INVESTIGATION FOR GENETIC DETERMINANTS OF CONGENITAL FLEXION CONTRACTURES AND CONTRACTED FOAL SYNDROME IN NEONATAL THOROUGHBRED FOALS

A Dissertation

by

JANA DENISE CALDWELL

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Bhanu P. Chowdhary
Committee Members,	Clare A. Gill
	Terje Raudsepp
	Christopher Seabury
	William Moyer
Head of Department,	Evelyn Tiffany-Castiglioni

December 2014

Major Subject: Biomedical Sciences

Copyright 2014 Jana Denise Caldwell

ABSTRACT

Musculoskeletal disorders are one of the leading causes of mortality in neonatal Thoroughbred foals. Contracted Foal Syndrome (CFS) has accounted for up to 48% of such disorders in foals submitted for necropsy according to the Kentucky Livestock Diagnostic Center and is reportedly a concern to clinicians and breeders. CFS is primarily characterized by limb contractures and other malformations of the appendicular and/or axial skeleton. Foals are often euthanized in severe cases and successful rehabilitation in moderate cases does not entirely negate secondary complications. Because of the economic implications associated with treatment costs, owners may opt to euthanize foals even though they potentially could have led productive lives.

A familial predisposition was observed in some cases. In addition, veterinarians reported increased incidence of contracted foals in one particular sire line. This, coupled with model genetic disorders in other species, prompted us to conduct the first molecular genetics study on congenital flexion contractures and CFS.

The inconsistent nature of clinical documentation and variable phenotypes pose a challenge to researchers investigating such complex conditions. We therefore conducted a detailed analysis of the phenotypes and used the data to propose a preliminary classification system that could be used by clinicians and researchers. The implementation of such a classification system will reduce ambiguity of clinical documentation and provide the basis for future study designs.

Our hypothesis states, that in some cases, flexion contractures and CFS are major gene disorders with the likelihood of genetic heterogeneity. Our first approach was to sequence the candidate gene, tropomyosin beta 2. This gene encodes a component of the skeletal muscle contractile apparatus and has been implicated in congenital distal limb contractures in humans. Next, we utilized the newly available Equine SNP50 Beadchip for a case/control population based genome-wide association mapping approach followed by a family validation study and family based genome-wide association study. These approaches resulted in the identification of associations between various subtypes of contracted foals and at least 3 disease susceptibility loci.

In summary, this study provides insight into the genetics underlying flexion contractures and CFS in the neonatal foal and has provided the first evidence for a genetic cause. Furthermore, it provides a solid foundation for future research targeting candidate genes for resequencing.

DEDICATION

This work is dedicated to my father, Dendy Scott and mother, Jo Eisenrich, who never relented in giving love, support and encouragement during the darkest of hours.

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

TABLE OF CONTENTS v

LIST OF FIGURES vii

LIST OF TABLES xi

CHAPTER I GENERAL INTRODUCTION TO CONGENITAL JOINT CONTRACTURES AND CONTRACTED FOAL SYNDROME IN NEONATAL THOROUGHBRED FOALS 1

- Disease Description 1
- Nomenclature 6
- Disease Frequency 8
- Pathoetiology 9
- Why are Flexion Contractures and CFS Important to Study? 10
- Rationale for a Genetic Study 12

CHAPTER II SEQUENCING AND ANALYSIS OF CANDIDATE GENE TROPOMYOSIN BETA 2 (TPM2): A SEARCH FOR VARIANTS CAUSING FLEXION CONTRACTURES IN THOROUGHBRED FOALS 28

- Introduction 28
- Methods and Materials 31
- Results 37
- Discussion 42

Page

CHAPTER III GENOME-WIDE ASSOCIATION ANALYSES IN THOROUGHBRED FOALS IDENTIFY CANDIDATE GENES IN A SKELETAL MUSCLE PROTEIN NETWORK ASSOCIATED WITH CONGENITAL JOINT CONTRACTURES 49

Introduction 49
Materials and Methods 52
Results 59
Discussion 86

CHAPTER IV CLASSIFICATION AND DELINEATION OF CONGENITAL JOINT CONTRACTURES AND CONTRACTED FOAL SYNDROME IN THE NEONATAL FOAL 94

Introduction 94
Methods 95
Results 96
Discussion 99

CHAPTER V CONCLUSION AND FUTURE WORK 111

REFERENCES 115

APPENDIX A 128

APPENDIX B 131

APPENDIX C 136

APPENDIX D 141

LIST OF FIGURES

Page

Figure 1 Normal joint angles of the forelimb and hindlimb in the horse. 2

Figure 2 Variable expression of flexion contractures and CFS with comparisons to normal conformations of the respective joints in newborn foals (N=normal). 4

Figure 3 (A) A stillborn, term foal with a severe case of CFS. 5

Figure 4 Two examples of mares (blue circles) producing more than one contracted foal with the same phenotype. 13

Figure 5 (A) Newborn diagnosed with amyoplasia presenting with bilateral hip and knee flexion contractures and bilateral clubfeet (Bamshad *et al.* 2009). 16

Figure 6 Heritable arthrogryposis multiplex congenita in a newborn lamb presenting with marked contractures of the carpi and tarsi and mild contractures of the distal joints. 20

Figure 7 A neonatal buffalo calf with multiple joint contractures affecting the proximal and distal limbs. 21

Figure 8 A neonatal calf presented with marked flexion contractures of the proximal and distal joints in the front limbs and of the hocks in the hind limbs. 23

Figure 9 Arthrogyposis multiplex congenita in Swiss Large White pigs. 24

Figure 10 Excision site for muscle tissue biopsies from *flexor carpi radialis*. 33

Figure 11 Fluorescence *in situ* hybridization confirmed the location of *TPM2* on homologous chromosomes (green signals) to be on ECA25q12-q13. 37

Figure 12 Graphical interpretations of 4 putative haplotypes and 8 diplotypes identified from exon and exon boundary resequencing of *TPM2* in 56 affected foals and 38 control animals. 38

Page

Figure 13 Alignment of horse and human *TPM2* CDS highlighting the location of the SNP discovered in exon 2. 39

Figure 14 Longitudinal measurements of fiber diameters from histological preparations in 2 affected and 2 normal foals. 40

Figure 15 Longitudinal measurements of fiber diameters from histological preparations in 2 affected and 2 normal foals. 41

Figure 16 Transmission electron microscopy of skeletal muscle taken from the *flexor carpi radialis* of a severely contracted foal (Affected Foal A). 42

Figure 17 (A) Nemaline rods from human skeletal muscle captured in a TEM image. (B) A type of benign myopathy characterized by degeneration of the Z-disks and actin filaments. Note the marked disorganization of the sarcomeres. 47

Figure 18 Pedigrees illustrating the family structure used in the family-based validation study and family based GWAS. 56

Figure 19 Association study stratification plots prior to correction. 60

Figure 20 Manhattan plot representing asymptotic *P*-values ($-\log_{10}$) from analysis of original cohort (23 cases and 23 controls) showing moderate associations to ECA5, 13, 18, and 20. 61

Figure 21 QQ plot of $-\log_{10}$ of *P*-values from basic chi-square association tests of the cohort of 23 affected foals and 23 control animals. 62

Figure 22 Manhattan plot representing asymptotic *P*-values ($-\log_{10}$) from the chi-square analysis of Phenotype Group 1 (7 foals with distal limb contractures) and 23 controls. 63

Figure 23 QQ plot of $-\log_{10}$ of *P*-values from the chi-square analysis of Phenotype Group 1 (7 foals with fetlock contractures) and 23 control animals. 64

Figure 24 Haplotype analysis of a 2.68 Mb region on ECA18 identifies 3 homozygous segments (in black boxes) observed in 4 foals with fetlock contractures and 3 foals from other Phenotype Groups. 67

Page

Figure 25 Homozygosity mapping results indicate a significant region of autozygosity on ECA18 (18:56863692-57022464) that corresponds with the associated genomic interval identified in the case/control Phenotype Group 1 analyses. 68

Figure 26 Plots representing the LD structures of genomic region ECA18: 56468723-58295606 using genome-wide genotype data. 70

Figure 27 Manhattan plot of $-\log_{10}$ representing permuted *P*-values from the chi-square analysis of phenotype Group 2 (3 affected foals with carpal contractures, angular deviations, and distal limb laxity. 71

Figure 28 QQ plot of $-\log_{10}$ of *P*-values from the basic chi-square analysis of Phenotype Group 2 (carpal contractures, angular limb deformities, and ligament/tendon laxity). 72

Figure 29 Haplotype analysis of a ~3.4 Mb region on ECA5 including the gene *TBX15* identified 3 unique segments (highlighted in orange and enclosed in black boxes) in 7 affected foals. 74

Figure 30 Haplotype analysis of a ~1 Mb region on ECA5 including the gene *CASQ2* identified a 24 marker haplotype (highlighted in yellow) found exclusively in 6 affected foals. 75

Figure 31 Haplotype analysis of a 1.6 Mb region on ECA5 including the gene *AMPD1* identified a 19 and 21 marker haplotype found exclusively in 6 affected foals (highlighted in orange and demarcated by black boxes). 76

Figure 32 Haplotype analysis of a ~1.3 Mb region on ECA5 including the gene *CAPZA1* identified a 23 marker haplotype (highlighted in orange) exclusively found in 5 affected foals. 77

Figure 33 LD structures of affected foals from Phenotype Group1 (top plots) and the control population of 23 unaffected foals (bottom plots). 78

Figure 34 Manhattan plot representing permuted *P*-values ($-\log_{10}$) from the DFAM test of association in a kindred of 60 individuals. 82

Page

Figure 35 Individual genotypes of 9 SNP discovered from exon sequencing of *ANKRD1*. Cells with a single letter are homozygous for that variant. 84

Figure 36 Individual genotypes of SNP discovered from exon sequencing of *ANKRD1* arranged by half-sibling relationships except where singletons are described. 85

Figure 37 Illustrations depict examples of mild, moderate, and severe contractures of the metacarpophalangeal joint measured in degrees from the zero position (assuming normal anatomical position). 98

Figure 38 Illustrations depict examples of mild, moderate, and severe contractures of the carpus measured in degrees from the zero position (assuming normal anatomical position). 99

LIST OF TABLES

Page

Table 1 A summary of oligonucleotide primers and standard conditions used for exon resequencing of *TPM2* in normal and affected foals. 34

Table 2 Phenotype classifications of affected foals used in case/control GWAS. 55

Table 3 Power to detect a completely additive DSL using markers with various r^2 and....... 59

Table 4 Affected foals, their respective phenotypes, and the genomic loci each foal is associated with as indicated by haplotype analysis. 80

Table 5 A summary of oligonucleotide primers and standard conditions used for exon and exon boundary resequencing of *ANKRD1* in normal and affected foals. 83

CHAPTER I

GENERAL INTRODUCTION TO CONGENITAL JOINT CONTRACTURES AND CONTRACTED FOAL SYNDROME IN NEONATAL THOROUGHBRED FOALS

Disease Description

Flexural limb deformities (FLD) are reported to be one of the most common congenital musculoskeletal disorders in Thoroughbred foals and one of the leading causes of foal mortality (Crowe & Swerczek 1985; Giles *et al.* 1993; Binanti *et al.* 2013) . The term "flexural limb deformity" can refer to joint hypermobility, a consequence of ligament and/or tendon laxity, or joint fixation in a hyperflexed position caused by a shortening of the muscle-tendon unit. Both conditions result in the inability to either flex or extend the distal and/or proximal joints normally (Adams 2000; Kidd & Barr 2002). The two types of FLDs may present concomitantly and are generally non-progressive. The degree of hyperflexion or hyperextension can vary from mild to severe. The most commonly affected joints are the distal joints of the forelimbs and hindlimbs, which include the metacarpophalangeals (MCP, also referred to as the fetlock), proximal interphalangeals (PIP), the distal interphalangeals (DIP), the carpi (carpus, *singular*) in the forelimb and the tarsi (tarsus, *singular)* in the hindlimb. The least affected areas are the most proximal joints including the elbow and stifle joints (Figure 1).

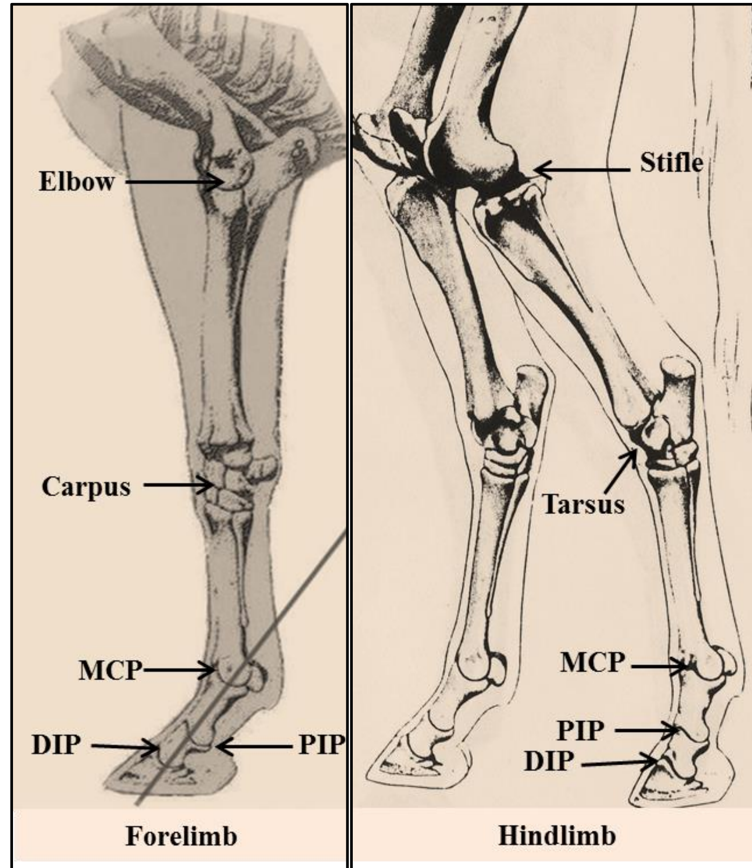


Figure 1 Normal joint angles of the forelimb and hindlimb in the horse. Distal joints of the forelimb and hindlimb include (1) metacarpophalangeal joint (MCP), (2) proximal interphalangeal (PIP), and (3) distal interphalangeal joint (DIP). The intermediate joints are the carpus (forelimb) and tarsus (hindlimb). The proximal joints of the forelimb and hindlimb are the elbow (forelimb) and the stifle (hindlimb). Figure courtesy of J. Eisenrich.

Contracted foal syndrome (CFS) is a condition in which joint flexion contractures are the primary clinical feature but a constellation of other defects may be present (Knottenbelt *et al.* 2004). According to the 242 case records we reviewed, other musculoskeletal abnormalities may include angular limb deformities (joints deviate medially or laterally in the frontal plane) and ligament or tendon laxity of the distal limbs causing hypermobility of

the joints. Entropions (inversion of the lower eyelid) and patent urachi are not uncommon and are frequently found to present in concurrence. The urachus is a tubular structure that is found within the umbilical cord and functions to drain the bladder into the allantois during fetal development and generally closes by birth. When this structure fails to obliterate at the time of birth, urine continues to drain outside the umbilicus but is often corrected surgically (Peyton 1981). Albeit more rare, deviations of the axial skeleton such as vertebral defects, wry nose (curvature of the muzzle), prognathism (extension of the lower jaw), and torticollis (asymmetrical positioning of the neck and head) can be associated (Binanti *et al.* 2013). Abdominal wall defects and bowel atresia have also been reported. Figure 2 illustrates the variable expression of flexion contractures and CFS with comparison images of normal joint angles of the forelimb and hindlimb and Figure 3 shows a stillborn foal with a severe case of CFS.

