. In an effort to collect random data, no specific Newfoundland population was targeted and no collected data were discarded. In addition, attempts were made to collect data from across the country so that specific families of Newfoundlands were not over-represented. The medical history of all dogs participating in the study was collected from the owners and if possible and relevant, the date of CCL injury was ascertained. Cheek swabs, and in some dogs, blood samples were obtained, from which DNA was extracted for future analyses. Five-generation pedigrees were collected from healthy and CCLR-affected dogs. All Newfoundlands identified in the recruitment study were examined by a study veterinarian (VLW) and classified as CCLR-affected on the basis of signs of pain during hyperextension of the stifle joint, stifle joint effusion, decreased range of motion, positive cranial drawer sign, or cranial tibial thrust. In addition, dogs that had a history of lameness (as reported by the owner), radiographic evidence of stifle effusion, osteoarthritis, and a diagnosis of CCLR based on either physical examination or surgical confirmation (as reported by the dog’s veterinarian) were considered CCLR-affected. Because the exact time of onset of lameness or diagnosis was not always available for investigation, mean age at the time of diagnosis was estimated by subtracting either the known date of CCLR diagnosis or the dog’s age at the time the information was reported for this study from the dog’s date of birth.

Statistical analyses

The ISU-CVM hospital prevalence rate was calculated on the basis of the number of dogs affected with CCLR divided by total number of Newfoundlands evaluated during that period. The Newfoundland pedigree generated from the recruitment study was used to determine mean level of inbreeding, heritability, and mode of inheritance for CCLR. Inbreeding coefficients of individual dogs were calculated from a standard relationship matrix based on the pedigree that was constructed. Heritability was determined by use of standard statistical software. The CCLR status was categorized as a binary trait because the dogs were considered either unaffected or affected. Heritability was computed by use of a single-trait model analysis of CCLR status via the restricted maximum likelihood method. The model used was: CCLR status = μ + a + s + e, where μ is the overall mean, a is the individual animal effect (random), s is the sex effect (fixed, male or female) and e is the random error. The effects a and e had a mean of zero and variances σ^2_a and σ^2_e, respectively. To estimate heritability, repeat analyses were performed that evaluated different prior heritability estimates as starting values until the maximum likelihood value, the measurement value for which the data best fit the model for heritability, was determined.
A different analysis was carried out by use of the method of Kinghorn\textsuperscript{10} to consider the possibility of a single major gene. If there are mutant (\textit{m}) and wild-type (+) alleles, the mode of inheritance can be described as the percentage penetrance for each of the 3 genotypes (+++, +m, mm). For example, a simple recessive mode of inheritance has penetrance values, or percent expression of the trait, of 0, 0, and 100 for homozygous CCLR unaffected, heterozygous carrier, and homozygous CCLR affected animals, respectively. The method makes an initial assumption for allele frequency and penetrance values, and uses complex segregation analysis of the CCLR data and the pedigree to calculate the probabilities of being ++, +m, or mm for each dog. These probabilities are used to make updated estimates of the frequency of allele \textit{m} and the 3 penetrance values. These updated estimates depend to some extent on the starting values, so the process is iterated until there is little change between iterations in the estimates. The Kinghorn method assumes that the single locus is the only locus affecting the trait concerned, so conclusions must be made with caution.

\textbf{RESULTS}

\textit{Hospital prevalence rate}

One hundred sixty three Newfoundlands (36 castrated males, 48 sexually intact males, 31 spayed females, and 48 sexually intact females) were evaluated at the ISU-CVM from January 1, 1996 through December 31, 2002. Twenty-two percent (15 castrated males, 5 sexually intact males, 12 spayed females, and 4 sexually intact females) had CCLR. Mean ± SD age at the time of definitive diagnosis was 3.53 ± 2.11 years (range, 1.09 to 10.62 years; median, 2.98 years).

\textit{Recruitment study}

The recruitment study included 411 Newfoundlands, of which 92 (22%; 53 females and 39 males) were CCLR-affected and 319 (182 females and 137 males) were unaffected. Mean ± SD age of all dogs in the study was 6.51 ± 3.22 years (range, 1.46 to 14.17 years; median, 5.71 years). Mean ± SD age at the time of diagnosis of CCLR was 5.17 ± 2.86 years (range, 0.84 to 13.88 years; median, 4.07 years).

The pedigree of all the study dogs consisted of 11 generations with a range of 1 to 5 offspring represented in each family. The mean inbreeding coefficient, defined as the probability that a mating pair's genes were identical because they were inherited from a common ancestor, was $1.19 \times 10^{-4}$ in the pedigree overall (\textit{n} = 411). The mean inbreeding coefficient for those dogs that were inbred (i.e. inbreeding coefficient > 0 \{n=17\}) was 0.05 (range, 0.004 to 0.17). Heritability, the degree of resemblance for CCLR classification between parents and offspring in this Newfoundland population, was calculated as 0.27. Segregation analysis predicted a major gene effect with a recessive pattern of inheritance. The frequency of the recessive allele was 0.60 with partial penetrance of 51%.
DISCUSSION

Dogs frequently develop CCLR secondary to a minor traumatic event. Owners often describe dogs developing lameness after events such as a running in the back yard, landing from a short jump, or standing up from a recumbent position. Because one finding of our study was that CCLR in Newfoundlands is a heritable condition, it is possible that Newfoundlands that develop CCLR from minor trauma may have a mutation in a gene or genes that predisposes the CCL to rupture. This may also be true in other breeds that are predisposed to CCLR and have a similar history. A mutation of one or more genes associated with structural components of the CCL or of anything that influences the mechanical or chemical environment of the CCL could predispose the dog to CCLR. It is important that genetic mutations can have varying degrees of phenotypic expression, some with minor clinical importance (e.g. hereditary clotting factor XII deficiency) and some with major clinical implications (e.g. systemic collagen mutations in Ehlers Danlos syndrome). Given the mechanical demand on the CCL, it is reasonable to consider that a mutation in a gene associated with, for example, collagen, that does not clinically affect skin strength may affect the CCL. Obviously, one motivation to investigate the heritability of such a common and economically important condition is to identify the etiology and reduce the prevalence of CCLR.

Prevalence of CCLR in the ISU-CVM hospital population was consistent with that reported in the literature. Of course, this represents dogs that were taken to veterinary hospitals and typically had a disease problem, although not necessarily CCLR. Dogs included in the recruitment population, which were obtained by communicating with owners of Newfoundlands, were free of this effect; nevertheless, prevalence of CCLR in the recruitment population was nearly the same as that in the hospital population. We believe that this similarity provides evidence that the prevalence rate was reasonably accurate, the method to classify affected and unaffected dogs was reasonable, and the recruitment technique was not skewed toward finding CCLR-affected dogs.

One limitation of the study was that dogs were evaluated at a variety of ages during the defined study period, and some of the dogs may have developed CCLR later. We attempted to calculate age at onset for both populations. However, the method to do so for the recruitment study was not as accurate and therefore the mean and median ages were older and likely biased upward, compared with the hospital population. However, another problem was that cases that occurred after the study period would have been missed; if this did not occur, prevalence of CCLR would have been higher. In addition, this could be a cause for error in the genetic analyses. For example, a dog that was 5 years old and unaffected at the time of the study may develop CCLR at 7 years of age. This would help explain the low value for penetrance, because dogs that were classified as unaffected
during the study period could still develop CCLR. Penetrance is typically calculated as the number of animals that express the phenotype divided by the total number expected to express the phenotype. Because the study was limited to a specific time period, a portion of the dogs may not have reached the critical period or event at which they were most likely to express the phenotype, making the number that were observed with the phenotype much lower than expected.

A second weakness of the study was that not all dogs included in the recruitment population had surgical confirmation of CCLR. Given the invasiveness of surgery, this was unavoidable. Magnetic resonance imaging (MRI) could have been used to non-invasively confirm CCL status but MRI requires general anesthesia and, given the cost and availability of MRI, we thought this was unreasonable. Perhaps the best way to consistently make the diagnosis would have been with a single veterinarian performing a physical examination on each dog and evaluating the stifle radiographs of each dog that did not have surgery. Although this would be possible it would likely limit other aspects of the study design. For example, pedigree data was collected from owners from all parts of the country. If data could only be collected by a single veterinarian, pedigree data would have been skewed to populations only within a reasonable travel distance, thus biasing the study population. In addition, this would have lessened the number of pedigrees that could have been studied. We believe this point makes the inclusion criteria defendable and allows for a reasonable estimate of heritability.