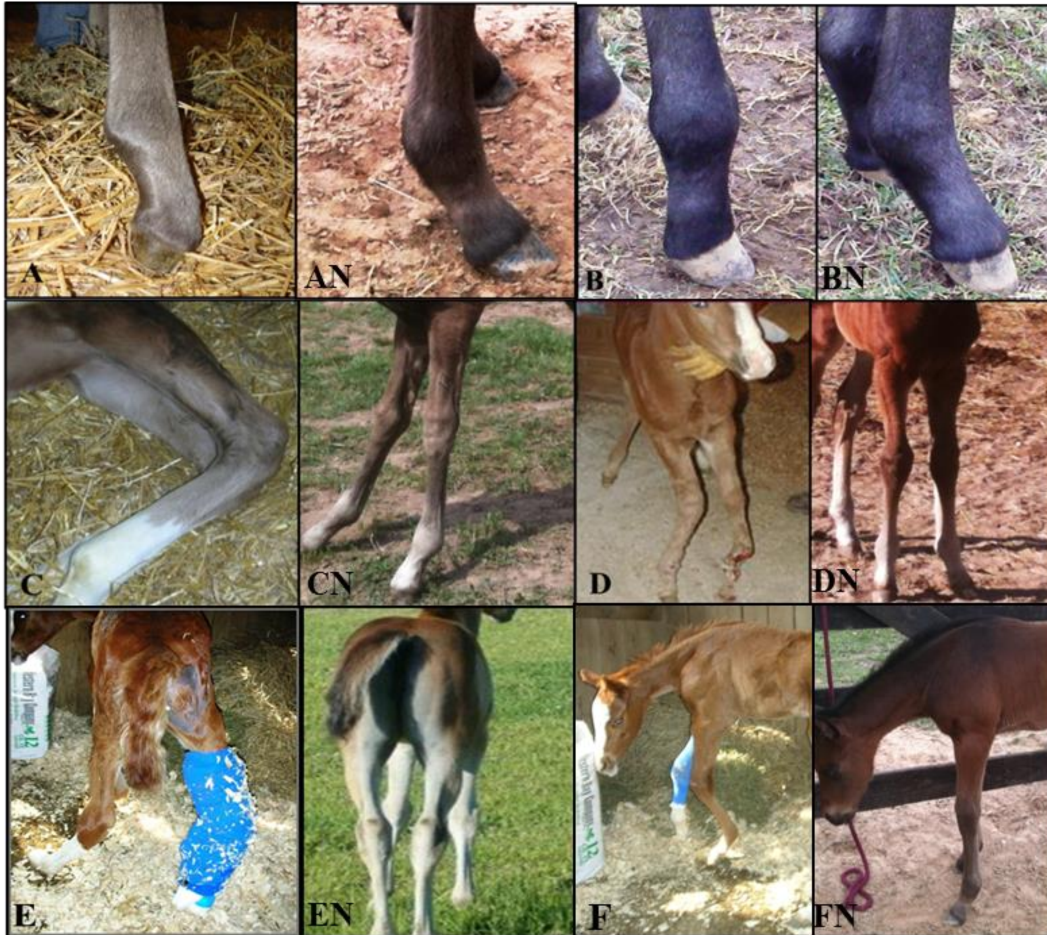


Figure 2 Variable expression of flexion contractures and CFS with comparisons to normal conformations of the respective joints in newborn foals (N=normal). (A) A unilateral distal contracture of the DIP joint and (AN) a normal DIP joint angle. (B) A unilateral flexion contracture affecting the MCP joint only and a (BN) normal angle of the MCP joint. (C) Severe bilateral carpal contractures (~90°) without involvement of the MCP, PIP, or DIP joints. The foal is unable to extend the carpi and is non-ambulatory and (CN) normal conformation of the carpi at time of birth. (D) A foal with bilateral MCP (fetlock) contractures and an angular limb deformity of the right carpus and tarsus. (DN) Normal conformation of the MCP and carpi joints in both the frontal and transverse planes (E) A foal presenting with angular deformities of the tarsi and ligament/tendon laxity in the lower hindlimbs and (EN) normal conformation of the hindlimbs in a newborn foal. (F) This foal presented with bilateral carpal contractures, a contracture of the front right MCP, PIP, and DIP, and laxity affecting the front left MCP, PIP, and DIP. (FN) Normal conformation of the front limbs in a newborn foal. Photographs by J. Caldwell

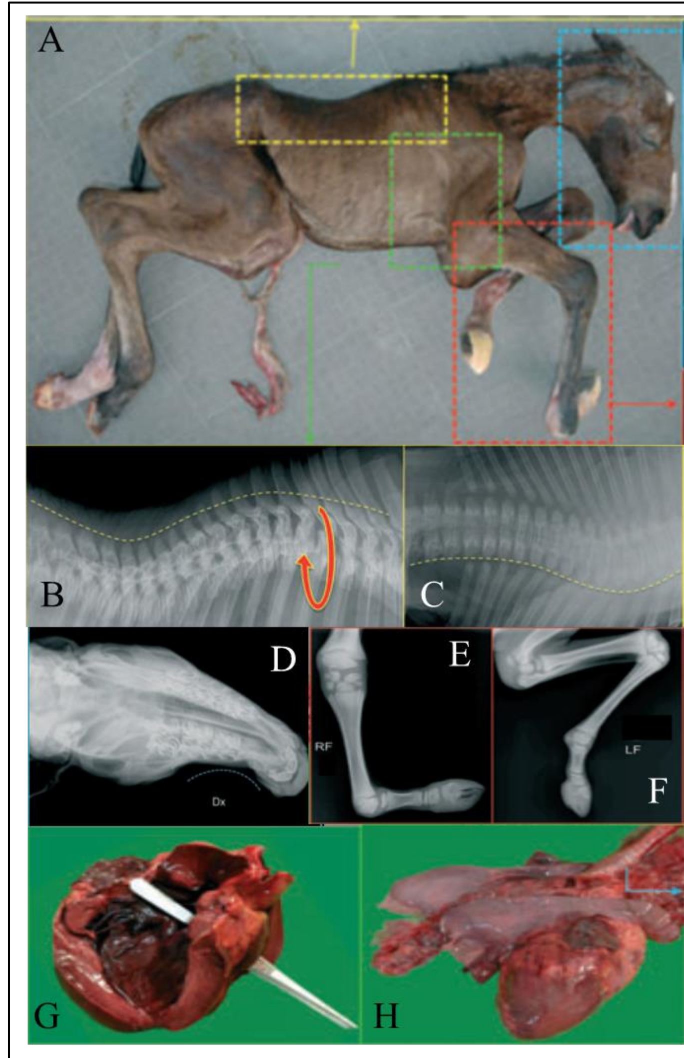


Figure 3 (A) A stillborn, term foal with a severe case of CFS. Axial defects included scoliosis (B and C) and wry nose (D). Angular deviations and flexion contractures were present in the limbs (E and F). A large interventricular septal defect (G) and pulmonary atelectasis and lung hypoplasia (H) were noted from necropsy. (Modified from Binanti *et al*. 2014; permission for reuse obtained from publisher).

Successful treatment depends on the severity and complexity of the condition. In moderate to severe cases, foals are often non-ambulatory and this presents many additional risks. Immobility can lead to secondary complications such as weakness, delayed development, compromised immune systems, and subsequent infections, which greatly increase the chances of foal death (personal communications with Nathan Slovis, DVM, Dipl. ACVIM, Hagyard Equine Medical Institute, Lexington, KY). Treatment methods for addressing limb deformities can be painful and may result in pressure sores further complicating rehabilitative efforts. In mild to moderate cases, treatment is often successful with surgical intervention, physical therapy, splinting, casting and/or drug therapy (Adams 2000; Greet 2000). However, in severe cases of joint contractures, aggressive surgical intervention is not recommended due to a generally poor outcome (Hunt 1997).

Nomenclature

There are few peer-reviewed publications reporting substantiated causes of joint contractures or CFS in equines. Most are outdated and generally refer to the condition as "arthrogryposis" or "arthrogryposis multiplex congenita" (Nes *et al.* 1982; Mayhew 1984). *Arthrogryposis* is of Greek origin meaning "curved or hooked joints" and is the term most often used to describe congenital joint contractures in other species including human neonates, lambs, calves, goats, and piglets (discussed in detail elsewhere). At present, this terminology is typically not applied to congenital joint contractures in foals. Generally, in practice, they are referred to as simply "contracted tendons" or if other abnormalities are present, then the diagnosis may be *contracted foal syndrome* (Knottenbelt *et. al* 2004).

However, there are some instances where CFS is used as a diagnosis for foals born with multiple contractures and no other anomalies. This may not be technically correct considering the definition of syndrome is *the association of several recognizable clinical features or characteristics that often occur together* (Merriam Webster; http://www.merriam-webster.com/dictionary/syndrome). In some opinions, "contracted tendons" is now considered to be somewhat of a misnomer due to the mounting evidence that points to muscle pathophysiology as the underlying cause in other species (Owen 1975). Moreover, some authors distinguish arthrogryposis in horses from "contracted foals" by the presence of cleft palate, brachygnathia, and torticollis (McKinnon 2011). These are clinical features often observed in other species with arthrogryposis. This suggests they may be distinctly different disorders. To date, few studies are reported that support the distinctions between contracted foal syndrome and *arthrogryposis* in horses. Both terms can refer to conditions in which joint contractures are the primary feature, and both indicate that other congenital abnormalities may be present. Therefore, it is clear that in equine medicine, the condition has not been well defined, nor does it have an established nomenclature. For our purposes, we considered all reports describing congenital joint contractures in foals regardless of terminology. This lack of definition and characterization prompted us to adapt a classification system for contracture disorders in human neonates for use in equines to help reduce ambiguity (Chapter 4).

Disease Frequency

The true frequency of joint contractures and CFS in the Thoroughbred population is not known, but the conditions are common enough to be a concern to both clinicians and breeders. Breeding farms in the U.S. have reported 10% to16% of their foal crops as having some degree of contracture over consecutive years (personal communications, anonymous). In 1985, 608 cases of congenital anomalies in Equidae were evaluated, and 202 (33%) were classified as CFS (Crowe & Swerczek 1985). The Livestock Disease Diagnostic Center in Lexington, KY reported that contracted foal syndrome was the most common congenital defect seen in fetuses and newborn foals submitted for necropsy between 1986 and 1991 (Giles *et al.* 1993). Contracted foal syndrome accounted for 188 out of 396 evaluated cases for that 6-year period. Breeds included Morgans, Saddlebreds, and Standardbreds. Thoroughbreds, however, constituted 94% of those cases. To assess the causes of reproductive loss in mares in the 1988 and 1989 foaling seasons, 1211 accessions were evaluated, and CFS represented 8.5% of all reproductive losses, ranking third after placentitis and dystocia-perinatal asphyxia. It was also reported that in 2004-2005, among 259 neonatal cases evaluated, 20% of deaths were caused by musculoskeletal defects (second to septicemia), including flexural deformities and fractures occurring during dystocia births (Sturgill 2008). Contractures and CFS occur on breeding farms globally. In response to increasing incidence of developmental orthopedic diseases in Thoroughbreds, the Rural Industries Research and Development Corporation in Hunter Valley, Australia conducted a workshop in 1997 to define the extent of the problem. They concluded that flexor tendon contractures were a major concern in neonates and were observed in 3-5% of foals. They

reported that flexural deformities, together with angular limb deformities and osteochondritis dissecans, cost breeders and owners more than $9.8 million dollars per year (Aldred 1998).

Pathoetiology

The pathogenesis of joint contractures and CFS is unknown but factors that are speculated to be causal include the following: (1) nutritional deficits or excesses in the mare, (2) malpositioning *in utero*, (3) infections, (4) ingestion of toxic plants or other teratogens, and (5) genetics (Munroe 1996; Hunt 1997; Kidd & Barr 2002). One study on dystocia births in horses suggested limb deformities, in combination with scoliosis and torticollis, were the result of a space constraint on the cranial half of the fetus due to the narrow tip of the uterine horn (Vandeplassche *et al.* 1984). In a report on "arthrogryposis" in Norwegian Fjord horses, pedigree analysis and a familial pattern of inheritance indicates a genetic contribution (Nes *et al.* 1984). In a case of "arthrogryposis multiplex congenita" in a Thoroughbred foal, histological evidence points to a neurological origin (Mayhew 1984). The most recent publication by Binanti *et al.* (2013) describes two cases of CFS with multiple severe congenital malformations. They state the cause is unknown but discuss the possibility of teratogenic mechanisms. During the second month of gestation, the mares were exposed to an insecticide used to treat a larval infestation of trees on the breeding farm. However, the agent used was a chitin synthesis inhibitor, which reportedly has low toxicity and is not known to be teratogenic in mammals. It may be noteworthy to add that assisted reproductive techniques were used with both pregnancies. One mare was artificially inseminated and the other mare was subjected to *in vitro* fertilization. In these reported cases, the causes are speculative and do not provide significant insight regarding the epidemiology

of flexion contractures in the Thoroughbred breed. To our knowledge, there have been no published results of molecular studies conducted on flexion contractures or CFS in any horse breed. Therefore, to gain better understanding of the pathogenesis of the condition, we undertook a molecular genetics study on flexion contractures and CFS in Thoroughbreds.

Why are Flexion Contractures and CFS Important to Study?

In the Broad Sense

Selection for particular traits in animals can result in a degree of inbreeding that reduces genetic diversity and increases the risk of inheriting genetic diseases. One of the best examples to exemplify this is the hyperkalemic periodic paralysis (HYPP) mutation that was traced back to the very popular Quarter Horse stallion, Impressive (Rudolph *et al.* 1992) (www.vgl.ucdavis.edu/services/**hypp**.php). This sire was known for producing offspring with heavy musculature. Increased muscle mass is a highly desirable characteristic in the Quarter Horse breed for sports such as sprint racing and other disciplines requiring powerful stops and quick turns. Due to his popularity as a sire that passed on this characteristic, the mutation became widespread. Additionally, several other Mendelian or major gene disorders affecting muscle metabolism and function (Finno *et Fal.* 2009) have gained much attention in the last decade as more research has brought the genetic causes to light. In Thoroughbreds, muscle mass and extreme efficiency in muscle metabolism are also very important attributes and have been artificially selected for because these individuals are expected to perform in sprint races (5-6 furlongs) and long distance races (up to1 ½ miles) at speeds up to 40 MPH (http://www.jockeyclub.com/pdfs/thoroughly_thoroughbred.pdf). The nature of inbreeding

practices in the Thoroughbred industry is not likely to change soon, and understanding the molecular and/or hereditary bases of current musculoskeletal (MS) disorders will better equip investigators to address novel and possibly more complex conditions that may arise in the future. More specifically, understanding the molecular bases of MS disorders is essential for dissecting the mechanisms underlying the pathophysiology leading to the disease. In terms of clinical relevancy, this can ultimately facilitate diagnosis and the discovery of therapeutic strategies.

In the Narrow Sense

The high mortality rate associated with moderate to severe cases of flexion contractures and CFS, coupled with the often painful process of rehabilitation in less severe cases warrant investigation into the etiology of the condition. Moreover, surviving foals can suffer from chronic conditions such as arthritis that may diminish the quality of life or impede future athletic potential (personal communications; Nathan Slovis, DVM, Dipl. ACVIM, Hagyard Equine Medical Institute, Lexington, KY). If treatment is an option, hospitalization may be required and the costs of treatment may exceed the value of the foal, and thus lead to an economic loss for the owners. With regards to the considerable disparity in the definitions of the conditions, it is essential we gain a better understanding of the etiologies before a delineation of the disorders can be achieved.

Rationale for a Genetic Study

Evidence Supporting a Genetic Contribution to Joint Contractures and Contracted Foal Syndrome in Thoroughbred Foals

Multiple etiological mechanisms have been proposed to cause congenital contractures in foals; however, we found that the most compelling evidence was in support of a genetic basis. Hagyard Davidson and McGee Equine Medical Institute in Lexington Kentucky provided us access to medical records from the years 2004-2009. Peterson and Smith Equine Hospital (Ocala, FL) and select Thoroughbred breeding farms also shared case information with us for the collection of preliminary data. We were able to document 242 cases of joint contractures and contracted foal syndrome. Interestingly, records revealed multiple examples in which there was a family history of contractures with siblings or half-siblings presenting with the same or similar phenotype. Two examples are illustrated in Figure 4.

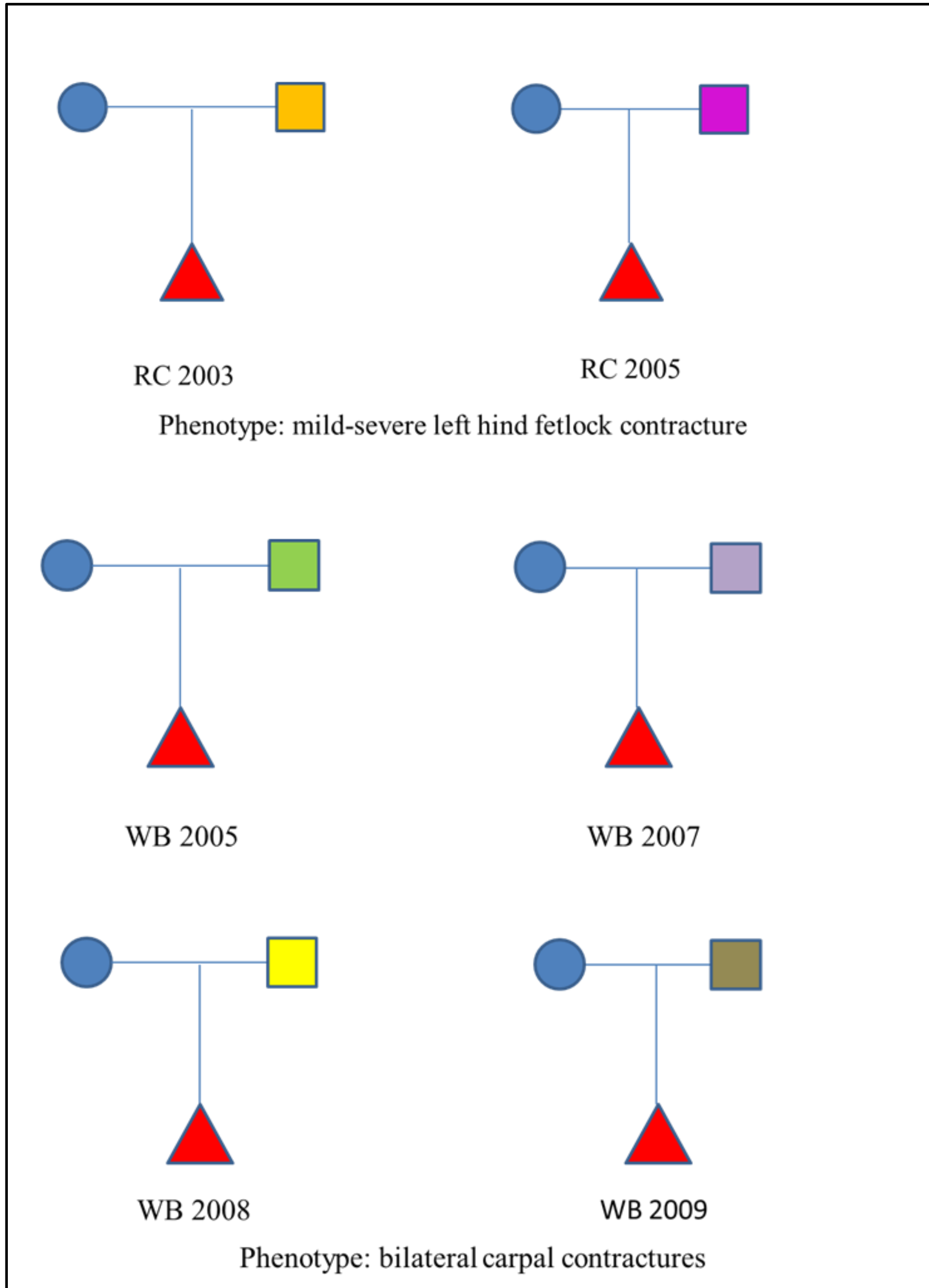


Figure 4 Two examples of mares (blue circles) producing more than one contracted foal with the same phenotype. The mare "RC" bore contracted half-sibling foals with left hind fetlock contractures in the years 2004 and 2006. The mare "WB" bore 4 half-sibling foals with bilateral carpal contractures in the years 2005, 2007, 2008, and 2009.

From personal communications with seasoned veterinarians in the Lexington, KY area, one particular sire line has been identified as having a higher incidence of contracted foals each year compared to other Thoroughbred lineages. From among our case histories, 44 foals were direct descendants of this sire. Moreover, one breeding farm standing several stallions from this line has reported 10-16% of foals having some degree of contracture in a given year (personal communications; Managing Director of a large Thoroughbred breeding farm). This lineage boasts winners of some of the most prestigious races in North America and exemplifies the kind of stock chosen for line breeding for performance. We were able to obtain DNA samples from 61 members of a kindred founded by this sire for the genome-wide association study described in Chapter 3.

Perhaps the most persuasive evidence for a genetic etiology comes from studies in other species with similar conditions with a proposed or known genetic basis. Congenital limb contractures have been documented in calves, lambs, and piglets but have been extensively studied in human neonates.

Congenital Joint Contractures and Arthrogryposis Dsorders in Humans (Homo sapiens)

Congenital joint contractures in humans are reported to occur in 1 in 3000 to 5000 births and limb contractures are a clinical finding in over 400 conditions (Hall 2014). There are sporadic forms as well as heritable forms exhibiting dominant, recessive, X-linked, and mitochondrial modes of transmission (Bamshad *et al.* 2009). A significant effort was initiated in the 1980s to understand their etiologies and the revision and extension of previous findings continues today as molecular studies reveal more information. In fact, to date, at

least 150 genes are associated with the various disorders involving congenital contractures (Hall 2014). Nonetheless, the consensus among key investigators is that there are multiple factors that can lead to congenital joint contractures (Hall & Reed 1982; Hall *et al.* 1982; Hall *et al.* 1983; Hall 1997; Bamshad *et al.* 2009).

The sporadic form is referred to as amyoplasia and has characteristic muscle pathology; generally, the muscle fibers are replaced by fibrous and fatty tissue (Hall *et al.* 1983). In the same study, the condition was characterized by flexion contractures of the most distal part of the limbs (hands/wrists and ankles) and distinct facial features. Considering morphological changes can be observed in some types of myopathies, a histological evaluation of tissue from contracted foals would be helpful in making a diagnosis or provide insight into the etiology. Additionally, one study on amyoplasia concluded that 2.7% of patients presented with abdominal abnormalities including bowel atresia (Reid *et al.* 1986). In some cases, bowel atresia and abdominal hernias have been reported in foals with limb contractures.

Arthrogryposis multiplex congenita (AMC) is a descriptive term used to describe the presentation of multiple congenital contractures potentially occurring in conjunction with other abnormalities (Hall 1997; O'Flaherty 2001; Fassier *et al.* 2009). In general, factors that are thought to cause AMC include (1) neuropathies, (2) muscle pathology, (3) uterine abnormalities, (4) intrauterine vascular compromise, (5) maternal diseases, (6) mutations or chromosomal aberrations, (7) connective tissue disorders, (8) a distal arthrogryposis, or (9) fetal crowding (Hall 1997; Bamshad *et al.* 2009). Many of these mechanisms are similar to the ones proposed to cause flexion contractures and CFS in foals.

Figure 5 shows examples of three forms of limb contractures in human neonates; amyoplasia, distal arthrogryposis, and AMC. The similarities among phenotypes only further emphasize the need for careful diagnostic evaluation using very precise clinical criteria.

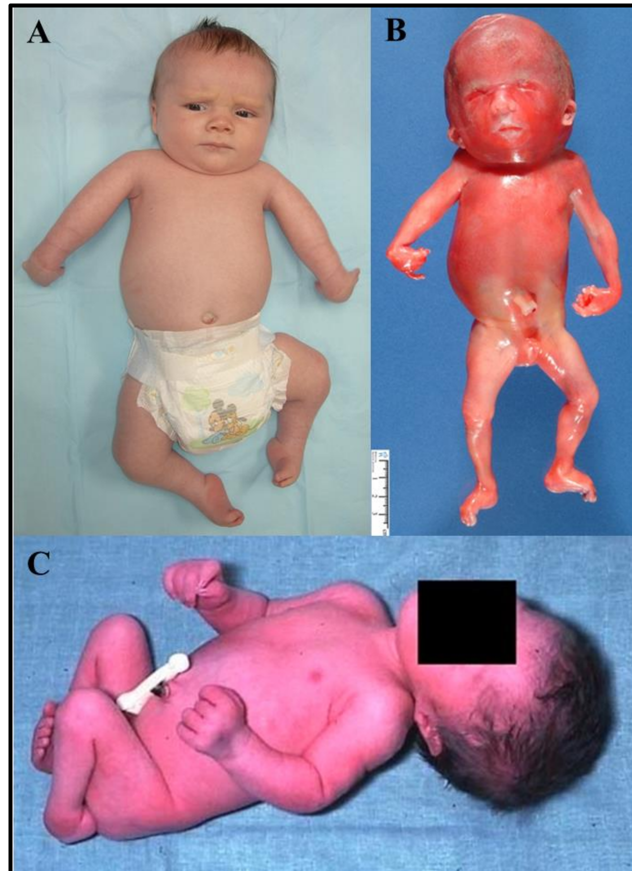


Figure 5 (A) Newborn diagnosed with amyoplasia presenting with bilateral hip and knee flexion contractures and bilateral clubfeet (permission for reuse obtained from publisher; Bamshad *et al.* 2009). (B) A 20 week old fetus with distal arthrogryposis presenting with contractures of the wrists, fingers, and toes. (C) A child diagnosed with AMC presenting with bilateral hip and knee contractures and bilateral clubfeet (permission for reuse obtained from publisher; Fassier *et al*. 2009).

In an effort to delineate these disorders in humans, some forms of multiple limb contractures were given classification groups based on strict diagnostic criteria. For example, the genetic distal arthrogryposis (DA) disorders are widely discussed in the literature (Bamshad *et al.* 1996; Hall 1997; Vanpaemel *et al.* 1997; Bamshad *et al.* 2009; Kimber *et al.* 2012) and serve as our primary disease model.

The distal arthrogryposes (DA) are a heterogeneous group of genetic disorders that have been classified into 10 categories based on clinical findings with joint contractures being the common feature (Bamshad *et al.* 1996). A number of these phenotypes such as DA1, DA2B, DA4, and DA5 are also seen in neonatal foals. Distal arthrogryposis type I (DA1) and distal arthrogryposis type IIB (DA2B) are the two most common classes and have significant overlap. DA1 in humans primarily involves contractures of the most distal limbs (i.e., hands/wrists and feet/ankles) and angular limb deviations with varying degrees of severity (Bamshad *et al.* 2009). DA2B is much the same but may be distinguished by facial contractures (Hall *et al.* 1982; Bamshad *et al.* 1996; Bamshad *et al.* 2009; Beck *et al.* 2013b). However, it should be noted that some features observed in humans are not seen in foals, presumably due to anatomical differences. DA4 features distal limb contractures and scoliosis, and DA5 describes distal contractures and defects of the extraocular muscles (Bamshad *et al.* 2009). These are all clinical features described in medical records of CFS provided to us by Hagyard Equine Medical Institute, Lexington, KY.

Currently, genetic mutations in at least 7 genes encoding key components of the contractile apparatus of skeletal muscle have been implicated in many of the human arthrogryposis subtypes indicating genetic heterogeneity (Sung *et al.* 2003; Tajsharghi *et al.*

2008). These include myosin binding protein C1 (*MYBPC1*) (Gurnett *et al.* 2010), beta-tropomyosin 2 *(TPM2*) (Sung *et al.* 2003; Tajsharghi *et al.* 2008), the embryonic myosin heavy chain 3 gene *(MYH3)* (Toydemir *et al.* 2006; Tajsharghi *et al.* 2008), the troponin I fast-twitch isoform *(TNNI2)* (Drera *et al.* 2006; Kimber *et al.* 2006), troponin T type 3 skeletal, fast *(TNNT3)* (Sung *et al.* 2003), and the perinatal isoform of myosin heavy chain 8 *(MYH8*) (Veugelers *et al.* 2004). Recently, mutations in the human endothelin-converting enzyme-like 1 (*ECEL1*) gene have been implicated in distal arthrogryposis type 5D (McMillin *et al.* 2013).

Genes of the troponin/tropomyosin complex such as *TPM2*, *TNNI2*, and *TNNT3* are potentially good candidate genes when investigating disorders of the muscle due their critical role in calcium mediated muscle contraction (Sung *et al*. 2003). These sarcomeric proteins have been implicated in several myopathies (Sung *et al.* 2003). Hence, these genes should be considered as candidates for targeted re-sequencing when there is a family history of congenital contractures in foals with phenotypes similar to human DA1 and DA2B.

It is likely that there are some cases of flexion contractures that are caused by non-genetic mechanisms, and when evaluating cases, it would be helpful if clinicians would first exclude a neurological basis as recommended by Bamshad and colleagues (2009) in humans, inquire about the clinical histories of the mares, and conduct a histological examination of the skeletal muscle tissue when possible. These diagnostic differentials can facilitate determining prognoses and treatment as well as assist with accurate selection of cases for research purposes. Spatial constraints in the uterus in some cases may contribute to the occurrence of flexion contractures as suggested by Vandeplassche *et al.* (1984) but the

mechanisms governing feto-maternal proportions are not well understood and to our knowledge, there have been no established parameters for mechanistically assessing this relationship. In a review of 59 medical records in which a description of size was given, 49% of foals were considered average size and 19% were considered small. Only 32% of contracted foals were described as large, which may indicate feto-maternal proportions are a factor but only in select cases. However, breeding farm management practices and routine veterinary care are generally consistent among mares at any one farm, yet only a small fraction of those mares produce contracted foals in a given year, suggesting that environmental exposures are not a significant factor.

In addition to the observations of a familial predisposition in some cases, another factor that lends favor to a genetic basis are the numerous reports of genetic forms of congenital joint contractures (arthrogryposis) in other mammalian species such as lambs, calves, and piglets.

Arthrogryposis in Lambs (Ovis aries)

There is accumulating evidence that some forms of arthrogryposis in lambs are genetic disorders with a recessive mode of inheritance. For example, Doherty *et al.* (2000) reported arthrogryposis in 58% (30/52) of a Suffolk lamb crop. In this report, primary clinical features were flexion contractures affecting the metacarpophalangeal and carpal joints. A newly introduced ram was found to be the only significant risk factor. Data analysis indicated that it was likely due to a recessively inherited “arthrogryposis gene”. Tejedor *et al.* (2010) describes a novel, heritable ovine arthrogryposis. In this study, lambs presented

with defects of the axial skeleton, distal arthrogryposis of the limbs, and cleft palate. A genetic study using DNA microsatellite markers coupled with a breeding management study suggests a genetic basis with recessive inheritance, thus ruling out environmental etiology. However, with this disorder, lambs die shortly after birth due to respiratory compromise and this is not typically a characteristic of CFS. Figure 6 shows a newborn lamb, post-humous, with arthrogryposis multiplex congenita. These examples clearly demonstrate that genetic heterogeneity in the lamb population is a possibility which may result in similar phenotypes with some distinctions.

Figure 6 Heritable arthrogryposis multiplex congenita in a newborn lamb presenting with marked contractures of the carpi and tarsi and mild contractures of the distal joints (Permission for reuse obtained from publisher; Tejedor *et al.* 2010).

Arthrogryposis in Murrah Buffalo Calves (Bubalus bubalis)

Schild *et al.* (2003) describe congenital joint contractures in the Murrah buffalo without associated defects, except for one case displaying brachygnathia (Figure 7). Histological evidence in this report showed a reduction of the motor neurons of the ventral horns of the spinal cord and hypoplasia of the limb muscles potentially indicating a neuromuscular etiology. A genetic basis with recessive inheritance was suggested according to their pedigree analysis.

Figure 7 A neonatal buffalo calf with multiple joint contractures affecting the proximal and distal limbs. (Schild *et al.* 2003)

Arthrogryposis in Calves (Bos taurus)

Arthrogryposis multiplex in calves is a lethal disorder featuring flexion contractures of the front limbs and flexion or extension of the hind limbs (Figure 8; Van Eenennaam 2009). Vertebral defects and/or cleft palate may be associated. This is a recessive Mendelian disorder that results from a deletion of a small segment of DNA, which includes a gene whose encoded protein is necessary for proper development (Van Eenennaam 2009). The gene name has not yet been disclosed. In addition, there are documented cases of contractures in calves that are caused by non-genetic mechanisms. One example is crooked calf disease, which is characterized by congenital arthrogryposis, ALDs, under- or over-extension of the interphalangeal joints, occasional cleft palate, and scoliosis. It is believed to be caused by teratogenic plants, specifically those from the *Lupinus* genus ingested between the 40th and 80th day of pregnancy (Abbott *et al.* 1986). Certain viral species including the Akabane (i.e., Aiono) virus are also known to cause congenital malformations including flexural deformities (Panter *et al.* 1998; Tsuda *et al.* 2004). However, no molecular investigations were conducted to rule out a genetic basis.

Figure 8 A neonatal calf presented with marked flexion contractures of the proximal and distal joints in the front limbs and of the hocks in the hind limbs. (http://animalscience.ucdavis.edu/animalbiotech/Outreach/Curly_Calf_test.pdf) (Van Eenennaam 2009).

Arthrogryposis Multiplex Congenita in Pigs (Sus scrofa)

It is also proposed that AMC in pigs has various etiologies but the most important ones are considered to be genetic factors, nutritional deficiencies, teratogens, viral agents, or restricted fetal limb movement (Genini *et al.* 2004). However, like in the other species previously described, there is evidence for a major genetic contribution when a familial pattern of inheritance is observed. For example, Genini and colleagues (2004) mapped a recessively transmitted disease susceptibility locus (DSL) to chromosome 5 in a kindred of Swiss Large White pigs using linkage analysis. Here, AMC is described as a lethal condition with characteristic limb contractures but also with brachygnathia (shortened lower jaw) and

vertebral column defects. Figure 9 shows a stillborn piglet with AMC compared to a newborn piglet without AMC. The proximal and distal joints of all four limbs are affected and brachygnathia and scoliosis is also present. More recently, fine-mapping using single nucleotide polymorphisms (SNP) and microsatellite markers has refined a region to a 5 mega-base (Mb) interval on pig chromosome 5 associated with porcine AMC in this breed, but a candidate gene has not yet been identified. However, two markers in complete linkage disequilibrium with the disease susceptibility loci (DSL) have been used for diagnostic testing (Haubitz *et al.* 2012). Like in some cases of arthrogryposis in lambs, it can cause death shortly after birth and therefore may differ in etiology from forms associated with foals.

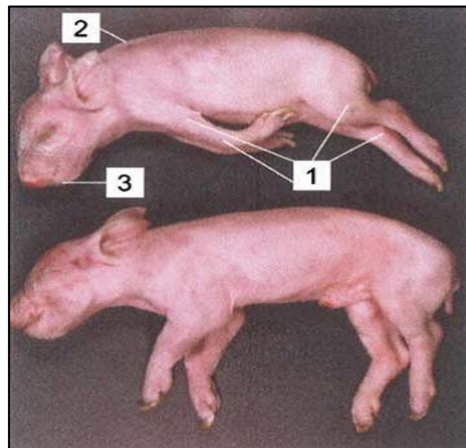


Figure 9 Arthrogyposis multiplex congenita in Swiss Large White pigs. In the top portion of the picture is a typical presentation for piglets with AMC. Joint contractures can be seen in all four limbs (1), scoliosis (2), and brachygnathia (3). The bottom portion of the picture shows a newborn piglet without AMC and normal conformation. (Permission for reuse obtained from publisher; Haubitz *et al*. 2012)

Arthrogryposis in Model Organisms: Zebrafish (Danio rerio) and Mouse (Mus musculus)

Other species such as the zebrafish and mouse have been used experimentally to gain a better understanding of congenital contractures. The effect of 2 mutations in the *MYBPC1* gene, reported to cause a subtype of distal arthrogryposis in human neonates, was investigated using the zebrafish. Injection of mRNA containing the corresponding human W236R and Y856H *MYBPC1* mutations into zebrafish embryos resulted in a mild bent body phenotype with decreased motor activity (Ha *et al.* 2013). This implies that perturbations in sarcomere proteins can result in impaired muscle function and supports the consensus from human studies that reduced fetal movements lead to contractures.

The peroneal muscular atrophy (PMA) strain of mouse presents with equinovarus (clubfoot) of the hind legs. It reportedly occurs due to failure of proper innervation of the common peroneal nerve resulting in atrophy of the crural muscles and is a recessively inherited condition (Nonaka *et al.* 1986). The *PMA* gene was mapped to a region on chromosome 5 in the mouse but no causative mutations have been discovered (Katoh *et al.* 2003). This mouse strain is proposed to be a good model for investigating arthrogryposis multiplex congenita and club foot deformities. Mouse studies supporting other plausible factors for congenital contractures include the role of maternal antibodies acting against fetal muscle or nerve antigens. For example, Polizzi *et al.* (2000) discusses the occurrence of arthrogryposis in fetuses born to mothers with myasthenia gravis (MG), an autoimmune disorder. In this report, human maternal serum containing MG antibodies against the nicotinic acetylcholine receptor was injected into pregnant mice. The transfer of these

antibodies to the developing pups resulted in clinical features characteristic of AMC including multiple contractures, craniofacial deformities, and scoliosis/kyphosis.

In summary, these reports infer that multiple factors may lead to flexural deformities and other developmental anomalies, and some forms are shown to be heritable. Many of the clinical features observed among the various arthrogryposis syndromes across species are present in foals with CFS, and although we hypothesize that there are heritable forms of flexion contractures, sporadic occurrences, whether of genetic or environmental origin, should not be ruled out when investigating cases. Developmental mechanisms are sensitive to exogenous agents and such factors could account for some incidences of the condition.

In conclusion, with the consanguineous nature of current animal breeding practices comes the inherent risk of inadvertently selecting for traits that negatively impact the overall health of the breed. Finno and colleagues (2009) observed this in the Quarter Horse with genetic diseases such as hyperkalemic periodic paralysis and glycogen branching enzyme disorder, lavender foal syndrome in Arabians, overo lethal white syndrome in paint horses, and polysaccharide storage myopathy that affects multiple breeds. Recently, using genome-wide SNP data, it has been noted that the Thoroughbred breed, when compared to other breeds, has low genetic diversity (Petersen *et al.* 2013) which may increase the frequency of rare deleterious alleles. If a genetic determinant can be identified, the utilization of this knowledge to promote better breeding practices could possibly reduce the occurrence of congenital joint contractures and thus reduce suffering, injury, and the number of foals euthanized. The mechanism by which this could be accomplished is to provide a genetic

screen that would not only prevent and diagnose the conditions but also negate potentially fatal outcomes.