The level of inbreeding was investigated because it increases the level of homozygosity in the population and increases the potential for deleterious recessive alleles to be expressed. Efforts were to make the recruitment population as complete as possible, but some individuals were missing. Several nearly complete families were included, which supported the level of inbreeding detected. The low level of inbreeding in the recruitment population suggested that the high prevalence of the disease was not attributable to inbreeding, although inbreeding is only one potential genetic explanation for high prevalence of a disease.

Heritability in the narrow sense is calculated as the additive genetic variance divided by the phenotypic variance. A higher value means that the phenotype can be primarily explained by the genotype of the individual, whereas a lower value indicates that other factors, such as environment, have a bigger influence on the trait. In effect, this is also a measure of how much the offspring resemble the parents, or the probability that an offspring will develop CCLR if one knows the CCLR status of the parents. In this population of Newfoundlands, the heritability coefficient was calculated as 0.27. This was considered a moderate value for heritability and thus amenable to change with selective breeding. This implies that 27% of the phenotypic expression of rupture of the CCLR is attributable to genetics and therefore, 73% of the phenotype is attributable to environmental effects.
Possible environmental effects include body condition score, level of exercise, diet, housing conditions, and neutering status. Aside from neutering status, the authors did not obtain information concerning other environmental factors of the dogs that participated in the recruitment study.

Fitting a single locus model resulted in an estimated frequency of 60% for the mutant allele m, and penetrance values of 0.0%, 4.1%, and 50.8% for genotypes ++, +m, and mm, respectively. This suggested a predominantly recessive mode of inheritance with 51% penetrance for the homozygous recessive genotype. On the basis of this assumption of monogenic inheritance, expression of CCLR would require 2 copies of the mutant allele, and the environment would permit only 51% of these dogs to express the condition. Therefore, approximately 96% of the heterozygotes, or carriers, would be clinically healthy yet could transmit the abnormal form of the gene to their offspring. However, the method used assumed that penetrance was random with respect to the rest of the genome, and this may not have been true. It is proposed that the results indicated a likely mode of inheritance that can be useful in designing more efficient gene mapping experiments and in making sensible breeding decisions.

Penetrance is related to the number of affected individuals that express the phenotype, but is not related to the level of expression. We classified CCLR as a binary trait, because the dogs were either unaffected or expressed the CCLR trait. Thus, it was considered an all-or-none trait. Penetrance of 51% suggests that a breeding program based solely on dogs that do not express the trait will have limited impact on reducing prevalence of the trait because many of these dogs may have the undesirable allele. This confirms the importance of identifying a genetic marker for the disease. In addition, because CCLR occurs at various ages, it presents a dilemma for the owner of a dog with a recessive genotype (e.g. having two copies of the CCLR allele) as to how best provide appropriate management of the dog to delay or even help prevent development of CCLR.

Quick elimination of carriers is not recommended because of the unknown effects the trait-causing gene may have on other traits (ie, pleiotropy). Furthermore, knowing the genetic basis for CCLR could lead to the development of a preventative treatment for dogs with the recessive genotype or curative treatment for dogs that have developed CCLR. In addition, a breed of dog that experiences CCLR with a high frequency would serve as an ideal animal model for studying the genetic cause for CCLR in young, female athletes. Rupture of the anterior cruciate ligament (ACL) occurs in approximately 38,000 women per year in the U.S. Athletic women are 2 to 8 times more likely to injure their ACL than athletic men and usually sustain the injury via minimal trauma through non-contact mechanisms (cutting or jumping). We feel that we have demonstrated the Newfoundland breed to fit this model due to the high prevalence, 22%, of CCLR both in our hospital
population and in our recruitment study population. In addition, CCLR in the pure-bred dog is similar to the injury in athletic females in that the injury is usually due to a minor traumatic incident, there are breed and sex predispositions to increased CCLR, and in comparison, this human cohort experiences a very similar clinical course of CCLR.

Medical and surgical management of CCLR in the dog costs the public over a billion dollars each year\textsuperscript{11} with many different causes for CCLR in the dog suggested. This is the first study that has defined a genetic basis of this disease in the Newfoundland breed. Based on this study, breeders should cautiously breed Newfoundland dogs with a diagnosis of CCLR.

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FOOTNOTES
\textsuperscript{a}PROC INBREED, SAS, version 8.2, SAS Institute Inc., Cary, NC.
\textsuperscript{b}Pedigraph, version 1.1, Department of Animal Science, University of Minnesota, St Paul, MN.
\textsuperscript{c}MTDFREML. U.S. Department of Agriculture, Agricultural Research Service, Lincoln, NE.

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CHAPTER 4. SNP DETECTION AND ASSOCIATION ANALYSES OF CANDIDATE GENES FOR RUPTURE OF THE CRANIAL CRUCIATE LIGAMENT IN THE DOG

A paper published in Animal Genetics

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ABSTRACT

Rupture of the cranial cruciate ligament (CCLR) is the most common cause of lameness in the dog and leads to arthritis formation. Previous studies have identified CCLR as a genetic disease with a possible partial penetrance, recessive mode of inheritance. Pedigrees, determination of cranial cruciate ligament (CCL) status (normal or affected with rupture of the CCL), and DNA were collected from Newfoundland dogs in a study performed at Iowa State University. Using a biological candidate gene approach, single nucleotide polymorphisms (SNPs) were identified in fibrillin 1 (FBN1), collagen Type 9 alpha 1 (COL9A1), collagen Type 9 alpha 2 (COL9A2), and cartilage oligomeric matrix protein (COMP). From this pedigree, affected and normal dogs were chosen for association analyses based on their likelihood of being genetically either homozygous normal (all unaffected, n=45) or homozygous recessive (all affected, n=45). A PCR-RFLP was performed for one SNP in each gene for all 90 dogs, and Chi-square analyses were performed to test for association of SNP genotype and allele frequency with CCLR status using JMP 5.1. No significant association was found between any SNP and CCLR status of the dogs although for COL9A1 the association with allele frequency and CCLR status was close to significance. Although there were no statistical associations between the identified SNPs and CCLR status, the PCR-RFLP tests described here may be useful for association studies involving dogs that are affected with other musculoskeletal diseases, such as hip or elbow dysplasia.

\(^{1}\)Reprinted with permission from An Gen 2005;36:519-521.
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SOURCE/DESCRIPTION
Rupture of the cranial cruciate ligament (CCLR) is the most common cause of lameness in the dog and leads to arthritis formation. Previous studies have identified CCLR as a genetic disease with a possible partial penetrance, recessive mode of inheritance. Using a biological candidate gene approach, polymorphisms in genes that were associated with joint hypermobility in cattle and primary arthritis formation in humans were tested for association with the CCLR phenotype. The genes chosen were fibrillin 1 (FBN1), collagen Type 9 alpha 1, (COL9A1), collagen Type 9 alpha 2 (COL9A2), and cartilage oligomeric matrix protein (COMP).

PRIMER SEQUENCE
Human FBN1 sequence (GenBank accession no. NM_000138) was used to design primers (Table 1), with the forward primer designed to force incorporation of the recognition sequence for the restriction enzyme HincII (New England Biolabs, Beverly, MA). To determine the canine sequence for COL9A1, COL9A2, and COMP, the respective human mRNA sequences NM_001851, NM_001852, and NM_000095 were compared using the BLAST algorithm against the whole genome shotgun sequence (National Center for Biotechnology Information, Bethesda, Maryland). The sequence was then aligned using human exonic/intronic boundaries as determined by Ensembl (European Bioinformatics Institute and Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK). Primers were designed using Oligo 6.0 software (Molecular Biology Insights, Cascade, CO) or Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA). All information is included in Table 1.