These factors, in addition to the known genetic causes for flexural limb deformities in other species, prompted us to perform the first molecular-based study on congenital joint contractures in Thoroughbred foals. Although the clinical complexity can vary, we hypothesize that joint contractures and contracted foal syndrome, in some cases, are major gene disorders with the likelihood of locus heterogeneity in the population (more than one major gene causing the same phenotype in different families).

In the following chapters, we report the results of the first genetic study on congenital joint contractures in Thoroughbred foals. A candidate gene approach was initially taken followed by a case/control genome-wide association study (GWAS), a family-based validation study, and a family-based GWAS. We conclude with an initiative to provide the first system for classifying and characterizing equine congenital joint contractures and CFS. The primary purpose of this system is to establish a consistent method for clinically documenting instances of disease that will ultimately facilitate future studies.

CHAPTER II

SEQUENCING AND ANALYSIS OF CANDIDATE GENE TROPOMYOSIN BETA 2 (TPM2): A SEARCH FOR VARIANTS CAUSING FLEXION CONTRACTURES IN THOROUGHBRED FOALS

Introduction

Congenital flexural limb contractures are a relatively common musculoskeletal condition in Thoroughbred foals. Contracted foal syndrome (CFS) is a general term describing congenital joint contractures but with additional physical anomalies such as wry nose, scoliosis, and torticollis (Binanti *et al.* 2013). To date, there have been only marginal attempts to accurately characterize and delineate flexural limb contractures and CFS cases that are congenital in nature, or understand the etiology and pathogeneses underlying them. The actual frequency of occurrence has not been determined; however, past reports and personal communications with clinicians and breeders indicate the frequency is substantial enough to be a concern, especially considering the high mortality rate associated with moderate to severe cases (Crowe & Swerczek 1985; Giles *et al.* 1993; Hong *et al.* 1993). Additionally, there is an increased risk to the mare from dystocia births caused by abnormal birth presentations (Binanti *et al.* 2013).

Although there are several factors that could result in flexion contractures in foals, we hypothesize that some cases are caused by a disease susceptibility locus (DSL) with large effect. However, it may be that there is more than one gene of major effect segregating in the Thoroughbred population causing congenital contractures. This is true for the congenital

distal arthrogryposis disorders (DA) in human neonates. The human DAs are characterized by variable and sometimes overlapping phenotypes with distal limb contractures being the clinical feature shared among all 10 classification groups (DA1-DA10) (Bamshad *et al.* 2009). A number of these phenotypes such as DA1, DA2B, DA4, and DA5 appear as similar conditions in neonatal foals. Mutations in at least 7 genes have been linked to DA subtypes, including myosin binding protein C1 *(MYBPC1*; Gurnett *et al.* 2010), tropomyosin 2 beta (*TPM2*; Sung *et al.* 2003; Tajsharghi *et al.* 2008), the embryonic myosin heavy chain 3 gene *(MYH3*; Toydemir *et al.* 2006; Tajsharghi *et al.* 2008), the troponin I fast-twitch isoform (*TNNI2;* Drera *et al.* 2006; Kimber *et al.* 2006), troponin T3 fast skeletal muscle (*TNNT3;* Sung *et al.* 2003), myosin heavy chain 8 *(MYH8*; Veugelers *et al.* 2004), and most recently, endothelin-converting enzyme-like 1 (*ECEL1*; McMillin *et al.* 2013). Some forms of human DAs are sporadic but many are heritable, and genetic (locus) heterogeneity has been well established (Bamshad *et al.* 2009). For example, genes *TPM2*, *TNNT3*, *TNNI2*, and *MYH3* all have mutations associated with both the DA1 and DA2B phenotypes (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov).

Candidate gene approaches enabled by comparative genomics have been used in the past to study conditions with similar phenotypes across species. We took this approach and selected *TPM2* as a putative candidate gene for CFS for two primary reasons. First, of the seven genes associated with DA disorders in humans, *TPM2* has been implicated in DA1 and DA2B, the two classifications that resemble the most common CFS phenotypes described in the 242 medical records we reviewed. Both forms in humans are characterized by distal limb contractures and angular limb deviations, but are distinguished primarily by the presence of

facial abnormalities (DA2B) (Bamshad *et al.* 2009). Medical records indicate that in foals, angular limb deviations are commonly seen with flexion contractures, and although the occurrence is less common, craniofacial malformations have been reported in cases of CFS (Binanti *et al.* 2013). Secondly, *TPM2* is a major component of the skeletal muscle contractile apparatus. It encodes a dimeric protein that runs along the major groove of the actin filament modulating the effect of Ca^{2+} binding with the troponin complex (Clayton *et al.* 1988; Schevzov *et al.* 2011; Clarke *et al.* 2012). Studies on mutations in contractile genes affecting intercostal flight muscles in *Drosophila* revealed that genes of the troponin-tropomyosin complex were often associated with myodysgenesis (Sung *et al.* 2003). This pre-empted investigations into genes such as *TPM2,* which ultimately led to the discovery of missense mutations linked to DA1 and DA2B I humans (Sung *et al.* 2003; Tajsharghi *et al.* 2008). Therefore, based on the role of *TPM2* in skeletal muscle physiology and its association with our model disorder, we set out to search for putative causal variants in equine *TPM2* by analyzing all 9 exons and their respective boundary regions in groups of affected (n=56) and unaffected foals (n=39). According to the Ensembl genomic database, (http://useast.ensembl.org/Equus_caballus/Info/Index), the equine *TPM2* gene has genomic coordinates of ECA25:917080-926112 and is a total of 9,032 bp.

TPM2 mutations have been implicated in some myopathies that in part, can be diagnosed by the histological evaluation of the myofibers and sarcomere ultrastructures. So in addition to sequencing, histological and transmission electron microscopy (TEM) exams were carried out on skeletal muscle tissue from 2 contracted foals with severe phenotypes to assess whether pathological features were present. The findings of this brief evaluation will

help determine whether *TPM2* is potentially involved in the clinical manifestation of CFS in horses, and will for the first time provide histo-pathological comparison between affected and unaffected foals with severe flexion contractures.

Methods and Materials

Fluorescence In Situ Hybridization Mapping of the Equine TPM2 Gene to ECA25 q12-13

BAC identification and DNA isolation

To confirm the location of *TPM2* on ECA25, a bacterial artificial chromosome containing *TPM2* was mapped to horse metaphase chromosomes by fluorescence *in situ* hybridization (FISH). BAC clone locations (118O23, 194H21, and 299J13) within the CHORI-241 equine BAC library (http://bacpac.chori.org/library.php?id=41) were identified by referencing the database http://www.tiho-hannover.de/index.php?id=1372 (Tosso Leeb and Ottmar Distl; now unavailable). *TPM2*-specific primers confirmed the presence of *TPM2* in the selected BACS. Growing BAC cultures and BAC DNA isolation was carried out as described in detail elsewhere (Gustafson *et al.* 2003). Briefly, BACs were cultured in 2YT media containing 0.5µg/ml of chloramphenicol and plated on LB agar plates which also contained 0.5µg/ml chloramphenicol. Single colonies were picked and inoculated into 250 ml of 2YT (with 0.5µg/ml chloramphenicol) and grown overnight at 37°C. BAC DNA was isolated using Plasmid Midi Kit (Qiagen) as per manufacturer's instructions. Gel electrophoresis was used to assess the quality of BAC DNA.

FISH was carried out by the protocol described in Raudsepp and Chowdhary (2008). Briefly, 1 µg of probe DNA was labeled with biotin or digoxigenin using nick translation and hybridized to metaphase chromosomes. Biotin-labeled probes were detected with avidin-FITC antibodies and digoxigenin labeled probes with anti-digoxigenin–rhodamine antibodies (Roche Molecular Biochemicals). A Zeiss Axioplan2 fluorescence microscope equipped with Isis v.5.2 software was used for imaging and analysis. At least 10 images per hybridization were captured and analyzed.

Sample Acquisition

Collection of tissue samples from affected foals

Under terminal anesthesia, skeletal muscle tissue samples were excised from the *flexor carpi radialis* (Figure 10) of 2 affected foals (Affected Foal A and Affected Foal B) presenting with severe contractures of the carpi. Tissue samples were immediately submerged in 1.5 ml RNA-later (Ambion) and stored at -80º C. It should be noted that these foals were not terminated for the purposes of this study. Due to the severity of contractures, and a poor prognosis, humane euthanasia was recommended by the attending veterinarian.

Collection of tissue samples from normal foals

Fresh tissue from 4 clinically normal neonatal Thoroughbred foals was obtained from the same muscle (*flexor carpi radialis*) under short-acting general anesthesia, immediately submerged in 1 ml RNA-later (Ambion) and stored at -80º C.

These tissue samples from normal and affected foals were used for histological evaluation of muscle structure and for scrutinizing sarcomere ultrastructure by transmission electron microscopy.

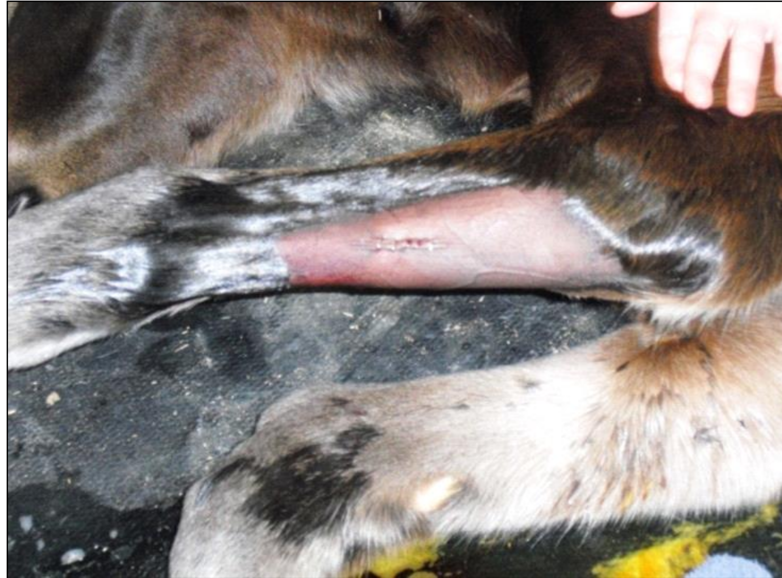


Figure 10 Excision site for muscle tissue biopsies from *flexor carpi radialis*.

Blood collection and DNA isolation from peripheral blood

Following standard procedures, approximately 20 ml of fresh blood was collected in EDTA Vacutainer® tubes from 56 newborn Thoroughbred foals diagnosed with congenital flexion contractures and 39 clinically normal foals. Clinical descriptions were provided by veterinarians or obtained from medical records of the foals (See Appendix A). DNA extraction from peripheral blood was carried out using a phenol/chloroform protocol (Sambrook 1998). The quality and quantity of DNA was assessed using gel electrophoresis

and NanoDrop spectrophotometry (Thermo Fisher Scientific). Extracted DNA was archived and stored at -80ºC.

Polymerase Chain Reaction (PCR) and Sequencing of TPM2 Exons

Forward and reverse primers were designed for 6 amplicons containing 9 exons and their respective 5' and 3' flanking sequence using Primer3 software (Rozen & Skaletsky 2000). Polymerase chain reactions were prepared in 10 µl volumes containing 20µM of each dNTP, 1x PCR buffer, 0.25 units of Jumpstart ™ Taq (Sigma-Aldrich), 1.5–2.5 mM $MgCl_2$ concentrations (dependent on primer pair), 0.3 µM forward primer, 0.3 µM reverse primer, and 50 ng of template DNA. Thermal cycling conditions were optimized for each primer set by changing annealing temperatures, extension times, or $MgCl_2$ concentration. Typical PCR conditions included: denature temperature of 95°C; extension temperature of 72°C; annealing temp from 56°C-64°C; and 30 cycles. The amplicons were visualized on a 2% agarose gel stained with ethidium bromide, purified using Sephadex-filled spin columns (Sigma Aldrich). The 6 amplicons were Sanger sequenced using the ABI BigDye v. 1.1 Terminator Kit (Applied Biosystems, CA) and Applied Biosystems 3730/3730xl DNA Analyzer. Six primer pairs were used for sequencing the 9 exons as summarized in Table 1.

Table 1 A summary of oligonucleotide primers and standard conditions used for exon resequencing of *TPM2* in normal and affected foals.

Primer Name	Left Primer	Right Primer	Annealing Temp.	Product Size (bp)	Coordinates:EquCab
TPM2 Block 1	TCTGCCCCTAAAAGGTTTG	TGTGCTCASTGTCCCACA	56°C	925	chr25:924560-925484
TPM2 Exon 2	CAGAGCCACGTTTCATCTCA	CTCCTCCTGCCTACAGCATC	65°C	780	chr25:924215-924994
TPM2 Block 2-4	AGCCTCTCTGATCCTCATCC	AGAAACTGCCTGCCCACT	64°C	652	chr25:920929-921580
TPM2 Exon 6	GCCCCACTTCCTTCTCATTC	CCTCCTCTAGGTCCCCACAT	62°C	620	chr25:920539-921158
TPM2 Block 5-7	ACTGTGCCTTCTCTGCTGTC	CCTAGCTGACTGTGGCAAAT	62°C	730	chr25:919898-920627
TPM2 Exon 9	CTGGTCAATCCGTCCATTCT	CTGCTCACAACCCCTCCTTA	60°C	771	chr25:917249-918019

Sequence Alignment, Analysis, and Haplotyping

Sequencher 4.8 software (Gene Codes Corporation, MI) was used for the alignment of sequences from normal and affected foals as well as for variant calling in coding (exons) and noncoding or intron boundary regions of exons. Genomic positions of 5 SNP identified from the sequencing analysis were assigned according to the *TPM2* coordinates in the Ensembl database from the EquCab2 assembly. See Appendix B for the *TPM2* sequence and genomic locations of the SNP. Putative haplotypes were deduced from sequencing data using loci in which both alleles were detected. Pedigree and map files were made for SNP genotypes in Microsoft Excel, and a Fisher's exact test in PLINK (Purcell *et al.* 2007) was performed for the allelic test of association to the disease phenotype. Tests for association under specific genetic models (dominant, recessive, and genotypic) were also carried out in PLINK using the --*model* command. Phenotypes were assigned as either "case" or "control" using the numbers 2 and 1, respectively. Taking into account the higher risk for false positive associations when performing multiple tests we set our significance threshold to α=0.01 (α=0.05/5 markers) after Bonferroni adjustment (Benjamini & Hochberg 1995). Also using PLINK software, we performed haplotype based association tests using 3, 4, and 5 marker sliding windows with a Bonferroni adjusted α=0.0125 (based on 4 haplotypes and thus 4 simultaneous tests).

Histological Preparations

Frozen skeletal muscle tissue from 2 affected foals and 2 clinically normal foals was prepared for light microscopy per the protocol provided by the Texas A&M University College of Veterinary Medicine Pathobiolgoy Department. Briefly, tissue processing included fixation in RNA-later (Ambion), dehydration with 95% and 100% ETOH, and clearing with Pro-par reagents (Anatech, LTD). The samples were then embedded into paraffin wax, sectioned, and stained with Hematoxylin and Eosin (H&E).

Transmission Electron Microscopy

Frozen skeletal muscle tissue from 1 affected foal and 1 clinically normal foal (preserved in RNA-later, Ambion) was prepared for electron microscopy as described in detail elsewhere (Rodrigues *et al.* 2010). Briefly, skeletal muscle samples were fixed in formalin and embedded in paraffin. Thereafter, excised segments were cleared with xylene and rehydrated with water. Tissue staining was carried out using 1% osmium tetroxide with 0.5% with potassium ferrocyanide. Tissue segments were then dehydrated with alcohol and embedded in epoxy resin. Ultrathin sections were cut with an ultramicrotome (MTX, RMC Products, Boeckeler Instruments, Inc, Tucson, AZ) and stained again with uranyl acetate and lead citrate. The samples were imaged with a Hitachi H-7000 transmission electron microscope at resolutions of 2800X, 7100X and 71,000X.

Results

Fluorescence In Situ Hybridization (FISH) Mapping of the Equine TPM2 Gene to ECA25 q12-13

In silico prediction methods are often used to annotate genes in the horse genome. Therefore, we decided to confirm the chromosomal location of *TPM2* to ECA25 using *FISH*. We were able to confirm that the equine *TPM2* gene was assigned correctly to the genomic location, ECA25q12-13 (Figure 11).

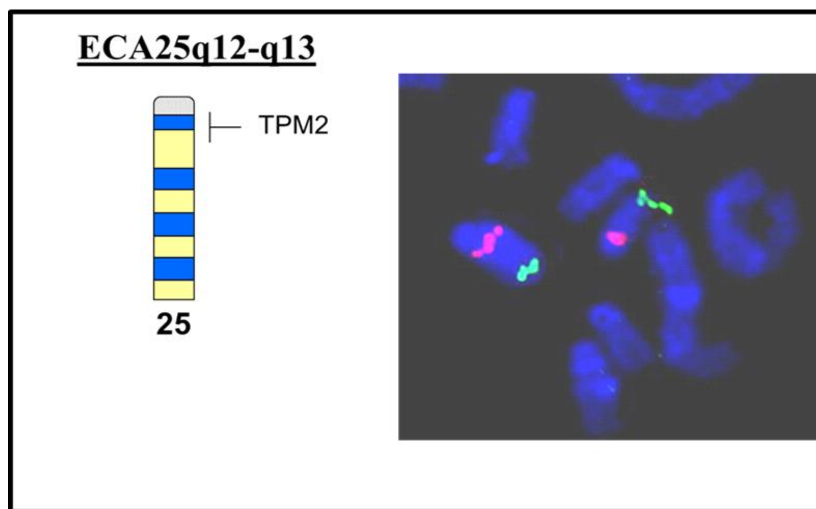


Figure 11 Fluorescence *in situ* hybridization confirmed the location of *TPM2* on homologous chromosomes (green signals) to be on ECA25q12-q13. Control DNA for ECA 25 is represented by the red signals.

Sequence Alignment and Analysis

Sequence analysis of all 9 *TPM2* exons and their respective 5´ and 3´ boundary regions revealed 5 SNP, 4 discrete haplotypes (deduced from sequencing data), and 8

corresponding diplotypes. Figure 12 provides a graphical representation of each of the 4 haplotypes (given numbers in red font) and the 8 diplotypes. In parentheses are the corresponding ratios of cases and controls carrying each diplotype. For example, the first pair of haplotypes (1,1) is homozygous for the alleles C-C-C-C-G, and was identified in 19 cases and 19 controls. The second set with haplotypes (2,1) was found in 22 cases and 11 controls. The *TPM2* sequence from the Ensembl database is given in the minus strand orientation; therefore, the coordinates of each locus in Figure 12 are shown to read in the 5' to 3' direction and were given the following location assignments: 25:924765; 25:924674; 25:92438225; 25:921308 and 25:917455.

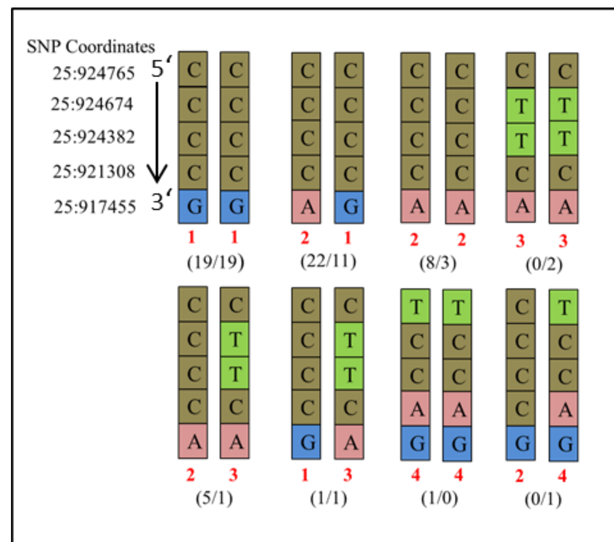


Figure 12 Graphical interpretations of 4 putative haplotypes and 8 diplotypes identified from exon and exon boundary resequencing of *TPM2* in 56 affected foals and 38 control animals. Haplotypes are enumerated in red font. The ratios of each diplotype found in case and control animals are presented in parentheses, respectively.

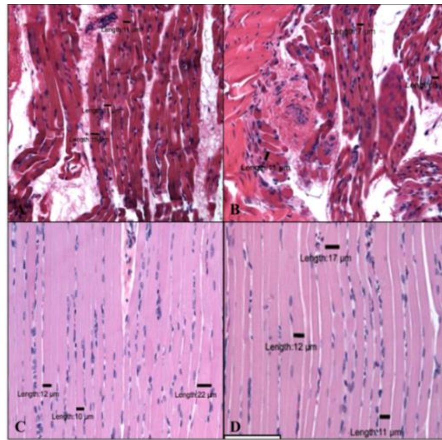
All SNP were found to be in introns except the C>T synonymous transition at 25:924674 which resided in exon 2. Figure 13 shows the alignment of horse and human CDS and the location of the exon 2 SNP (highlighted in red). A higher frequency of the *T* allele in exon 2 was noted in cases (6 cases; 2 controls). However, the test for association using a basic allelic test (two-tailed Fisher's exact test) did not reveal any SNP to be significantly associated with the disease phenotype nor did the genotypic tests under all modes of inheritance (recessive, dominant, and additive). Haplotype 2 in Figure 12 appeared to be overrepresented in case animals (35 cases/3controls) and when sliding windows of 3, 4, and 5 marker haplotypes were tested for association in PLINK using the command *–hap-window 3. 4 or 5*, the haplotype approached statistical significance (P_{Raw}=0.03) but α=0.0125 (Bonferroni adjusted *P*-value) was not attained.

```
>H = Human TPM2, variant 1 CDS [CCDS 6587.1]

>E = Equine TPM2 (Ensembl Database,EquCab2)


>H ATG GAC GCC ATC AAG AAG AAG ATG CAG ATG CTG AAG CTG GAC AAG GAG AAC GCC ATC GAC CGC
>E ATG GAC GCC ATC AAG AAG AAG ATG CAG ATG CTA AAG TTG GAC AAG GAG AAT GCC ATC GAC CGC

>H GCC GAC AAG AAG CAA GCT GAG GAC CGC TGC AAG CAG CTG GAG GAG GAG CAG CAG GCC CTC CAG
>E GCC GAC AAG AAG CAA GCT GAG GAC CGC TGC AAG CAG CTG GAG GAG GAG CAG CAG GCC CTC CAG

>H ACA GAG GAT GAG GTG GAA AAG TAT TCT GAA TCC GTG AAG
>E ACG GAG GAC GAG GTG GAG AAG TAT TCT GAG TCA GTG AAG
```

Figure 13 Alignment of horse and human *TPM2* CDS highlighting the location of the SNP discovered in exon 2. Most variation between horse and human CDS is in the 3rd base pair wobble position.

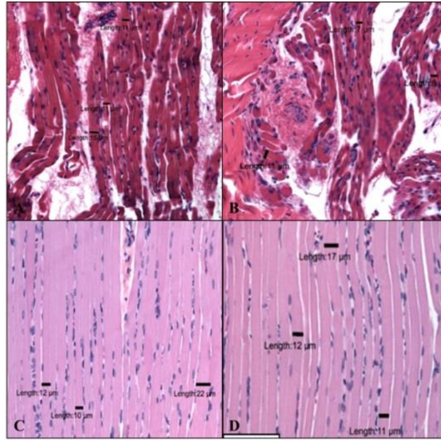
Histology

We observed minor fiber size disproportion between two samples of severely contracted foals. Average estimated fiber diameters from cross-sections of the *flexor carpi radialis* in the two affected foals were 23.8µm and 9µm, and control foals presented with 12µm and 12.5µm fiber diameters. Average fiber diameters from longitudinal sections of the same muscle in the two affected foals were 12.3µm, and 8.7µm, respectively and 14.7µm and 13.3 µm in 2 control animals (Figures 14A-D; 15A-D). According to the pathologist who reviewed the slides, there was no gross pathology noted.



Longitudinal	Measurement 1	Measurement 2	Measurement 3	Average
Affected A	11µm	11µm	15µm	**12.3µm**
Affected B	8µm	7µm	11µm	**8.7µm**
Normal C	10µm	12µm	22µm	**14.7µm**
Normal D	17µm	12µm	11µm	**13.3µm**

Figure 14 A-D Longitudinal measurements of fiber diameters from histological preparations in 2 affected and 2 normal foals. Average diameters in the affected foal A and control animals were largely consistent (12.3µm, 14.7µm, and 13.3µm, respectively). Average diameters of affected foal B were slightly smaller in comparison to the other foals (8.7µm).



Longitudinal	Measurement 1	Measurement 2	Measurement 3	Average
Affected A	11µm	11µm	15µm	**12.3µm**
Affected B	8µm	7µm	11µm	**8.7µm**
Normal C	10µm	12µm	22µm	**14.7µm**
Normal D	17µm	12µm	11µm	**13.3µm**

Figure 15A-D Longitudinal measurements of fiber diameters from histological preparations in 2 affected and 2 normal foals. Average diameters in the affected foal A and control animals were largely consistent (12.3µm, 14.7µm, and 13.3µm, respectively). Average diameters of affected foal B were slightly smaller in comparison to the other foals (8.7µm).

Transmission Electron Microscopy

In Figure 16A, the myofibers of Affected Foal "A" from the histology evaluation are seen at a resolution of 2800x and are organized and uniform. Two nuclei can be seen at the periphery of the cell which is expected by this stage of development. The images in Figure 16B and 16C were taken at a resolution of 7100x and 71,000x, respectively and show remarkable organization of the Z-disks and filaments. In this particular foal, no obvious defects in the myofibrils or sarcomere ultrastructures were observed; however, this does not

infer that there is normal muscle morphology in all cases of flexion contractures or CFS.

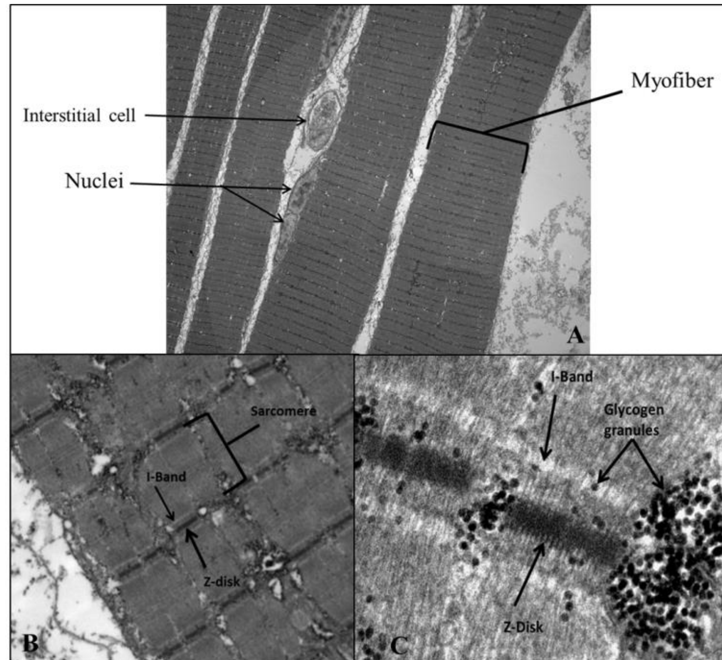


Figure 16A-C Transmission electron microscopy of skeletal muscle taken from the *flexor carpi radialis* of a severely contracted foal (Affected Foal A). There are no obvious abnormalities in the sarcomeres or Z-disks at these magnifications. (A=2800x, B=7100x, and C=71,000x)

Discussion

There is genetic (locus) heterogeneity among human populations with distal limb contractures and within select individual skeletal muscle contractile gene (Sung *et al.* 2003; Kimber *et al.* 2006; Robinson *et al.* 2007; Bamshad *et al.* 2009). We surmised that the same could be true for the occurrence of similar phenotypes observed in the Thoroughbred horse population. Tropomyosin beta-2 has proven to be a strong candidate for resequencing in human neonates with limb contractures and muscle disease (Sung *et al.* 2003; Kee &

Hardeman 2008; Tajsharghi *et al.* 2008; Marttila *et al.* 2014); therefore, we selected this gene for resequencing in case (n=56) and control populations (n=39) of neonatal Thoroughbred foals. We performed exon and exon boundary sequencing of *TPM2* to search for evidence that variants in this gene cause perturbations in the expression or function of the encoded protein.

One SNP, a C>T transition, was found to be in the coding sequence of exon 2 but doesn't cause an alteration in the amino acid sequence. The minor allele *T* was in higher frequency in cases animals than in control animals but an allelic test of association in PLINK failed to provide evidence of an association with the disease phenotype. There is a large degree of conservation between the *TPM2* DNA coding sequence of the horse and other mammals (Schevzov *et al.* 2011) and most variation is in the wobble position (3rd base of each codon) which is where the C>T exon 2 SNP exists (see Figure 13).

The 4 remaining SNP identified reside in introns and the 3' untranslated region (UTR), specifically, the 5' and 3' flanking regions of exon 2, the 5' flanking region of exon 4, and the proximal 3' UTR of the gene. Individually, they did not meet the significance threshold for association to the disease. However, when haplotypes of the SNP were evaluated, a sliding window association analysis resulted in one haplotype (haplotype 2 in Figure 12) approaching statistical significance (p_{raw}=0.03 with α=0.0125). Collectively, 35 affected foals and 3 clinically normal foals carried at least one copy of this 5 marker haplotype.

Although no protein-altering variants were discovered in this case population, mutations in promoters or other regulatory elements can also cause disease by altering the expression of genes (de Vooght *et al.* 2009; Epstein 2009; Laurila & Lahdesmaki 2009). Regulatory sequences, such as promoters, are not well-characterized for much of the horse genome but it is plausible that there are variants not yet identified in these intronic regions of *TPM2* that may be contributing to the disease phenotype. One limitation of this study is that only exons and exon boundaries were sequenced (4,478 bp, ~50% of the gene). Therefore, supplementary sequencing of introns and 5' upstream and 3' regions may yield more substantial evidence as to the regulation of *TPM2* and the potential role it plays in flexion contractures and CFS. It is reported that 1% of nucleotide substitutions causing disease are in promoters (de Vooght *et al.* 2009). A recent report by Schevzov *et al.* (2011) on tropomyosin isoforms states that the greatest source of primary sequence divergence results from alternative promoter use and alternative splicing. Furthermore, *TPM2* is known to have an alternative, internal promoter in other species, as discovered by Libri *et al.* (1989) who described a second promoter between the 2nd and 3rd exons of the chick *TPM2* gene. This promoter produces a lower molecular weight transcript found in undifferentiated muscles cells and nonmuscle cells. It is therefore reasonable to postulate that internal promoters may also exist in the equine *TPM2* gene and may potentially be active during certain developmental stages but this has yet to be investigated.

Promoter identification methods are often labor intensive and complex. *In silico* prediction methods can be used to identify sequence motifs characteristic of regulatory elements, but functional assays are often still required to confirm their role and to assess the

implications of mutation(s) in these elements. Additionally, there has been limited transcript profiling of many equine genes, particularly during developmental phases. For instance, according to the Ensembl database (http://useast.ensembl.org/Equus_caballus/Info/Index) there has been one transcript reported for the equine *TPM2* gene, but 8 transcript variants have been identified in humans, and 6 in the mouse. Moreover, with the advent of RNA-seq (Wang *et al.* 2009), it has been shown that many of the annotated equine genes are incomplete (Coleman *et al.* 2013). This method for isoform characterization would facilitate the identification of alternative transcription start sites and putative alternative promoters by identifying the 5' end of the transcript. Although the equine *TPM2* may not adhere to the properties of the gene in other mammalian species, alternative isoforms should be evaluated in the event that alternative promoters are governing expression in a temporal and spatial manner. Due its critical role in muscle physiology (Marttila *et al.* 2014), further investigation of *TPM2* may yield important data for understanding flexion contractures and other muscle phenotypes in Thoroughbred horses. Promoter identification assays and isoform characterization for this gene will be potential studies for the future.

Histological evaluation

Pathological conditions can result in a variety of morphological changes in skeletal muscle tissue (Goebel *et al.* 1997; Laing & Nowak 2005; Selcen & Bushby 2005; Lamont *et al.* 2006; Owji *et al.* 2010; Goebel & Bonnemann 2011). H&E staining of histological preparations is a commonly used technique that can clearly show aberrant ratios in fiber sizes and types, the presence of fatty or fibrotic tissue, and abnormal nuclei arrangement. This method can greatly facilitate the differential diagnosis of muscle diseases (Owji *et al.* 2010).

In a study conducted by Kimber *et al.* (2012), 12 out of 17 human patients with distal arthrogryposis displayed varying degrees of muscle pathology. Two patients with DA2B and a *TPM2* mutation had Type I fiber predominance. Atrophy or hypertrophy of muscle fibers can also be indicative of muscle pathology (Jackson 2008). There was marked variability in fiber diameter sizes in the samples from two severely contracted foals when compared to muscle morphology of two normal age-matched foals. It is unclear if these were indications of muscle pathology or natural variability, but clearly, the case ranges appeared to deviate from the controls using our small sample size. Moreover, it is not uncommon for there to be heterogeneity of muscle pathology among cases of congenital contractures in humans (Dastur *et al.* 1972). Therefore, analysis of additional samples to establish the mean muscle fiber diameter among normal neonatal foals would further elucidate whether our cases clearly represent an unequivocal deviation from the expected normal fiber diameter range.

Transmission Glectron Oicroscopy

Tropomysosin beta-2 mutations have been implicated in some myopathies that in part, can be diagnosed by the evaluation of the sarcomere ultrastructures (Goebel, H 2007). For instance, mutations in *TPM2* cause nemaline rod myopathy in humans, which is partially characterized by intra-sarcolemma or intra-nuclear rods as well as Z-disk defects (Ilkovski *et al.* 2001). These biological artifacts can be easily identified from images generated by TEM. For example, Figure 17 (Paciello & Papparella 2009) shows the presence of nemaline rods in human skeletal muscle caused by a deletion mutation in *TPM2* and an instance of Z-disk and actin filament degeneration caused by a benign congenital myopathy (Hudgson 1970; Mokbel *et al.* 2013).

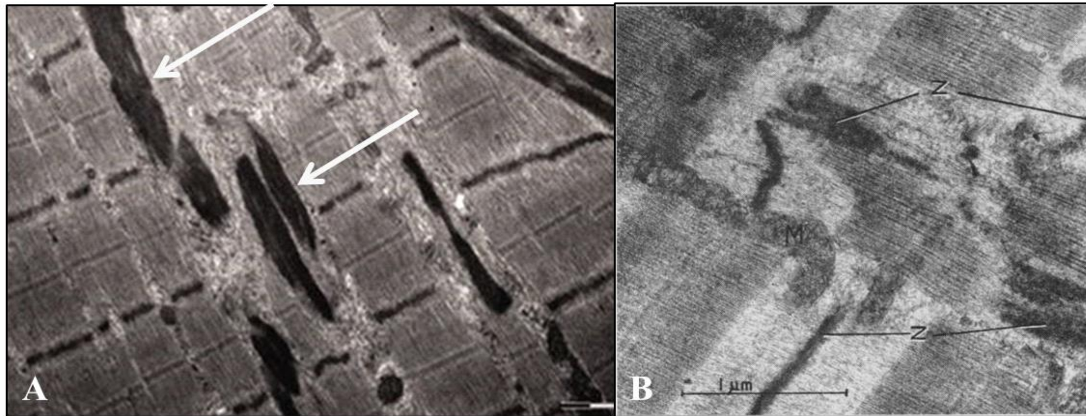


Figure 17 (A) Nemaline rods from human skeletal muscle captured in a TEM image. (B) A type of benign myopathy characterized by degeneration of the Z-disks and actin filaments. Note the marked disorganization of the sarcomeres

Because this is considered an important diagnostic criterion for human myopathies and for establishing an initial baseline phenotype, we evaluated muscle tissue from the caudal aspect of the upper forelimb of a severely contracted foal (Affected Foal A) for evidence of sarcomere ultrastructure disorganization. In the one case evaluated, no gross morphological abnormalities in the muscle architecture were observed. However, this does not imply that there are no pathological changes in the skeletal muscle in other cases of foals with CFS. Flexion contractures with neuromuscular etiologies may show distinguishing features which can be observed histopathologically (Paciello & Papparella 2009) and therefore, when possible, histological evaluation should be performed as a diagnostic criterion in foals with severe flexion contractures.