PCR CONDITIONS
For determination of sequence polymorphisms, a 10 µL PCR volume contained 12.5 ng genomic canine DNA, 2.5 pmol of each primer, 1.25 mM dNTP, 1 µL 10X PCR buffer (Promega, Madison, WI) for FBN1 or 2 µL GoTaq buffer (Promega) for COL9a1, COL9a2, and COMP, the respective amount (see Table 1) of MgCl2 (Promega), either 0.35 U Taq polymerase for FBN1 (Promega) or 0.25 U GoTaq polymerase (Promega), and distilled water to equal 10 µL. The thermal cycling protocol was 3 min. at 94° C, 35 cycles of 94° C for 30 sec., respective primer annealing temperature (see Table 1) for 45 sec., and 72° C for 45 sec., with a final extension of 72° C for 10 min.
SNP DETECTION AND POLYMORPHISMS

Single nucleotide polymorphisms were detected using pooled DNA samples from normal (n=3) and CCLR-affected Newfoundlands (n=3). The sequence data were analyzed using Sequencher software (Gene Codes Corporation, Ann Arbor, MI). A PCR-RFLP test for each SNP was designed for a 10 μL reaction by incubating 3 μL of PCR product with the respective restriction enzyme and secondary substrates (see Table 2) according to manufacturer’s directions for at least 5 hr. The digested PCR products were separated on agarose gels (see Table 2) stained with ethidium bromide.

ASSOCIATION ANALYSES

Pedigrees, determination of cranial cruciate ligament (CCL) status (normal or affected with rupture of the CCL), and DNA were collected from Newfoundland dogs in a study performed at Iowa State University. Newfoundland dogs were classified as CCLR affected based on signs of pain on hyperextension of the knee, knee effusion, decreased range of motion, positive cranial drawer sign or cranial tibial thrust, radiographic evidence of stifle effusion and osteoarthritis, and/or surgical confirmation of a ruptured cranial cruciate. From this pedigree, affected and normal dogs were chosen for association analyses based on their likelihood of being genetically either homozygous normal (all unaffected, n=45) or homozygous recessive (all affected, n=45). The PCR-RFLP was performed for one SNP in each gene for all 90 dogs, and Chi-square analyses were performed to test for association of SNP genotype and allele frequency with CCLR status using JMP 5.1 (SAS Institute Inc., Cary, NC). No significant association was found between any SNP and CCLR status of the dogs (see Table 3) although for COL9A1 the association with allele frequency and CCLR status was close to significance (power greater than .7).

COMMENTS

Although there were no statistical associations between the identified SNPs and CCLR status, the PCR-RFLP tests described here may be useful for association studies involving dogs that are affected with other musculoskeletal diseases, such as hip or elbow dysplasia. Mapping was not conducted because the chromosomal location of the genes was determined through the use of the previously mentioned whole genome shotgun sequence. FBN1, COL9A1, COL9A2, and COMP are expected to map on dog chromosomes 30, 12, 15, and 20, respectively, based upon the human-dog comparative map (http://idefix.univ-rennes1.fr:8080/Dogs/RH3270-page.html). In addition, further sequencing of these genes is needed for identification of additional SNPs that can then be tested for association with CCLR.
ACKNOWLEDGEMENTS

The authors thank Susan Shen and Alex Nisthal for their contributions in SNP detection. Current funding provided by American Kennel Club Canine Health Foundation, Iowa State University Orthopedic Research Laboratory, the Department of Animal Science, Special Research Initiation Grant and the Iowa Agriculture and Home Economics Experiment Station is appreciated.

REFERENCES

Table 1. Primers, conditions for polymerase chain reaction (PCR) amplification and SNP location of canine genes tested for involvement in CCLR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence, 5’→3’ (forward/reverse)</th>
<th>Annealing temp. (°C)</th>
<th>MgCl₂ (mM)</th>
<th>Fragment size (bp)</th>
<th>Location of primers</th>
<th>Location of SNP, type</th>
<th>dbSNP local identifier: NCBI ss#</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBN1</td>
<td>TATATTTATAATGCTTAAGAG ATATGGTCAA/CAGATGGTTTT TGTTGGAT</td>
<td>55.4</td>
<td>15</td>
<td>303</td>
<td>Intron 19 to exon 20</td>
<td>Intron 19, C→T</td>
<td>FBN1 (dbSNP: ss38343205)</td>
</tr>
<tr>
<td>COL9a1</td>
<td>CTGGAGTCAGGGGAGGTGA/ TGGATTTTCTGGAGAGCAG</td>
<td>60</td>
<td>0a</td>
<td>466</td>
<td>Intron 2 bp 40</td>
<td>A→T</td>
<td>COL9A1 (dbSNP: ss38343206)</td>
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<td>COL9a2</td>
<td>TAGTGGTGCCCCGTAACAT/C CCCCCTTACAGATGAGAA</td>
<td>57</td>
<td>0a</td>
<td>176</td>
<td>Intron 30 bp 121, 122</td>
<td>T→G</td>
<td>COL9A2-1 (dbSNP: ss38343207) and COL9A2-2 (dbSNP: ss38343208)</td>
</tr>
<tr>
<td>COMP</td>
<td>GCCCTAGGGTACCCCACTCA/GTTGAGCACCACCCAGTTG</td>
<td>62</td>
<td>10a</td>
<td>1131</td>
<td>Exon 12 to exon 15 bp 631</td>
<td>T→G</td>
<td>COMP (dbSNP: ss38343209)</td>
</tr>
</tbody>
</table>

*a15mM MgCl₂ included in GoTaq buffer
Table 2. PCR restriction fragment length polymorphism (RFLP) conditions and SNP patterns.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme, amt. (U/reaction)</th>
<th>Substrates</th>
<th>Digestion temp. (°C)</th>
<th>Fragment sizes (bp) based on SNP</th>
<th>Gel %&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBN1</td>
<td>Hinc I&lt;sup&gt;a&lt;/sup&gt; (5)</td>
<td>BSA, NEB3</td>
<td>37</td>
<td>C: 274, 29 T: 303</td>
<td>4</td>
</tr>
<tr>
<td>COL9a1</td>
<td>Fok I&lt;sup&gt;a&lt;/sup&gt;, (3)</td>
<td>NEB4</td>
<td>37</td>
<td>T: 283, 129, 54 A: 283, 183</td>
<td>4</td>
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<tr>
<td>COL9a2</td>
<td>BstN I&lt;sup&gt;a&lt;/sup&gt; (3)</td>
<td>BSA, NEB2</td>
<td>60</td>
<td>T,C: 144, 33 G,G: 119, 33, 25</td>
<td>4</td>
</tr>
<tr>
<td>COMP</td>
<td>BstE II&lt;sup&gt;a&lt;/sup&gt; (3)</td>
<td>BSA, NEB3</td>
<td>60</td>
<td>G: 1130, 90 T: 631, 500, 90</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>New England Biolabs, Beverly, MA

<sup>1</sup>Agarose
Table 3. Pearson Chi-square probability results.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Status</th>
<th>1,1 genotype (no.) animals</th>
<th>1,2 genotype (no.) animals</th>
<th>2,2 genotype (no.) animals</th>
<th>Chi-square value, prob</th>
<th>1 allele (no.) animals</th>
<th>2 allele (no.) animals</th>
<th>Chi-square value, prob</th>
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<tbody>
<tr>
<td>FBN1</td>
<td>normal</td>
<td>5</td>
<td>15</td>
<td>22</td>
<td>1.14, 0.56</td>
<td>25</td>
<td>59</td>
<td>1.30, 0.25</td>
</tr>
<tr>
<td></td>
<td>affected</td>
<td>3</td>
<td>13</td>
<td>27</td>
<td>1.30, 0.25</td>
<td>19</td>
<td>67</td>
<td>1.30, 0.25</td>
</tr>
<tr>
<td>COL9a1</td>
<td>normal</td>
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<td>19</td>
<td>18</td>
<td>2.97, 0.23</td>
<td>31</td>
<td>55</td>
<td>2.76, 0.10</td>
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<tr>
<td></td>
<td>affected</td>
<td>2</td>
<td>17</td>
<td>24</td>
<td>2.76, 0.10</td>
<td>21</td>
<td>65</td>
<td>2.76, 0.10</td>
</tr>
<tr>
<td>COL9a2</td>
<td>normal</td>
<td>10</td>
<td>31</td>
<td>4</td>
<td>1.44, 0.49</td>
<td>51</td>
<td>39</td>
<td>0.02, 0.89</td>
</tr>
<tr>
<td></td>
<td>affected</td>
<td>12</td>
<td>26</td>
<td>7</td>
<td>0.02, 0.89</td>
<td>50</td>
<td>40</td>
<td>0.02, 0.89</td>
</tr>
<tr>
<td>COMP</td>
<td>normal</td>
<td>10</td>
<td>27</td>
<td>7</td>
<td>0.55, 0.76</td>
<td>47</td>
<td>41</td>
<td>0.36, 0.55</td>
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<tr>
<td></td>
<td>affected</td>
<td>7</td>
<td>27</td>
<td>8</td>
<td>0.36, 0.55</td>
<td>41</td>
<td>43</td>
<td>0.36, 0.55</td>
</tr>
</tbody>
</table>
CHAPTER 5. CHROMOSOMAL ASSOCIATIONS WITH CRANIAL CRUCIATE LIGAMENT INJURY IN THE NEWFOUNDLAND DOG

V.L. Wilke,¹,⁵ A. Ruhe,² R.B. Evans,³ M.G. Conzemius,¹ M.F. Rothschild⁴

A paper to be submitted to Mammalian Genome

ABSTRACT

Cranial cruciate ligament rupture (CCLR) is considered a very common cause of hind limb lameness in the dog. While trauma is one cause of this disorder there is evidence that some breeds of dogs may have a genetic predisposition. A pedigree was constructed using Newfoundland dogs with CCLR status based on veterinary examination. From this pedigree, affected and normal dogs were chosen for genotyping based on their predicted statistical likelihood of being genetically either homozygous normal (all unaffected, n=45) or homozygous recessive (all affected, n=45).

Genotyping was performed for 417 MSATs. A total of 374 markers were informative in the selected population with an average interval of 7.2 cM between markers. Comparisons of genotypes and allele frequencies were made between affected and unaffected dogs. Ninety one markers were considered statistically significant based on the nominal P < 0.05 while 14 markers were significant using a false discovery rate. Several chromosomes had more than one significant marker. Fine mapping these chromosomal regions should further narrow the list of potential CCLR candidate genes.

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⁵Author for correspondence
INTRODUCTION

Cranial cruciate ligament rupture (CCLR) is considered a very common cause of hind limb lameness in the dog. In a study of Newfoundland dogs seen at a veterinary teaching institution in a six year period, 22% had a diagnosis of CCLR (Wilke et al. 2006). A recent publication highlighted the economic impact of medical and surgical treatment for CCLR which was estimated at more than 1.3 billion dollars for the year 2003 (Wilke et al. 2005). The mechanical instability created by loss of the cranial cruciate ligament (CCL) inevitably leads to osteoarthritis (Vasseur et al. 1992; Elkins et al. 1991; Chauvet et al. 1996), however severity of osteoarthritis (OA) does not correlate with lameness grade (Gordon et al. 2003). Although multiple different surgical techniques are described as treatment for CCLR, no technique is considered superior to another for the treatment of CCLR (Conzemius et al. 2005). The expected outcome of surgical treatment is clinical improvement in 85% to 90% of dogs; unfortunately, less than 50% of dogs will achieve complete soundness (Doverspike et al. 1993; Smith et al. 1985). In fact, one study determined that less than 15% of dogs return to 80% of normal function by six months after surgery (Conzemius et al. 2005).

CCLR has a variable age at onset and the gold standard for diagnosis, surgical confirmation, is invasive. Therefore, animals may be bred before onset of clinical signs or confirmation of diagnosis, allowing transmission of the disease to offspring and no reduction in the overall prevalence of the disease. Proper categorical ascertainment of CCLR status is important for determining potential causes. For instance, an animal that experienced CCLR after having been hit by a car would be unlikely to have it as a result of a genetic predisposition. This would be a simple case of categorical status ascertainment based on trauma. Other cases of “trauma” are not as straightforward. One case would be a dog that sustained CCLR from a minor traumatic event, such as running in the backyard. The question becomes if this dog had a genetic predisposition that allowed it to sustain a rupture of the CCL, or was it a traumatic event because it experienced an excessive load to the CCL?

Specific breeds of dogs have an increased incidence of CCLR while other breeds are considered protected against development of CCLR. This indicates a genetic basis for CCLR. Recent studies suggest that CCLR in the Newfoundland is autosomal recessive with incomplete penetrance (Wilke et al. 2006). Heritability was reported to be 0.27, which indicates that a moderate to high environmental factor may impact the expression of CCLR. The animal that has the genetic predisposition to CCLR must also have the right environmental conditions to express the phenotype. A similar clinical course of CCLR occurs in young female athletes; an estimated 38,000 women/year sustain injury to the anterior cruciate ligament (ACL) (Toth et al. 2001). The significance of this problem becomes more evident when one considers that athletically active females are 2 to 8 times
more likely to injure their ACL than athletic males (Anderson et al. 2001; Arendt et al. 1995; Charlton et al. 2002; Fagenbaum et al. 2003; Gwinn et al. 2000; Lohmander et al. 2004; Sciore et al. 1998; White et al. 2003; Wojtys et al. 2002; Wojtys et al. 2002).

Although phenotypic causes for CCLR have been studied in both humans and dogs, no comparative candidate genes are available to study for association to the condition. Our hypothesis for this study was that there is a chromosomal region(s) associated with CCLR status. We performed a microsatellite based genome scan in an attempt to identify chromosomal region(s) that may be associated with the trait in a population of Newfoundland dogs.

MATERIALS AND METHODS

Selection of dogs: Pedigrees, determination of CCL status (normal or affected with CCLR), and DNA were collected from Newfoundland dogs in a study performed at Iowa State University (ISU) (Wilke et al. 2006). Newfoundland dogs were classified as affected with CCLR based on signs of pain on hyperextension of the knee, knee effusion, decreased range of motion, positive cranial drawer sign or cranial tibial thrust, radiographic evidence of stifle effusion and osteoarthritis, and/or surgical confirmation of a ruptured CCL (Moore et al. 1996). From this pedigree, affected and normal dogs were chosen for genotyping based on their predicted statistical likelihood of being genetically either homozygous normal (all unaffected, n=45) or homozygous recessive (all affected, n=45) (see Table 1). Details for the method are found in Macrossan et al. (2005).

Selection of markers: Two different and complimentary genome scans were conducted. An initial broad genome scan was performed utilizing 130 microsatellite markers (MSATs). Most of the MSATs were selected from Minimal Screening Set 2 (Guyon et al. 2003). Initially, primer optimization for the 130 microsatellite markers was performed on 8 dogs. Final selection of MSATs for the broad genome scan was based on ease of scoring and informativeness of marker, as determined by polymorphic content of alleles. The second phase allowed for the density of the genome scan to be increased by the inclusion of an additional 344 MSATs. This list was generated from MSATs currently utilized in the Veterinary Genetics Laboratory (VGL), University of California at Davis. Similarly, markers were selected for this second stage of the genome scan based on ease of scoring and ability to amplify. All MSATs were multiplexed based on established protocols of the laboratory performing the genotyping.

Statistical analysis. Initial analysis of the MSATs included number and frequency of alleles and genotypes, followed by a chi square analysis performed on each marker. Such analyses compared the allele and genotypic frequencies for each marker for each group of dogs; those assumed to be homozygous normal and those assumed to be homozygous susceptible. Some of the
contingency tables (CCLR status by allele and CCLR status by genotype) had cell counts
(observations) of less than five, a violation of an assumption of classical chi square tests. Instead,
randomization chi square tests were used for all the markers. Briefly, the null hypothesis of the
randomization test is that CCLR status is independent of allele or genotype, so that the chi square
statistic from the data should be consistent with chi square statistics generated after randomly
shuffling the CCLR status relative to the markers. If the original chi square statistic is an outlier
(infrequent) relative to the distribution of randomly generated chi square statistics (less than 5% are
greater than the original statistic, i.e.; \( P<0.05 \)), then the statistical test is considered nominally
statistically significant. For each marker, 5000 random chi square statistics gave a stable distribution
to compare with the original chi square statistic. Statistical tests were performed (one for each
marker) so that the conservative Bonferroni and less conservative false discovery rate (FDR)
adjustments were used to account for type I error inflation and fix the final cutoff for statistical
significance.