In summary, we identified 5 previously unreported SNP in the *TPM2* gene from a case/control population of Thoroughbred foals. Presently, there is nothing to suggest that these SNP are risk alleles for flexion contractures or CFS, but considering two SNP reside

only a few bases upstream from exons, promoter identification and further characterization of *TPM2* transcripts in developing muscle is warranted for the investigation of effects these variants may have on gene expression. Future work may also include gene expression analysis to evaluate how the exonic SNP modulates the speed or translation of transcription.

CHAPTER III

GENOME-WIDE ASSOCIATION ANALYSES IN THOROUGHBRED FOALS IDENTIFY CANDIDATE GENES IN A SKELETAL MUSCLE PROTEIN NETWORK ASSOCIATED WITH CONGENITAL JOINT CONTRACTURES

Introduction

Congenital flexion contractures in the neonatal foal are musculoskeletal conditions that may result in euthanasia or chronic conditions such as osteoarthritis thus potentially compromising long-term athletic ability and soundness. If non-ambulatory, the foal is at risk for secondary complications such as weakness, delayed development, and an increase susceptibility to infectious disease (personal communications; Nathan Slovis, DVM, ACVIM; Hagyard Equine Medical Institute, Lexington, Kentucky). The degree of contracture can vary from mild to severe and may differ between joints in the same foal. Symmetrical or asymmetrical presentations may also be observed (Kidd and Barr 2000; Adams *et al.* 2002). Although foals presenting with mild to moderate degrees of flexion contractures may be successfully rehabilitated, it is unknown if the mechanisms leading to contractures *prenatally* increase the risk of musculoskeletal problems as adults. Furthermore, severe contractures causing abnormal presentations may lead to dystocia births resulting in injury or even death in the mare (Binanti *et al.* 2013). In some cases, additional congenital defects may be observed resulting in a much more complex condition, which is often referred to as contracted foal syndrome (CFS) (Binanti *et al.* 2013). According to the 242 medical cases of contracted foals we reviewed, the most commonly documented associated defects

included: angular limb deformities, ligament/tendon laxity of the distal limbs, scoliosis, entropions, cranio-facial malformations, and abdominal wall defects. Although CFS exists across breeds, there seems to be a relatively high prevalence in Thoroughbreds over other breeds with a familial predisposition in some cases. To our knowledge, there are no established etiological mechanisms causing flexion contractures and CFS and there have been limited reported attempts to identify the physiological or molecular basis. Hence, novel approaches are needed to delineate this potentially life-threatening condition.

The Illumina Equine SNP50 Beadchip, a high density SNP array for association mapping, genetic diversity and phylogeny studies in the domestic horse and extant Perissodactyla, became available in 2008 and properly tested and characterized by McCue and colleagues in 2012. The utility of this platform for simple and complex conditions has been demonstrated with several recent studies. Some examples include: mapping of a locus associated with lavender foal syndrome (Brooks *et al.* 2010); identification of a 2Mb candidate region associated with dwarfism in Friesian dwarf horses (Orr *et al.* 2010); the identification of a major gene associated with guttural pouch tympany in Arabian and German Warmblood horses (Metzger *et al.* 2012); identification of putative quantitative trait loci associated with osteochondrosis in Norwegian Standardbred trotters (Lykkjen *et al.* 2010); the identification of candidate loci associated with wither height in German Warmblood horses (Tetens *et al.* 2013); and the identification of a susceptibility locus for impaired acrosome reactions in stallions (Raudsepp *et al.* 2012).

The GWAS approach is dependent on several factors including SNP density, LD structure of the species or breed under study, accurate genotyping and phenotyping, and heritability of the trait of interest because these impact experimental power (Klein 2007; Spencer *et al.* 2009; Feng *et al.* 2011). Power is the probability that the experiment will correctly reject the null hypothesis when a disease susceptibility locus (DSL) of a given size of effect exists in the population. Specifically, power to detect an association between a SNP and a DSL depends upon: (1) linkage disequilibrium (r^2) between the marker and DSL; (2) the proportion of total phenotypic variance explained by the DSL (h^2); (3) number of phenotypic records, n; (4) minor allele frequency (p) of the marker and the unobserved DSL (q); (5) dominance ratio at the locus (0 for completely additive to 1 for completely dominant), and (6) the significance level, α (Luo 1998; Ball 2005).

A careful initial analysis of these parameters suggested that a genome-wide association approach would be favorable for investigating flexion contractures and CFS. Our hypothesis states that there is a major locus for susceptibility to contractures. Therefore, our objective was to identify a CFS susceptibility locus by GWAS using a case-control design and to validate findings using genome-wide genotype data from a kindred featuring multiple probands. We concluded with a family based GWAS although the sample size was a limitation for this model. Our results, for the first time, provide insight into genetic factors underlying congenital flexion contractures and CFS in Thoroughbred foals.

Materials and Methods

Sample Acquisition for Case/Control Study

All procedures involving animals were approved by the Texas A&M Institutional Animal Care and Use Committee (AUP # 2011-234). Control animals (n = 43) were randomly selected from 3 Thoroughbred breeding farms in the Lexington, KY area during the 2008 foaling season. Samples were taken from clinically normal foals from 0 to 3 weeks of age. We personally observed all foals used as controls and corroborated the absence of any evidence of congenital defects. Case samples from neonatal Thoroughbred foals diagnosed with congenital limb contractures (n = 57) were obtained from 5 Thoroughbred farms and Hagyard Davidson McGee Equine Medical Institute in Central Kentucky and Peterson and Smith Equine Hospital in Ocala, Florida. A criterion for selection of case animals was the contracture had to be severe enough to require medical intervention. See Table 2 for a list of affected foals and their respective phenotypes.

Approximately 20 mL of peripheral blood was collected in 10 mL EDTA Vacutainer® tubes by veterinarians or licensed veterinary technicians following standard medical procedures. DNA extraction was carried out using a phenol/chloroform protocol for peripheral whole blood DNA isolation (Sambrook 1998). The quality and quantity of DNA was assessed using gel electrophoresis and NanoDrop spectrophotometry (Thermo Fisher Scientific, CA). Extracted DNA was archived and stored at -80º C.

Calculating Relationships among Cases and Controls

To identify a subset of cases and controls with minimal relationship among them for a population-based GWAS, we computed pairwise relationship coefficients (R) using the Multiple Trait Derivative Free REML program from 5-generation pedigrees (Van Vleck & Boldman 1993). A set of cases (n = 24) and controls (n = 24) with R ≤ 0.1 were selected.

Power Calculations

Power calculations were performed in R using ld.power based on the formulas from Luo– (1998) and Ball (2005). We performed the calculations iteratively for D values equivalent to r^2 = 0.1 to 1.0, h^2 = 0.1 to 1.0, $p = q = 0.2$ (i.e. average MAF for the commercial array), no dominance, $\alpha = 0.05$, and n = 48.

Genotyping and Quality Control

Affected and unaffected foals selected for GWAS (n = 48) were genotyped for 54,602 SNP using the Illumina Equine SNP50 Beadchip (Illumina, CA). Genotyping was performed by Genotyping Shared Resource at the Mayo Clinic (Rochester, MN), according to the manufacturer's protocol. Approximately 200 ng of genomic DNA per sample was used for hybridizations to probe DNA. Genotype calling and sample clustering was carried out by GeneSeek (Neogen Corp., NE) using Illumina's BeadStudio analysis software.

Default settings of PLINK (Purcell *et al.* 2007) were used for quality control (QC) filtering of genotypes. Those individuals with a genotyping completion rate of ≤ 90%, SNP with minor allele frequency < 0.05, SNP with a genotyping call rate of ≤ 90%, and SNP that deviated from Hardy-Weinberg Equilibrium proportions ($P < 0.001$) were filtered. SNP from

the X chromosome were used to verify sex of the samples based on heterozygosity rates. After these QC steps were completed, a pairwise identity-by-descent matrix was produced in PLINK to evaluate sample contamination or duplication and to verify that genomic relationships among samples were similar to pedigree relationships. Finally, population substructure was assessed using multi-dimensional scaling (MDS) in PLINK.

Genome-wide Association Study

Model-free genome-wide association tests were performed in PLINK using a basic case-control chi-square association test (Purcell *et al.* 2007). The genomic inflation factor was calculated in PLINK and quantile-quantile (QQ) plots were generated in R to detect inflation of test statistics due to population stratification. We applied a conservative Bonferroni correction assuming 43,392 independent tests to determine the genome-wide significance threshold ($P = 1.15 \times 10^{-6}$). Association data were visualized as Manhattan plots created in R.

Sub-classification of Disease Phenotypes

After the initial case-control study, based on the presence or absence of contractures, we performed additional GWAS based on further sub-classification of the phenotype. Affected foals were grouped by the anatomical location of the joint contracture or sharing of more definitive clinical features, similar to the approach taken by Teyssedre *et al.* (2012). The original cohort of 24 affected foals was subdivided into 4 phenotype groups of cases (Table 2) and basic chi-square association tests were performed against the same 23 control animals as previously.

Table 2 Phenotype classifications of affected foals used in case/control GWAS.

Phenotype Group 1		
JC59	F	3 fetlocks mild
JC91	M	Hind fetlock contracture
JC108	F	Severe bilateral front fetlock contractures
JC114	M	Mod-severe bilateral front fetlock contractures
JC116	F	Mod-severe bilateral front fetlock contractures
JC118	M	Left front and right hind fetlock contractures
JC134	M	Bilateral front fetlock contractures
JC136	F	Bilateral front fetlock contractures
Phenotype Group 2		
JC4	F	Severe bilateral carpal contractures; pastern laxity x3, bilateral carpal valgus; bilateral hock deviations
JC34	F	Severe bilateral carpal contractures; laxity in hind limbs
JC36	F	Severe bilateral carpal contractures; hind pastern laxity; bilateral carpal valgus
Phenotype Group 3		
JC38	M	Severe bilateral carpal contractures
JC101	F	Moderate bilateral carpal contractures
JC102	M	Moderate bilateral carpal contractures
JC120	F	Moderate bilateral carpal contractures
JC126	M	Moderate bilateral carpal contractures
JC89	M	Moderate bilateral carpal contractures
Phenotype Group 4		
JC32	F	Moderate bilateral carpal and fetlock contractures
JC51	F	Left carpal and fetlock contracture
JC65	M	Moderate bilateral carpal and fetlock contractures
JC86	M	Bilateral forelimb contractures
JC93	M	Bilateral carpal, front fetlocks with DDFT contractures and mild hind limb fetlock contractures
JC98	F	Bilateral carpal and fetlock contractures
JC106	M	Bilateral carpal and and left hind fetlock contractures

Sample Acquisition for Family-based Validation Study

Peripheral blood (20 mL), for extraction of DNA as previously described, was collected from a kindred of 60 individuals from a sire line known for producing contracted foals. All samples were collected in Lexington, KY during the 2010 breeding season from 5 Thoroughbred farms and Hagyard Equine Medical Institute. Ages of the affected and control foals were 4 months or less at the time of collection. Clinical descriptions were obtained from the farm personnel or records and medical records. All records and reports describe the

contractures as being present at the time of birth. The cohort consisted of paternal half-sib trios from 6 closely related sires and one maternal half-sib trio all from the same founder. In addition, we included 1 mare/affected foal pair and one affected singleton from the same lineage. Two dams included in this group also had contracted foals by sires from different lineages in other years. We were able to include these foals as well as their respective stallions. Pedigree structure of these individuals as well as the phenotypes can be seen in Figure 18.

Figure 18 Pedigrees illustrating the family structure used in the family-based validation study and family based GWAS**.** The blue square represents the sire hypothesized to be a carrier of predisposing alleles. Red circles (females) and squares (males) represent affected foals from the sire line. Half-sibling controls are depicted in grey. Only individuals with an ID were included in the study.

Genotyping, Quality Control and Association Analysis

The Illumina Equine50 Beadchip (Illumina, CA) was also the genotyping platform used for the family-based study. GeneSeek (Neogen Corp., NE) performed DNA hybridizations as well as genotype calling and sample clustering using Illumina's BeadStudio analysis software. In addition to all the quality control measures applied in the case-control study, parentage was verified. SNP spanning regions that were significant or approached significance in the case-control studies were extracted and the family-based association test, DFAM, was performed on these subsets of SNP using PLINK.

Family-based Genome-wide Association Studies

In addition to using the family material to validate the findings from the case-control studies, we chose to perform family-based GWAS due to the robustness of this design against genetic (locus) heterogeneity. However, we also had expectations that the study would be underpowered given the limited number of trios. We used TDT and DFAM in PLINK as well as FBAT software (Laird & Lange 2009) to generate the test statistics. As with the case-control studies, the Bonferroni correction for multiple tests was applied to determine significance thresholds.

Homozygosity Mapping

We also used Homozygosity Mapper (HM) software (http://www.homozygositymapper.org) to identify regions of extended homozygosity within the families. See methods described by (Seelow *et al.* 2009). Briefly, filtered PLINK files were reformatted to specifications appropriate for HM and uploaded to the HM database which also contains maker data from

the Illumina Equine50 Beadchip (Illumina, CA). In the analysis phase, HM reads the length of homozygous blocks for each affected animal and generates a score for each SNP. The observed frequency of homozygous markers is then compared to the homozygosity frequency in control animals. The homozygosity scores are then plotted against physical location. A bar chart is generated for visualization of the data and the *genotype view* output provides the single genotypes of all samples marked as cases or controls.

Phasing and Linkage Disequilibrium Structure

Haplotypes generated for the chromosomal regions associated with the disease phenotypes in both the case/control and family studies were constructed using FastPhase1.3.0c and Phase v2.1.1 (Stephens & Donnelly 2003) from input files created in PLINK. LD plots were created in Haploview for visualizing LD structure (Barrett *et al.* 2004).

Sequencing of the ANKRD1 Promoter Region and Exon

The sequence of ankyrin repeat domain containing protein 1 (*ANKRD1*), a candidate gene identified from the family GWAS, was retrieved from the EquCab2 (2007) assembly (http://www.ensembl.org/Equus_caballus/Info/Index) in the UCSC database (http://genome.ucsc.edu/). Sanger sequencing of *ANKRD1* exons was performed using the ABI BigDye v. 1.1 Terminator Kit (Applied Biosystems, CA) and Applied Biosystems 3730/3730xl DNA Analyzer. Sequencher 4.8 software (Gene Codes Corporation, MI) was used for alignment of DNA sequence from all family members and for the identification of sequence variants.

Results

Based on an estimated r^2 between SNP on the Equine50 beadchip in Thoroughbreds of 0.4 (Corbin *et al.* 2010; Corbin *et al.* 2012), and our hypothesis that there is a susceptibility locus of large effect for flexion contractures ($h^2 \geq 0.6$), we expect to be able to correctly reject the null hypothesis at least 93% of the time if an effect of the expected size exists in the population of 48 samples selected for the case-control study (Table 3).

Table 3 Power to detect a completely additive DSL using markers with various r^2 and h^2 (0.1 to 1.0)[1]. The estimated r^2 for Thoroughbreds is 0.4 and with a gene of large effect (h^2=0.6-0.9) the corresponding power is ≥ 93% (red box).

	Heritability									
r^2	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
0.1	0.088	0.133	0.182	0.235	0.289	0.345	0.400	0.456	0.511	0.563
0.2	0.127	0.221	0.324	0.429	0.530	0.625	0.709	0.781	0.841	0.888
0.3	0.168	0.314	0.465	0.608	0.729	0.825	0.895	0.942	0.970	0.987
0.4	0.210	0.404	0.592	0.749	0.862	0.933	0.972	0.990	0.997	0.999
0.5	0.254	0.496	0.708	0.857	0.942	0.982	0.996	0.999	1.000	1.000
0.6	0.301	0.584	0.803	0.928	0.981	0.996	1.000	1.000	1.000	1.000
0.7	0.347	0.663	0.874	0.968	0.995	1.000	1.000	1.000	1.000	1.000
0.8	0.392	0.732	0.923	0.987	0.999	1.000	1.000	1.000	1.000	1.000
0.9	0.439	0.794	0.957	0.996	1.000	1.000	1.000	1.000	1.000	1.000
1.0	0.483	0.844	0.977	0.999	1.000	1.000	1.000	1.000	1.000	1.000

[1] Calculated using formulas from Luo, 1998 and Ball, 2005 with n = 48, p = q = 0.2, no dominance, α = 0.05.

After filtering genotypes, 43,392 SNP remained for the association analysis with an overall call rate of 0.994. Genomic relationships were consistent with pedigree relationships for all but two individuals. JC232 and JC233 were genetically identical indicating a

duplication of DNA samples; therefore, JC233 was removed from further analysis. Multidimensional scaling analysis (Figure 19) showed that one affected foal from Phenotype Group 1 (JC116) was an outlier and was subsequently removed from the study.

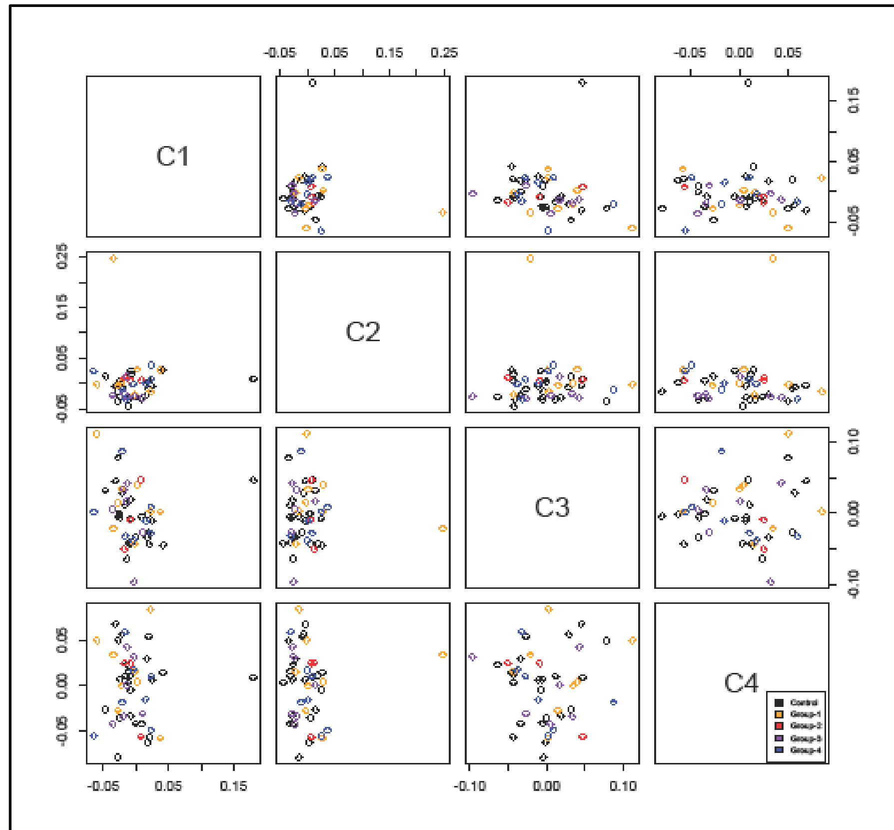


Figure 19 Association study stratification plots prior to correction. The relationship between all individuals used in the association study was examined by plotting components C1 to C4 from the multidimensional scaling analysis. Foals were grouped into five classes, controls unaffected by contractures and 4 phenotype groups of cases based on location of the contractures.

We performed a genome-wide association study to map the susceptibility locus for flexion contractures using foals classified as affected (n = 23) and unaffected (n = 23). Moderate associations were detected to regions on equine chromosome ECA13 (13: 2032780-2038953) with $P_{raw} = 1.36 \times 10^{-5}$-$1.38 \times 10^{-4}$, ECA 5 (5:38654516-38858506 and 5: 72847537) with $P_{raw} = 6.25 \times 10^{-5}$, ECA 18 (18: 58623185) and ECA 20 (20: 27782384) with $P_{raw} = 1.47 \times 10^{-4}$ (Figure 20), but none of these nominal probabilities reached the genome-wide significance threshold after Bonferroni correction for multiple testing. The QQ plot in Figure 21 does not support the presence of population stratification within this study population.

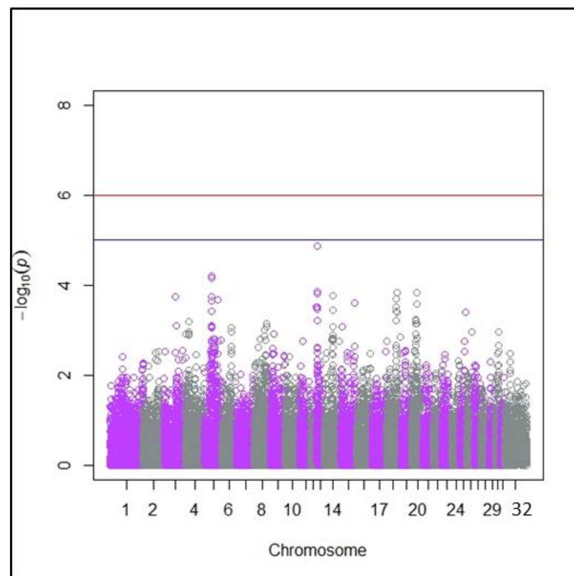


Figure 20 Manhattan plot representing asymptotic *P*-values ($-\log_{10}$) from analysis of original cohort (23 cases and 23 controls) showing moderate associations to ECA5, 13, 18, and 20.

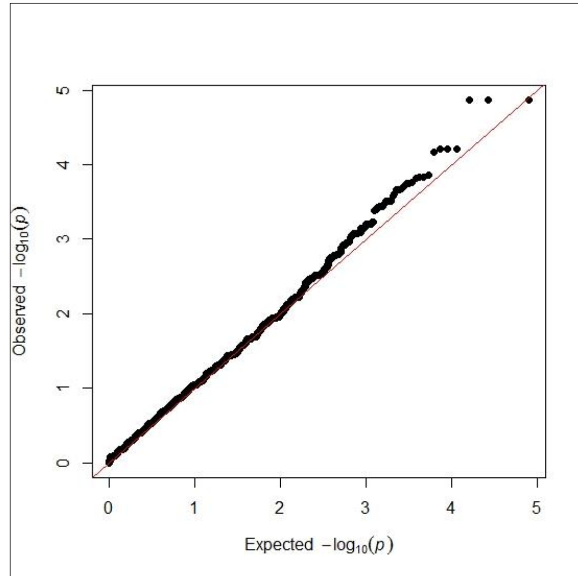


Figure 21 QQ plot of $-\log_{10}$ of *P*-values from basic chi-square association tests of the cohort of 23 affected foals and 23 control animals.

The results of this association study suggest that rather than a single susceptibility locus, there may instead be genetic (locus) heterogeneity because of phenotypic heterogeneity among our CFS cases. We reclassified the case animals based on specific phenotypic criteria and ran separate basic chi-square association analyses for each group against a common group of controls.

Phenotype Group 1 Analysis

Phenotype group 1 included 7 foals with fetlock contractures. After a chi-square analysis (Figure 22) and correction for multiple testing, 7 SNP in a 1.25 Mb region of ECA18 (18: 57022464-58268945) approached significance at the genome-wide level (P_{raw} = 1.18 x 10^{-6}; P_{genome} = 0.0502; genomic inflation factor = 1.24). The QQ plot in Figure 23 suggests some possible inflation of the test statistics as suggested by a relatively early departure from the expected distribution.

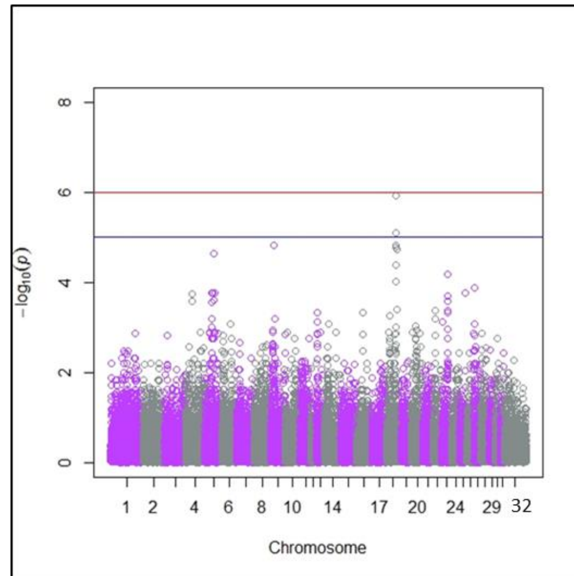


Figure 22 Manhattan plot representing asymptotic *P*-values ($-\log_{10}$) from the chi-square analysis of Phenotype Group 1 (7 foals with distal limb contractures) and 23 controls.

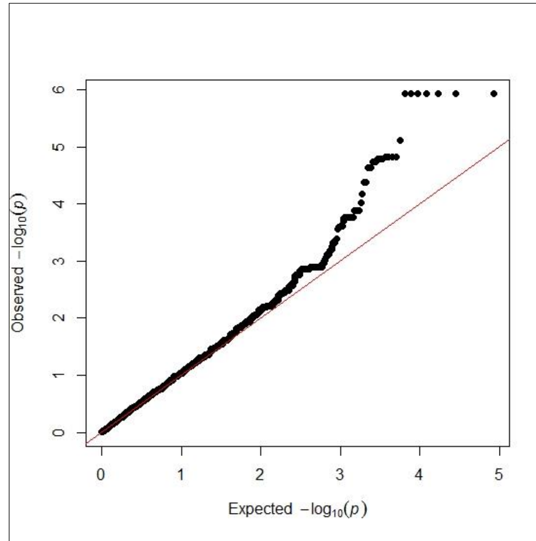


Figure 23 QQ plot of $-\log_{10}$ of *P*-values from the chi-square analysis of Phenotype Group 1 (7 foals with fetlock contractures) and 23 control animals.

Haplotypes were constructed for a ~2.68 Mb region on ECA18 spanning the associated SNP and ~1Mb of the 5' and 3' flanking sequence (59 markers; 18:56007908-58690725). Haplotypes were first evaluated in the 7, Group 1 phenotype case animals and all controls then in the remaining 16 affected animals from the initial study group. In all, 4 of the 7 affected foals from the Phenotype Group 1 analysis (all presenting with fetlock contractures) were identified as having two copies of 3 segments of one haplotype which was only present as one copy in 4 of 23 control animals. This haplotype is highlighted in orange in Figure 24 and the segments are demarcated by black boxes. Three affected foals from the remaining cohort of 16 case animals (JC36, JC51, and JC65) had two copies of at least smaller sections of these segments but the phenotypes were not consistent with the Phenotype Group 1 foals. Instead of fetlock contractures, JC36 presented with bilateral

carpal contractures, hind pastern laxity, and bilateral carpal valgus. JC51 was described as having a unilateral left fetlock and carpal contracture and JC 65 had bilateral carpal and fetlock contractures. The frequency of this haplotype in the 7 Phenotype Group 1 case animals is f = 0.786 and f = 0.174 in control foals. Overall, the frequency is f = 0.457 in the case population of 23 affected animals.

When this region was tested in the family data, 25 SNP were captured in the analysis. Of the 25 SNP captured, BIEC2-416030 (18: 58026623) and BIEC2-416035 (18:58029997) were notable with P_{raw} = 0.057, but they did not meet the Bonferroni adjusted threshold of P_{genome} = 0.002 (α = 0.05/25 SNP). We tested these 2 SNP individually to abate an increased Type II error rate that is characteristic of multiple testing and designated a standard significance threshold to $\alpha = 0.05$. The P-values for both SNP, tested independently, were $P = 0.057$ which suggests a possible association to this genomic region. Phasing of the region in the kindred did not reveal any haplotype to predominate in the probands when compared to the related unaffected foals (See Appendix C for haplotypes).

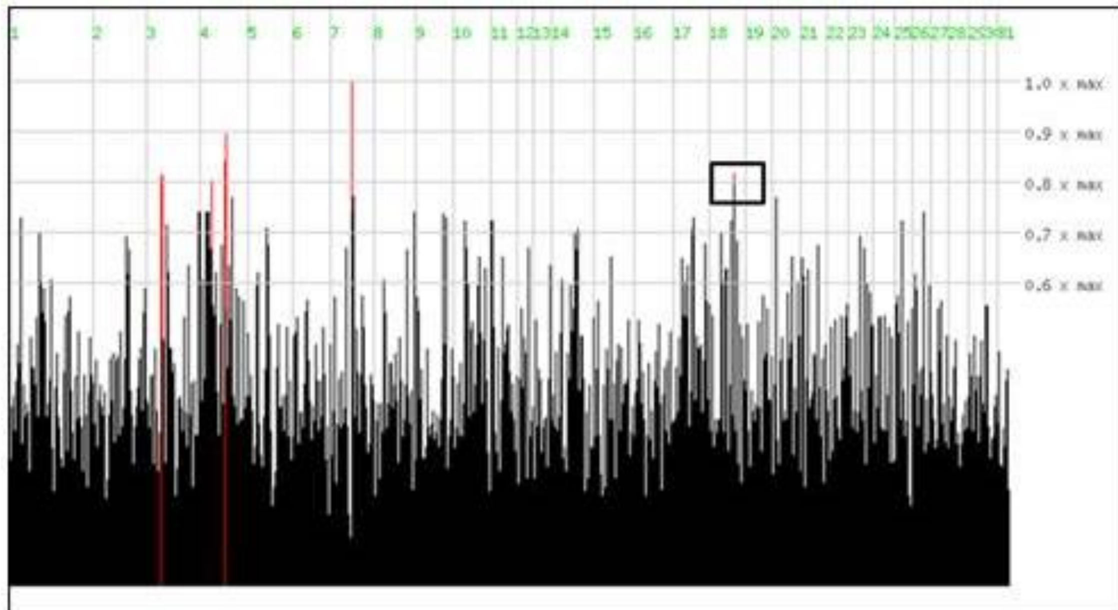
A GWAS analysis of the family data failed to yield an association to ECA18 but low statistical power may have been a factor. Considering this, and because homozygosity mapping can be more robust in the face of a genetically heterogeneous condition and with small sample sizes (Alkuraya 2010), we looked for regions of extended autozygosity using our genome-wide genotyping data from the kindred of 60 animals. A region on ECA18 with coordinates 56863692-57022464 scored greater than 80% of the maximum score which indicates a significant region of homozygosity (see Figure 25). This autozygous region overlaps with the significant SNP marker, BIEC2-415468 from the case/control study at the genomic location 18: 57022464.

A candidate gene search revealed that titin (*TTN*) (18:56684352-56950669) was within 71.8 kb of the associated SNP, BIEC2-415468, from the Phenotype Group 1 analysis and within 1.1 Mb of BIEC-416030 from the validation test in the family data. This gene also overlaps with a segment of the autozygous region from the homozygosity mapping of the family data. Titin plays a major structural and signaling role in the sarcomeres of skeletal muscle.

Foal ID	Affection Status	56567378	56735837	56779448	56785443	56843481	56859971	56861571	56863586	56863692	56889413	56889525	56990492	57022464	57022573	57087413	57117832	57122419	57125689	57139212	57246288	57299129	57318584	57318586	57434860	57503825	57523800	57529010	57532286	57578847	57579230	57703985	57943621	58026623	58029997	58060985	58186973	58268935
JC4	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC4	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC32	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC32	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC34	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC34	CASE	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC36	CASE	1	1	0	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	1	1
JC36	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC38	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC38	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC51	CASE	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1
JC51	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC59	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC59	CASE	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC65	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC65	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC86	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC86	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC89	CASE	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1
JC89	CASE	1	1	0	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	1	1
JC91	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC91	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC93	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC93	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC98	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC98	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC101	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC101	CASE	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC102	CASE	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC102	CASE	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC106	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC106	CASE	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	0	0	1	0	[1]	0	0	1	1	0	1	1	1	1	0	0
JC108	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC108	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC114	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC114	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC118	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC118	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC120	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC120	CASE	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC126	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC126	CASE	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1
JC134	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC134	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC136	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC136	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0

Foal ID	Affection Status	56567378	56735837	56779448	56785443	56843481	56859971	56861571	56863586	56863692	56889413	56889525	56990492	57022464	57022573	57087413	57117832	57122419	57125689	57139212	57246288	57299129	57318584	57318586	57434860	57503825	57523800	57529010	57532286	57578847	57579230	57703985	57943621	58026623	58029997	58060985	58186973	58268935
JC210	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC210	CONTROL	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC212	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC212	CONTROL	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1
JC213	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC213	CONTROL	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC214	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC214	CONTROL	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC215	CONTROL	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC215	CONTROL	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC216	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC216	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC218	CONTROL	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC218	CONTROL	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC220	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC220	CONTROL	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC222	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC222	CONTROL	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC224	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC224	CONTROL	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC226	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC226	CONTROL	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC228	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC228	CONTROL	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1
JC230	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC230	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC232	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC232	CONTROL	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC234	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC234	CONTROL	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1
JC235	CONTROL	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC235	CONTROL	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC236	CONTROL	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC236	CONTROL	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
JC237	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC237	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
JC238	CONTROL	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1
JC238	CONTROL	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
JC240	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC240	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC241	CONTROL	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	1	1
JC241	CONTROL	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC242	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC242	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC243	CONTROL	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
JC243	CONTROL	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1

Figure 24 Haplotype analysis of a 2.68 Mb region on ECA18 identifies 3 homozygous segments (in black boxes) observed in 4 foals with fetlock contractures and 3 foals from other Phenotype Groups. The foal IDs highlighted in dark green are those bearing the Group 1 Phenotype and IDs in light green are foals from other phenotype groups with 2 copies of at least a segment of the haplotype of interest. At the top of the figure, associated SNP from the case/control study are highlighted in orange, the SNP in blue are within *TTN* or its untranslated regions, and the SNP highlighted in green are those approaching significance from the family validation study.



Score	Chr	from (bp)	to (bp)	from SNP	to SNP
223	7	48711437	49208360	#51957	#51973
199	4	57758145	58495503	#45529	#45542
182	18	56863692	57022464	#22096	#22100
181	3	34267860	35084304	#40452	#40475
181	4	58700536	58889420	#45549	#45554
179	4	27550868	28333817	#44771	#44788
179	4	58656153	58700536	#45546	#45549
179	18	57125689	57299129	#22106	#22109

Figure 25 Homozygosity mapping results indicate a significant region of autozygosity on ECA18 (18:56863692-57022464) that corresponds with the associated genomic interval identified in the case/control Phenotype Group 1 analyses. A score of 182 was determined for this interval resulting in greater than 80% of the maximum score (shown by red line enclosed by black box).

To visualize the LD structure for our subpopulation of Phenotype Group 1 foals (7 affected foals and 23 control foals) for the region 18:56468723-58295606, a LD plot was generated in Haploview for case and control animals independently (Figure 26). Among the 7 affected foals, strong to complete LD is indicated between 5 associated SNP markers (BIEC2-415468, BIEC2-415469, BIEC2-415501, BIEC2-415509, BIEC2-415700) and the candidate locus, *TTN* (Figure 25; top plot). There is notably more decay in LD within this region in the control population (Figure 26; middle plot) and weak or no LD between these SNP and markers associated with *TTN*. The two SNP from the family validation test that approached significance (Figure 26; bottom plot) do not appear to be in LD with one another, nor with any SNP in *TTN,* which may be a reflection of spurious associations or low power. Black diamonds in this figure represent pairwise LD of 1 (complete LD). The dark grey diamonds represent moderate LD, and the light grey and white diamonds indicate weak to no pairwise LD, respectively, between SNP.