Since we could not completely determine those that were expected to be genetically normal
and those to be genetically susceptible we also selected a subset of dogs. This stratification of the
dogs was based on age and a more specific known cause of CCLR. This generated a list of 8 young,
CCLR affected dogs (expected to have genetic susceptibility) and 9 old, CCLR normal dogs which
are more likely to be genetically normal. Recalculation of the chi square statistic as described above
was repeated for genotypes of these 17 dogs.

RESULTS

The original optimization results revealed that 107 of the 130 chosen MSATs could be
reliably scored and of these 107 MSATs, 97 were polymorphic. These 97 MSATs provided genome
coverage of the 38 autosomes in the canine genome with an average spacing of 28 cM. Initial
analyses of the genotyping results for the selected 90 Newfoundland dogs revealed no association for
a chromosomal region(s) and CCLR status. For the additional 344 MSATs, 320 were selected for
genotyping based on ease of scoring and ability to amplify. This provided additional genome
coverage with spacing of markers approximately every 6.7 cM (see Figure 1). Due to problems that
were encountered in amplifying the original DNA at the VGL, new samples had to be collected.
Unfortunately, new samples were only collected from 66/90 dogs (the remaining dogs were either
deceased (\( n = 11 \)) or the owners failed to respond (\( n=13 \)). DNA was successfully amplified for the
first 97 MSATs in the 90 dogs, but genotyping for the remaining 320 MSATs was completed for
66/90 dogs. Cumulatively, genotyping was performed for 417 MSATs for which 10 were duplicated
to allow for error checking. Fifteen markers failed to amplify in the majority of dogs, and 18 markers
were monomorphic, leaving 374 informative markers. Of these 374 markers, there was an average of 5 alleles per marker with a range of 2 to 20 alleles per marker.

Based on the nominal P < 0.05, 91 markers were considered nominally statistically significant (see Table 2). The 91 markers were located on 31 autosomes. Seven chromosomes (CFA 3, 5, 10, 14, 18, 23, and 27) had 4 or more markers with statistical significance. Based on FDR and Bonferroni correction, 14 markers (located on CFA 1, 3, 5, 9, 10, 11, 13, 16, 24, 30, and 31) were considered to be significantly associated with CCLR. All of the chromosomes with markers considered statistically significant for CCLR status based on FDR and Bonferroni correction had one marker considered significant except for three markers that were located on chromosome 10 and two markers located on chromosome 13.

Based on the stratification of young, CCLR affected dogs and old, unaffected with CCLR dogs, 72 markers were determined to be nominally statistically significant with P < 0.05 (see Table 2). These 72 markers were located on 33 autosomes. Five chromosomes had 4 or more markers with statistical significance; chromosomes 3, 8, 10, 23, and 24. Based on FDR correction, 6 markers were considered significant; located on chromosomes 5, 9, 13, 24, and 30. All of the chromosomes with markers considered statistically significant for CCLR status based on FDR correction had one marker considered significant except for the same two markers located on chromosome 13 that were also considered significant in the full data set.

DISCUSSION
Rupture of the cranial cruciate ligament causes stifle instability and results in osteoarthritis and lameness. Degenerative lesions tend to be concentrated in the central core of the CCL, the area with the poorest blood supply (Amockzy et al. 1979) and the most common site of rupture (Paatsama 1952). This is also the site that the two functional bands of the CCL (the craniomedial and the caudolateral bands) twist upon each other. It has been noted that midsection rupture of the CCL often occurs during low impact activities or as a result of daily mechanical wear (Hayashi et al. 2004). Our previous study indicated that CCLR in the dog has an autosomal recessive mode of inheritance with 51% penetrance (Wilke et al. 2006). The frequency of the recessive allele was 0.60. Clearly this makes diagnosis of those with genetic susceptibility very difficult.

Rupture of the CCL in dogs has a very similar clinical course to ACL injury noted with increasing frequency in young female athletes (Moore et al. 1996; Baker 1998). The ability to identify an underlying genetic basis for CCLR in the dog would allow huge advances in understanding the etiopathogenesis of the disorder in humans as well as provide a large animal species to study therapeutic and preventative treatment options before their use in humans.
The process of biologic candidate gene analysis is based on analyzing genes that may play a role in the biological pathway leading to CCLR. The genes we have analyzed, selected based on their involvement in primary arthritis formation in humans, includes Cartilage Oligomeric Matrix Protein (COMP), Matrilin-3 (MATN), Collagen Type 9 alpha 1, 2 and 3 (COL9A1, COL9A2, COL9A3), Fibrillin-1 (FBN1), and Interleukin Receptor 4 (IL4R). Single nucleotide polymorphisms (SNPs) were identified in COMP, COL9A1, COL9A2, and FBN1. No significant association was found between the SNPs and CCLR status (normal and unaffected), although some suggestion of an association was found between COL9A1 (located on CFA 12) and CCLR affected status (P=0.10) (Wilke et al. 2005).

The positional candidate gene approach uses first the identification of a chromosomal region that is associated with a disorder, and then identification of all genes located in that region. The genes are then organized and selected according to different roles based on gene ontology, such as cellular component, molecular function, or physiologic function. Since the dog genome has been recently sequenced one can use that information, in consultation with the human genome and the comparative map, to choose the best positional candidates to study for further association to a trait. We previously predicted a simple recessive mode of inheritance. However in this study several chromosomal regions are statistically associated with CCLR status. This may come about by false identification of certain regions or, given our heritability estimate of 0.27 for this trait, it is possible that there are modifying genes that are being expressed that may explain trait variability.

A list of possible candidate genes based on adjacent location to statistically significant markers located on chromosomes 3, 10, and 23 is noted in Table 3. These chromosomes were chosen because they contained the largest number of significant markers in common based on analyses of both the total population and the stratified population. The Map Viewer program of NCBI (National Center for Biotechnology Information, Bethesda, Maryland) was used to identify genes located in specific canine chromosomal regions identified as statistically significant from the genome scan analysis. Genes were selected as potentially involved in etiopathogenesis of CCLR based on known comparative function available in the Online Mendelian Inheritance of Man (OMIM, NCBI).

Potential candidate genes include several related growth factor receptor genes: fibroblast growth factor binding protein, fibroblast growth factor receptor substrate 2, insulin like growth factor-1 receptor, transforming growth factor alpha, transforming growth factor beta type II receptor, and the related bone morphogenetic protein 10 precursor. In addition, several proteoglycan related genes (versican, proteoglycan link protein 2 precursor, aggrecan core protein precursor, and chondroitin synthase 1) and interleukin receptors (interleukin-1 receptor type I and II and interleukin 2 receptor beta) were identified on these chromosomes. Fibroblast growth factor (FGF) has a prominent role in
skeletal development and is known to bind to cell-surface heparan-sulfated proteoglycans (Ornitz et al. 2001). Insulin like growth factor I (IGFI) responds to growth hormone and exerts its effect on cellular function, specifically bone growth (Mohan et al. 1991). The IGFs cause hypertrophy of all cells involved in osteogenesis. Transforming growth factors (TGFs) are involved in wound healing and activation of signal transduction (Lawrence 1996). Bone morphogenic proteins (BMPs) are known as bone growth-regulatory factors and are osteoinductive (Mohan et al. 1991). Proteoglycans are major components of extracellular matrix of cartilage (Schwartz et al. 2002), whereas the interleukins are involved in immune modulation. Cartilage link protein stabilizes aggregates of aggrecan and hyaluronan, giving cartilage its tensile strength and elasticity (Watanabe et al. 1999).

One potential limitation of our study is CCLR status ascertainment; the ability to accurately classify a dog as homozygous unaffected or homozygous affected with CCLR. Rupture of the CCL is known to have a variable age of onset and incomplete penetrance (Wilke et al. 2006), making it difficult to characterize an animal as unaffected with CCLR until it is at least 8 years of age (Rooney et al. 2002). Due to the nature of the study, it was impossible to include only animals over eight years of age, and even then, they could still be homozygous affected yet not expressing clinical signs due to the incomplete penetrance of the disorder. Therefore, even though dogs were selected for the genome scan based on their potential to be homozygous affected or unaffected, an inaccurate classification of some dogs would lower the opportunity to find specific markers that were significant. In addition, there is a potential that there are several different pathologic causes of CCLR, and this effect may be breed or age specific. Recent studies have noted an increased incidence of CCLR in young female dogs (Duval et al. 1999); however classical studies of CCLR have concentrated on degenerative changes of the CCL that are associated with aging (Vasseur et al. 1985). We therefore reanalyzed the data through stratification of the dogs based on age and defined cause for CCLR to narrow the list of CCLR associated chromosomal regions and potentially point to more obvious candidate genes. An interesting point is that there is agreement between the two data sets and the list of nominally statistically significant markers in common for both analyses has markers located on chromosomes 3, 10, and 23.

Rupture of the cranial cruciate ligament has a genetic basis in the Newfoundland dog (Wilke et al. 2006). Using a microsatellite based genome scan in a population of Newfoundlands, we have determined several chromosomal regions that are associated with CCLR status. Fine mapping these chromosomal regions should further narrow the list of potential CCLR candidate genes.
ACKNOWLEDGEMENTS

The authors appreciate the support received by the American Kennel Club Canine Health Foundation; Orthopedic Research Laboratory, College of Veterinary Medicine, Iowa State University; Department of Animal Science, Iowa State University; Special Research Initiation Grant, the Iowa Agriculture and Home Economics Experiment Station; and Hatch and State of Iowa funds. Assistance by K. Glenn and all the students involved in the Max Rothschild laboratory is appreciated.

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Figure 1: Name and chromosomal position of microsatellites used in this study. The 417 MSATs provide markers at approximately 6.7 cM intervals.
Table 1. Summary statistics for dogs used in genome scan.

<table>
<thead>
<tr>
<th></th>
<th>No. Males</th>
<th>No. Females</th>
<th>Avg Age (yrs)</th>
<th>Avg Age at CCLR (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCLR unaffected</td>
<td>8</td>
<td>37</td>
<td>6.34</td>
<td>0</td>
</tr>
<tr>
<td>CCLR affected</td>
<td>16</td>
<td>29</td>
<td>7.15</td>
<td>4.7</td>
</tr>
</tbody>
</table>
Table 2. List of MSATs significantly associated with CCLR status.

<table>
<thead>
<tr>
<th>CFA</th>
<th>Location (Mb)</th>
<th>Marker</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.4</td>
<td>FH3325</td>
<td>0.0278</td>
</tr>
<tr>
<td>1</td>
<td>9.0</td>
<td>FH2663</td>
<td>0.0049</td>
</tr>
<tr>
<td>2</td>
<td>51.2</td>
<td>REN70M14</td>
<td>0.0051</td>
</tr>
<tr>
<td>2</td>
<td>60.8</td>
<td>FH2848</td>
<td>0.0322</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>FH3396</td>
<td>0.0171</td>
</tr>
<tr>
<td>3</td>
<td>41.7</td>
<td>FH2541</td>
<td>0.0062</td>
</tr>
<tr>
<td>3</td>
<td>64.0</td>
<td>LEI1E12</td>
<td>0.0129</td>
</tr>
<tr>
<td>3</td>
<td>68.5</td>
<td>CPH19</td>
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</tr>
<tr>
<td>3</td>
<td>75.0</td>
<td>REN153P03</td>
<td>0.0322</td>
</tr>
<tr>
<td>3</td>
<td>#</td>
<td>PEZ12</td>
<td>0.0442</td>
</tr>
<tr>
<td>4</td>
<td>#</td>
<td>G07704</td>
<td>0.0133</td>
</tr>
<tr>
<td>4</td>
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Bold: significant based on FDR
Italics: significant based on Bonferroni
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<td>10</td>
<td>Interleukin 2 receptor, beta</td>
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<tr>
<td>10</td>
<td>Interleukin-1 receptor type I</td>
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<tr>
<td>10</td>
<td>Interleukin-1 receptor type II</td>
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<tr>
<td>23</td>
<td>Transforming growth factor beta type II receptor</td>
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CHAPTER 6. GENERAL CONCLUSIONS AND DISCUSSION

GENERAL CONCLUSIONS

The research objectives of this thesis were to perform an economic impact survey for CCLR treatment in the dog, perform genetic analyses of CCLR in a population of Newfoundland dogs, and perform biological and positional candidate gene analyses. The ultimate goal of all these objectives was to attempt to identify an associated or causal mutation for CCLR in the dog. Chapter 2 addresses the economic impact of medical and surgical management of CCLR, chapter 3 determines a genetic basis for CCLR, and chapters 4 and 5 address biological and positional candidate gene studies in the dog, respectively.

The main objective of the economic impact survey presented in chapter 2 “Estimate of the annual economic impact of cranial cruciate ligament injury in dogs in the United States” was to determine the overall economic impact of medical and surgical management of CCLR in dogs in the U.S. in the year 2003. The survey compiles responses from small animal veterinary practitioners and small animal veterinary orthopaedic surgeons.

The findings of this work were:

- The survey had a higher than expected response. The ACVS study population had a response rate of 51.1% and the AVMA study population had a 27.1% response rate.
- Based on survey responses, on average ACVS responders perform surgery for CCLR on 186.24 cases per year and AVMA responders perform 29.16 cases per year. In addition, ACVS responders medically manage 23.88 and the AVMA responders medically manage 12.24 cases of CCLR per year.
- The ACVS response group reported an average of $1,840.50 per surgery for CCLR and the average cost for medical management was $241.20. The AVMA response group reported an average of $898.00 per surgery and the average cost for medical management was $265.40.
- Within the AMVA response group, responders included 739 males and 344 females. A comparison of the mean cost of surgery from veterinarians that reported that they performed CCLR surgery noted that males reported a mean ± SE charge of $1,023.43 ± 18.54 and females reported a mean ± SE charge of $1,095.04 ± 33.86. This difference was significant (P < 0.05).
- Overall, owners spent $1.32 billion for the treatment of CCLR in the United States in the year 2003.
The main objective of the genetic analyses in chapter 3 “Inheritance of rupture of the cranial cruciate ligament in Newfoundlands” was to determine an underlying genetic basis for CCLR in a population of Newfoundland dogs and propose a potential mode of inheritance for the trait. Findings of this study were:

- Analysis of one hundred sixty three Newfoundlands evaluated at the ISU-CVM from January 1, 1996 through December 31, 2002 revealed that 22% had CCLR. Mean age at the time of definitive diagnosis was 3.53 years.
- Analysis of 411 recruited Newfoundlands revealed 92 (22%) were CCLR-affected and 319 were unaffected with CCLR. Mean age of all dogs in the study was 6.51 years. Mean age at the time of diagnosis of CCLR was 5.17 years.
- In the pedigree generated from the recruited Newfoundlands, the mean inbreeding coefficient was 1.19 x 10⁻⁴. Heritability was calculated as 0.27. Segregation analysis predicted a major gene effect with a recessive pattern of inheritance. The frequency of the recessive allele was 0.60 with partial penetrance of 51%.

The main objective of the biological candidate gene analyses in chapter 4 “SNP detection and association analyses of candidate genes for rupture of the cranial cruciate ligament in the dog” was to determine if SNPs identified in biological candidate genes were associated with CCLR status. The findings of this work were:

- Single nucleotide polymorphisms (SNPs) were discovered in four biological candidate genes: fibrillin, (FBN1), collagen Type 9 alpha (COL9A1), collagen Type 9 alpha 2 (COL9A2), and cartilage oligomeric matrix protein (COMP).
- The SNP discovered in FBN1 was located in intron 19 and was a C to T transition (dbSNP local identifier: NCBI ss#: FBN1 (dbSNP: ss38343205)).
- The SNP discovered in COL9A1 was located in intron 2 at position 40 from the start of the intron and was an A to T transversion (dbSNP local identifier: NCBI ss#: COL9A1 (dbSNP: ss38343206)).
- Two SNPs were discovered in COL9A2 located in intron 30 at positions 121 and 122 from the start of the intron and were a T to G transversion and a C to G transversion, respectively (dbSNP local identifier: NCBI ss#: COL9A2-1 (dbSNP: ss38343207) and COL9A2-2 (dbSNP: ss38343208)).
- The SNP discovered in COMP was located at position 631 from the ATG site and was a T to G transversion (dbSNP local identifier: NCBI ss#: COMP (dbSNP: ss38343209)).
• No significant association was found between any SNP and CCLR status of the dogs although for \textit{COL9A1} (CFA 12) the association with allele frequency and CCLR status was close to significance (P = 0.10).

The main objective of positional candidate gene analyses in chapter 5 “Chromosomal associations to cranial cruciate ligament injury in the Newfoundland dog” was to identify chromosomal region(s) that may be associated with the CCLR status. The findings of this work were:

• Four hundred and seventeen microsatellite markers were genotyped in our population providing genome coverage with a marker approximately every 6.7 cM. Of those markers, 10 were duplicated to allow for error checking, 15 markers failed to amplify in the majority of dogs, and 18 markers were monomorphic, leaving 374 informative markers.

• Based on the nominal P < 0.05, 91 markers were considered statistically significant. The 91 markers were located on 31 autosomes. Six chromosomes had 4 or more markers with statistical significance. These six chromosomes were chromosomes 3, 5, 14, 18, 23, and 27. Based on FDR and Bonferroni corrections, 14 markers were considered significant; located on chromosomes 1, 3, 5, 9, 10, 11, 13, 16, 24, 30, and 31.

• Based on the stratification of young, CCLR affected dogs and old, CCLR unaffected dogs, 72 markers were determined to be nominally statistically significant with P < 0.05. These 72 markers were located on 33 autosomes. Five chromosomes had 4 or more markers with statistical significance; chromosomes 3, 8, 10, 23, and 24. Based on FDR correction, 6 markers were considered significant; located on chromosomes 5, 9, 13, 24, and 30.

**GENERAL DISCUSSION**

Rupture of the cranial cruciate ligament (CCLR) in the dog is the most common cause of hind limb lameness. It causes stifle instability, which leads to progressive, debilitating osteoarthritis and results in lameness. This lameness may necessitate either surgical intervention and/or life long medical therapy, the sum of which has an estimated economic cost to owners of approximately $1.32 billion per year (Wilke \textit{et al.} 2005). Rupture of the CCL in the dog has been reported to be associated with trauma, immune-mediated mechanisms (Galloway \textit{et al.} 1995; Lawrence \textit{et al.} 1998; Niebauer \textit{et al.} 1987), age related degeneration of the CCL (Vasseur \textit{et al.} 1985), obesity (Whitehair \textit{et al.} 1993), conformational abnormalities such as a patella luxation (Aiken \textit{et al.} 1995) narrowed femoral intercondylar notch (Aiken \textit{et al.} 1995; Shelbourne \textit{et al.} 1998) and the tibial plateau angle (Morris \textit{et al.} 2001). Approximately 20% of dogs develop CCLR from a single high-load traumatic event; the majority of the remaining dogs develop CCLR with a history of mild trauma (the effect of daily
mechanical wear) (Moore et al. 1996). Traditionally, these dogs are considered to have age related degeneration as the cause of CCLR (Duval et al. 1999; Moore et al. 1996; Whitehair et al. 1993; Vasseur et al. 1985).

Particular breeds of dogs have an increased incidence rate of CCLR (Duval et al. 1999; Whitehair et al. 1993; Wilke et al. 2006). Additional studies report that within these predisposed, large breed dogs, CCL injuries occur at a younger age than expected for age related degeneration (Duval et al. 1999; Harasen 1995; Moore et al. 1996; Whitehair et al. 1993). We hypothesized that these young, large breed dogs had a genetic predisposition whereby they developed CCLR with a history of only mild trauma. We therefore recruited a population of Newfoundland dogs in order to study the underlying genetic basis of CCLR. In this population, we reported a 22% incidence rate of CCLR and a potential recessive mode of inheritance with partial penetrance. Based on these findings, we initiated a microsatellite based genome scan in an attempt to identify chromosomal region(s) associated with CCLR.

Analysis of the microsatellite genotypes for the Newfoundlands was performed first in the entire population (based on information we have acquired to date) and then in a subpopulation of dogs. This subpopulation of dogs was chosen to minimize errors associated with status ascertainment, or the variability created in analysis that could be due to a dog having an incorrect identified cause of CCLR. Other potential causes for CCLR include true trauma (strain to the CCL that exceeds four times the body weight of the dog) or from age related degeneration. The subpopulation was selected based on young, less than 2 years old, Newfoundlands affected with CCLR from a cause that we knew was consistent with our hypothesis and old, greater than 8 years old, Newfoundlands unaffected with CCLR.

Comparison of the statistically significant markers between the two analyses revealed three chromosomes with a large number of markers in common, chromosomes 3, 10, and 23. We elected to concentrate on these three chromosomes for the positional candidate gene selection. The Map Viewer program of NCBI (National Center for Biotechnology Information, Bethesda, Maryland) was used to identify genes located on these chromosomes within regions that contained the statistically significant markers. Genes were selected as potentially involved in etiopathogenesis of CCLR based on known comparative function as available in the Online Mendelian Inheritance of Man (OMIM, NCBI). This list of positional candidate genes includes several related growth factor genes, proteoglycan related genes, and interleukin receptors.

The positional candidates include growth factors and their related molecules: fibroblast growth factor binding protein, fibroblast growth factor receptor substrate 2, insulin like growth factor-
1 receptor, transforming growth factor alpha, transforming growth factor beta type II receptor, and the related bone morphogenetic protein 10 precursor. Fibroblast growth factor (FGF) has a prominent role in skeletal development and is involved in regulation of cell proliferation, migration, and differentiation (Ornitz et al. 2001). FGFs have a high affinity for heparan-sulfated proteoglycans and activation of one of the four FGF cell surface receptors requires heparin sulfate (Ornitz et al. 2001). Insulin like growth factor I (IGFI) responds to growth hormone and its primary action is mediated by binding to specific IGF receptors present on many cell types in many tissues. Almost every cell in the human body is affected by IGF1, especially cells in muscle, cartilage, bone, liver, kidney, nerves, skin, and lungs. It exerts its effect on cellular function, including bone growth. The effect is the promotion of cell growth and multiplication (Mohan et al. 1991). Transforming growth factors (TGFs) are involved in wound healing and activation of signal transduction (Lawrence et al. 1998). They are known to enhance the formation of connective tissue (Sporn et al. 1986). Bone morphogenetic proteins (BMPs) are known as bone growth-regulatory factors and belong to the TGF-β super family with a strong ability to induce new bone and/or cartilage formation (Mohan et al. 1991).

Several proteoglycan related genes were identified as positional candidate genes. These include versican, proteoglycan link protein 2 precursor, aggrecan core protein precursor, and chondroitin synthase 1. Proteoglycans (PGs) are major components of articular cartilage matrix (Schwartz et al. 2002). They consist of a core protein with one or more covalently bound glycosaminoglycan chains (GAGs). Aggrecan is one of the largest PGs, approximately 2500 kDa in size, and is known for its ability to resist forces of compression in articular cartilage due to its large number of GAG chains. Aggrecan is named by the formation of aggregates of aggrecan and hyaluronan which are stabilized through the binding of a cartilage link protein (Yanagishita et al. 1992). Thus, cartilage link proteins give cartilage its tensile strength and elasticity (Watanabe et al. 1999). Versican is smaller than aggrecan, approximately 1000 kDa, but has a wider range of tissue distribution and also binds to hyaluronan (Wight et al. 1991). Chains of GAGs are able to hold a large number of water molecules and occupy enormous hydrodynamic space in solution. The list of GAGs includes chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, and keratan sulfate.

Additional positional candidate genes, the interleukin-1 receptors type I and II and interleukin 2 receptor beta, were selected based on their involvement in immune modulation. In particular, some studies suggest that CCLR has an immune mediated component. One study noted that cases with partial CCLR had inflammatory changes present in the synovial fluid (Griffin et al. 1992). Another
study determined the presence of anticollagen antibodies in the synovial fluid of dogs with CCLR (Niebauer et al. 1987).

One limitation of this work is the large number of incomplete families in the pedigree used to determine the mode of inheritance. We did have several complete families that allowed us to consider our prediction was accurate. It would have been ideal to perform the segregation analysis only in families with complete family history. Unfortunately, dog breeders sell puppies when young, approximately two months old, and do not maintain long term relationships with the new owners. Since CCLR can occur at any time, up to an average of eight years of age, the majority of owners participating in our recruitment study did not have CCLR information for complete litters. Without knowing the CCLR status of all dogs in a family, it is possible that a study could be biased towards selection of a particular trait, such as enrolling more CCLR affected dogs vs. CCLR unaffected dogs. We attempted to minimize this risk by stressing the enrollment of both CCLR affected and unaffected dogs in the study. However, it is impossible to recruit without impartiality of those participating and their perception of the study. Therefore, based on the nature of dog breeding, the difficulty of CCLR status ascertainment which can be complicated by many initiating factors, and the fact that CCLR has a variable age of onset and can occur throughout life, a planned breeding colony would be the best solution to elucidate the true underlying genetic basis for CCLR in the Newfoundland.

We suggest that the dog will serve as an excellent model to study the increased incidence of ACL injury that occurs in young female athletes. First, there are over 350 reported inherited canine diseases, of which greater than 50% are also experienced by humans (Nicholas 2001; Nicholas 2003; Ostrander et al. 2000). This combined with the sequence information gained from completion of sequencing of the canine genome opens many doors into comparative genomic studies. Second, humans and dogs share high sequence homology, much higher than humans share with the mouse (Kirkness et al. 2003). Domestication of the dog into several breeds has led to a high inbreeding ratio within each breed, increasing the frequency of recessive diseases that are experienced within that breed. Thus, each breed serves as a selectively purpose bred colony that is an excellent source of comparative diseases. Third, the dog is a large animal model and is better suited to testing therapeutic and interventional therapies that are targeted for human use than a smaller animal model such as the mouse. And finally, naturally occurring CCLR in the purebred dog is similar to the injury in athletic females in that increased incidence is: 1) usually found in specific breeds of dogs more commonly than other breeds (i.e. predisposed population), 2) found in female and neutered (female and male) dogs more commonly than in sexually intact male dogs, 3) occurs at a young age in the predisposed
populations and, 4) usually linked to an activity that places only a modest mechanical demand on the CCL that shouldn't lead to failure.

Another point of discussion is the mode of inheritance and heritability that we calculated for our population. Heritability is calculated as the additive genetic variance divided by the total phenotypic variance. This implies that any value less than one has non-genetic factors that contribute to the trait. It also implies the trait is quantitative. This is in part a contradiction to our segregation analysis that predicted a recessive mode of inheritance with partial penetrance. One explanation for this disparity could be that the trait is controlled by a major gene effect with modifier genes rather than a polygenic or monogenic mode of inheritance. A major gene is a single locus that has a large effect on a trait. Modifier genes have small quantitative effects on the level of expression of another gene. This could be due to individual mutations of large effect or, a more recent hypothesis associated with high linkage disequilibrium in a population, multiple associated polymorphisms (Stam et al. 1996). For comparison, a recent segregation analysis of canine hip dysplasia (CHD) in a population of German shepherd dogs revealed that a major gene effect is a better fit for the model than the previously accepted polygenic mode of inheritance (Janutta et al. 2005). This implies that significant advances can be made in the reduction of CHD incidence based on selective breeding and that researchers should concentrate on major gene loci that may affect the incidence of CHD.

We opted to perform the positional candidate gene approach beginning by identifying a chromosomal region(s) associated with CCLR. Once a chromosomal region is identified, refinement of the region(s) can be performed through the process of fine mapping. Fine mapping is based on the same premise as the genome scan but instead of scanning the entire canine genome containing 38 autosomes and the sex chromosomes for disease associated chromosomal regions, scanning is only performed between markers in a narrower range. This can narrow the potential positional candidate gene list even more, allowing the search for the disease causing mutation to proceed more quickly. This approach has been used successfully in other disease studies (Lowe et al. 2003; van de Sluis et al. 2002). An alternative approach to identifying potential candidate genes for CCLR would be gene expression arrays. Gene expression profiling provides information on gene regulation and function for tens of thousands of genes simultaneously. Classifying the differentially expressed genes allows identification of molecules that may be involved in disease pathophysiology that may not have been detected using traditional, less high-throughput methods. Unfortunately, microarray analysis can be extremely complex. A solution would be to combine the information obtained from the genome scan concerning ACL injury associated chromosomal regions and the gene expression array to allow one to hone in on the causal gene(s) more proficiently than either technique alone.
In conclusion, we report that medical and surgical treatment for CCLR in the dog in the U.S. costs an estimated $1.32 billion. The CCLR disorder has a potential recessive mode of inheritance with 51% penetrance in a population of Newfoundlands and that also has an incidence rate of CCLR of 22%. The SNPs from a list of biological candidate genes that we examined were not associated with CCLR status and chromosomes 3, 10, and 23 have a large number of statistically significant microsatellite markers associated with CCLR status.

**RECOMMENDATIONS FOR FUTURE RESEARCH**

*Fine mapping*

Based on analysis of all the microsatellite genotypes in the whole population, 91 markers were considered statistically significant based on a nominal P value < 0.05. Six chromosomes had four or markers that were statistically significant. This is a wide range of regions that needs to be studied further. In attempting to narrow the range of regions even more, further stratification of the population and subsequent analysis and comparison to the original analysis revealed chromosomes 3, 10, and 23 to have a large number of microsatellite markers that are statistically associated with CCLR status. This still presents an experimental challenge as the markers span large regions of the chromosomes, such as from 4 to 70 Mb on chromosome 10. In order to narrow the associated chromosomal region(s) even more and refine the list of positional candidate genes, we recommend to fine map these regions by genotyping additional markers in close proximity to and between the already genotyped microsatellite markers.

The obvious next step after fine mapping and generation of a list of positional candidate genes is to perform sequence analysis and SNP discovery in those genes. Association analyses of the SNPs and CCLR status may reveal an associated or causal mutation for CCLR. Based on those results, proof of causality via functional studies are necessary to confirm the role the potential causative mutation has in the development of CCLR.

*Mode of Inheritance*

As previously mentioned, a large number of incomplete families were used in the pedigree to determine the mode of inheritance. Ideal would have been to perform the segregation analysis only in families with complete family history. Therefore, based on the nature of dog breeding, the difficulty of CCLR status ascertainment which can be complicated by many initiating factors, and the fact that CCLR has a variable age of onset and can occur throughout life, a planned breeding colony would be the best solution to elucidate the true underlying genetic basis for CCLR in the Newfoundlands.
**Gene Expression Profiling**

Another potential approach to identifying candidate genes is gene expression arrays, looking for expression differences between CCL harvested from CCLR affected and CCLR unaffected dogs. Gene expression profiling provides information on gene regulation and function for tens of thousands of genes simultaneously. Classifying the differentially expressed genes allows identification of molecules that may be involved in disease pathophysiology that may not have been detected using traditional, less high-throughput methods. The analysis for this method is more complex than the traditional candidate gene approach and still relies on validation of differentially expressed genes and SNP discovery to determine an associated or causal mutation. Therefore, we suggest that combining the information from a traditional positional candidate gene approach with a gene expression array may be more beneficial towards identifying the causal mutation than either technique alone. Based on the information gained from this current stage of the project, performing fine mapping of associated chromosomal regions and combining that information with data from gene expression profiling would be recommended as the most efficient method to identify the causal mutation for CCLR in the Newfoundland dog.

**REFERENCES**