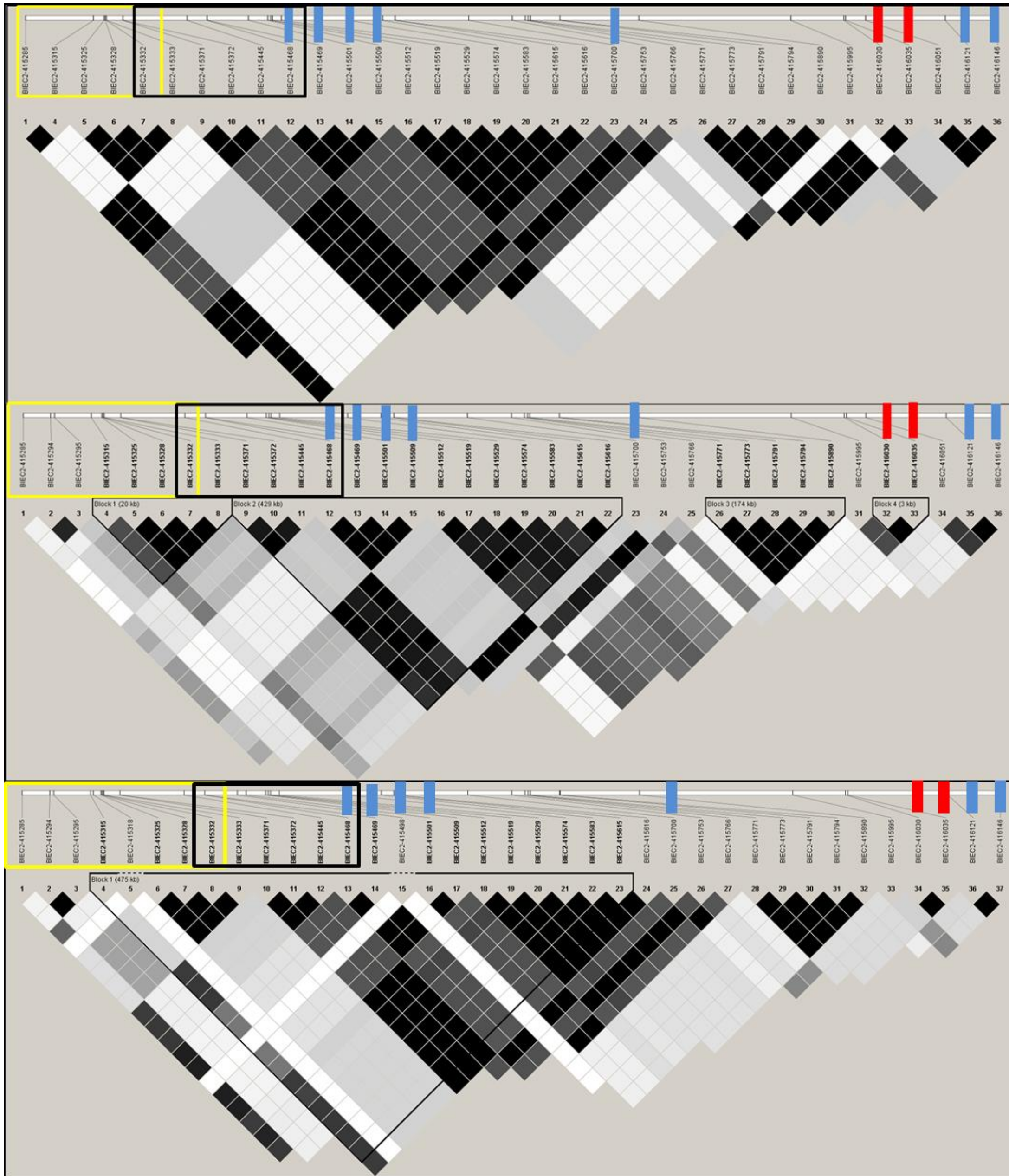


Figure 26 Plots representing the LD structures of genomic region ECA18: 56468723-58295606 using genome-wide genotype data. The top plot represents the LD structure of 7 affected foals with fetlock contractures. The middle plot represents the LD structure of 23 unaffected foals and the bottom plot represents the LD structure of the kindred of 60 individuals. The 7 significant SNP from the Phenotype Group 1 analysis are indicated by the blue bars. The red bars indicate SNP approaching significance from the family validation test. The SNP outlined by the yellow box are those residing within or flanking *TTN* and SNP within the black box are those spanning the region of autozygosity identified in the family data.

Phenotype Group 2 Analysis

Phenotype Group 2 included 3 foals (JC4, JC34, and JC36) with carpal contractures, ligament laxity, and angular limb deformities. Figure 27 represents a plot of the asymptotic *P*-values. There was a strongly associated SNP on ECA26: BIEC2-677817(P_{raw} = 5.51 x 10^{-9}, P_{genome} = 2.3 x 10^{-4}. Additionally, 26 significant SNP spanning an 11.2 Mb region on ECA5 (5:4851904-59705304) were also associated (P_{raw} = 7.79 x 10^{-7}, P_{genome} = 0.033). The genomic inflation factor for this analysis was 1.24. The QQ plot in Figure 28 indicates the possibility of an inflation of the test statistic.

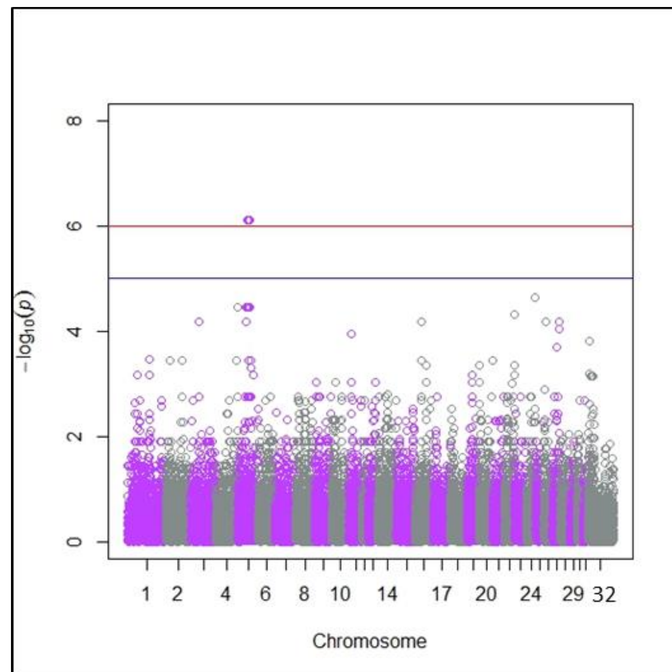


Figure 27 Manhattan plot of $-\log_{10}$ representing permuted *P*-values from the chi-square analysis of phenotype Group 2 (3 affected foals with carpal contractures, angular deviations, and distal limb laxity.

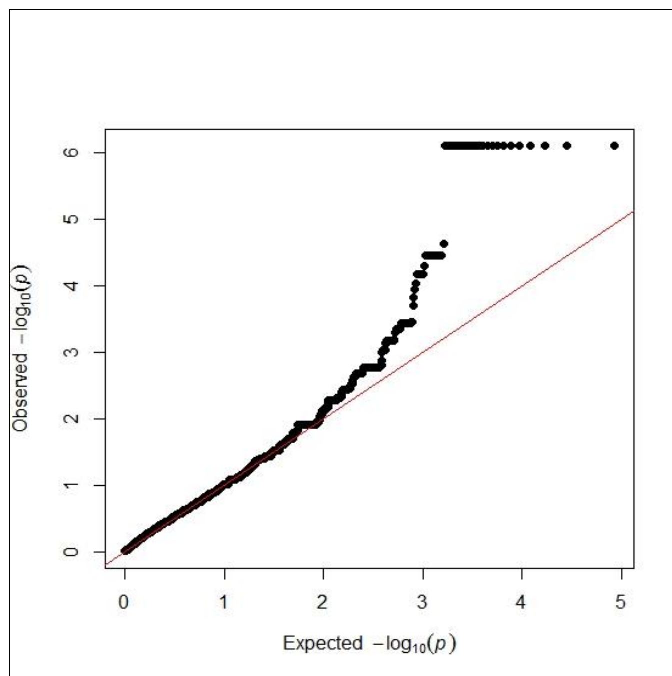


Figure 28 QQ plot of $-\log_{10}$ of *P*-values from the basic chi-square analysis of Phenotype Group 2 (carpal contractures, angular limb deformities, and ligament/tendon laxity).

Haplotype analysis of a 1.6 Mb segment including the one SNP on ECA26 did not support a true association and was likely spurious. We also tested the region from ECA26 in the family data using the DFAM test and no SNP from this region reached significance. When the 11.2 Mb region on ECA5 was tested for association in the family data, 190 SNP were captured in the analysis. From this region, 2 SNP (BIEC2-908677 and BIEC2-908711) with genomic locations of 5:49860757 and 5:50393996, respectively, had *P*-values of < 0.05 ($P = 0.0067$ and $P = 0.0196$) and 7 SNP ranging from 5: 50398104-59394245 had *P*-values of 0.0522-0.0588. Each of these SNP were tested individually with a significance threshold of $\alpha = 0.05$. The *P*-values remained unchanged. A gene search in the 11.2 Mb region

revealed T-Box protein 15 (*TBX15; 5:50,433,744-50479498*), calsequestrin 2, a cardiac isoform (*CASQ2; 5:53134872-53195934*), adenosine monophosphate deaminase 1 (*AMPD1; 5:54062569-54083314*) and actin capping protein-alpha1 (*CAPZA1; 55853345-55897158*) as putative candidates genes.

Haplotypes were generated for the putative candidate genes, *TBX15 (5:48100327-51499985)*, *CASQ2* (*5:52690753- 53728115), ADMP1 (5:53133920- 54885914),* and *CAPZA1* (5:55184021- 56446872), which included approximately 1 Mb of their respective 5' and 3' sequences. Two of the 3 foals included in the Phenotype Group 2 analysis (JC34 and JC36) were found to carry a unique haplotype in all 4 candidate gene regions (highlighted in orange in Figures 29-32). This haplotype was not observed in any control animal. Three additional affected foals from other phenotype groups (JC32, JC59 and JC136) also carry at least one copy of a segment of these haplotypes in all 4 candidate regions. In the region encompassing *TBX15* and *CASQ2*, JC114 also bears a copy of the haplotype of interest and JC98 has one copy of the haplotype spanning *AMPD1*. The affected foal JC91 also had 3 smaller segments of the unique haplotype near the gene *TBX15* (see Figures 29-32 for haplotypes). These particular haplotypes were not observed in the family data. The haplotypes constructed for all 4 candidate regions in the kindred were unremarkable in that all haplotypes observed in the probands were also observed in the unaffected foals (See Appendix C for family based haplotypes of all candidate regions of ECA5).

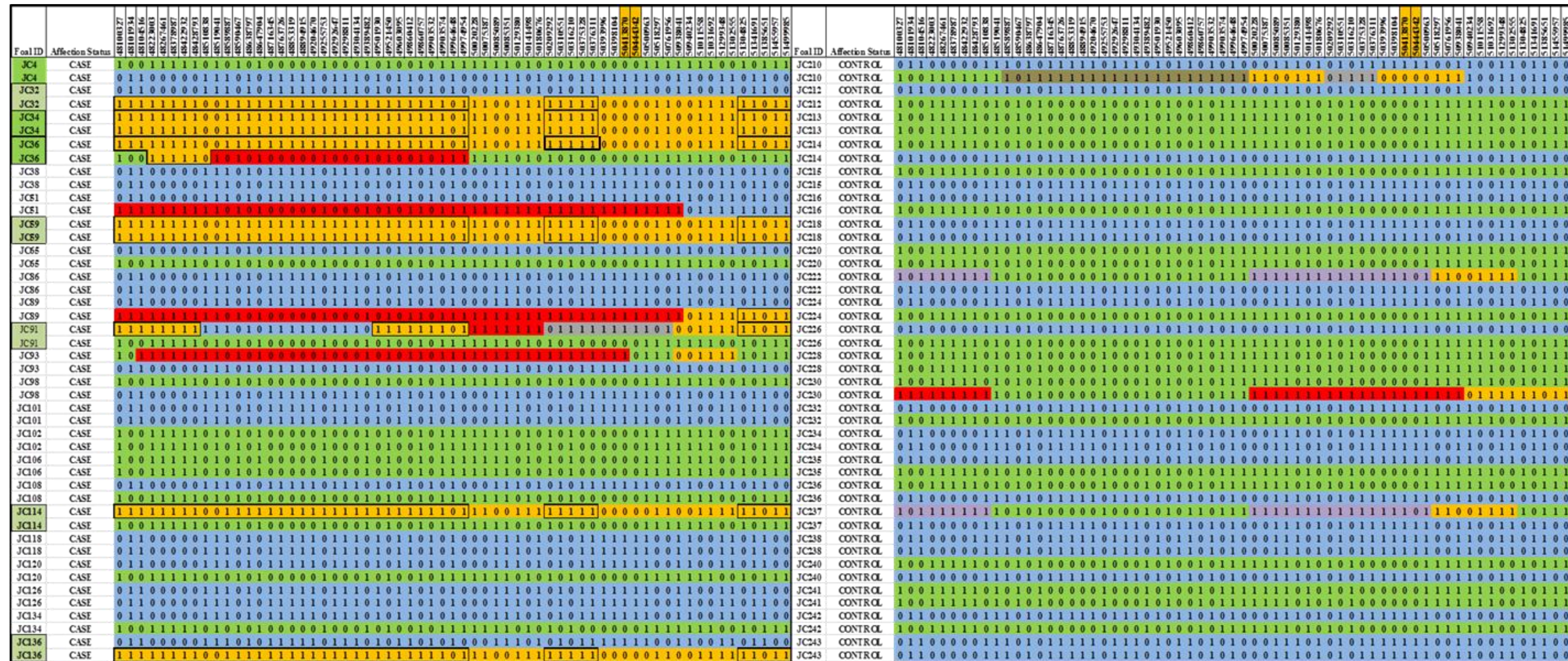


Figure 29 Haplotype analysis of a ~3.4 Mb region on ECA5 including the gene *TBX15* identified 3 unique segments (highlighted in orange and enclosed in black boxes) in 7 affected foals. Foal IDs highlighted in dark green are affected foals from the Phenotype Group 2 analysis. Foal IDs highlighted in light green are foals from other phenotype groups who also have at least partial segments of this haplotype that are not in common with any control animal. At the top of the figure, the SNP coordinates highlighted in orange are those within or closest to the candidate gene.

Foal ID	Affection Status	55184021	55290030	55293804	55294311	55304751	55307605	55317182	55319162	55503606	55519047	55628374	55728630	55758920	55776139	55836086	55898566	56110634	56110906	56177960	56265761	56348491	56392983	56446872
JC4	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC4	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC32	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC32	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC34	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC34	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC36	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC36	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC38	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC38	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC51	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC51	CASE	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC59	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC59	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC65	CASE	1	1	1	F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC65	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC86	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC86	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC89	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC89	CASE	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC91	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC91	CASE	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0	1	1	1	0
JC93	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC93	CASE	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC98	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC98	CASE	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC102	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC102	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC106	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC106	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC108	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC108	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC114	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC114	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC118	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC118	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC120	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC120	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC134	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC134	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC136	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC136	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1

Foal ID	Affection Status	55184021	55290030	55293804	55294311	55304751	55307605	55317182	55319162	55503606	55519047	55628374	55728630	55758920	55776139	55836086	55898566	56110634	56110906	56177960	56265761	56348491	56392983	56446872
JC210	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
JC210	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC212	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC212	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC213	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC213	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC214	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC214	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC215	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC215	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC216	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC216	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC218	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC218	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC220	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC220	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC222	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC222	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC224	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC224	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC226	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
C226	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC228	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC228	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC230	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC230	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC232	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC232	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC234	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC234	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC235	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC235	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC236	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC236	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC237	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC237	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC238	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC238	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC240	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC240	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC241	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC241	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC242	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC242	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC243	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC243	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1

Figure 30 Haplotype analysis of a ~1 Mb region on ECA5 including the gene *CASQ2* identified a 24 marker haplotype (highlighted in yellow) found exclusively in 6 affected foals. Foal IDs highlighted in dark green are affected foals from the Phenotype Group 2 analysis. Foal IDs highlighted in light green are foals from other phenotype groups who also have at least one copy of this haplotype. The haplotype highlighted in orange was not observed in any control animal (left). At the top of the figure, the SNP coordinates highlighted in orange are those within or closest to the candidate gene.

Foal ID	Affection Status	53133920	53136645	53192988	53198662	53206673	53218124	53253842	53346217	53538898	53644387	53728115	53851924	53875750	54025556	54029966	54084267	54195545	54251377	54253387	54312432	54335720	54370558	54502557	54539683	54561516	54611785	54823158	54845379	54885914
JC4	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC4	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC32	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC32	CASE	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	0	0
JC34	CASE	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	0	0
JC34	CASE	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	1	0
JC36	CASE	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	1	0
JC36	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC38	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC38	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC51	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC51	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1
JC59	CASE	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	1	0
JC59	CASE	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	0	0
JC65	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC65	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC86	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC86	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC89	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC89	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1
JC91	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC91	CASE	0	1	1	0	0	1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
JC93	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC93	CASE	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1
JC98	CASE	1	1	1	0	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	0	0	1	1	1	1
JC98	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC101	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC101	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC102	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC102	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC106	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC106	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC108	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC108	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC114	CASE	0	0	0	1	1	1	1	0	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC114	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC118	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC118	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC120	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC120	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC126	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC126	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC134	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC134	CASE	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC136	CASE	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC136	CASE	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	0	0

Foal ID	Affection Status	53133920	53136645	53192988	53198662	53206673	53218124	53253842	53346217	53538898	53644387	53728115	53851924	53875750	54025556	54029966	54084267	54195545	54251377	54253387	54312432	54335720	54370558	54502557	54539683	54561516	54611785	54823158	54845379	54885914
JC210	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC210	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC212	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC212	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC213	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC213	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC214	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC214	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC215	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC215	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC216	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC216	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC218	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC218	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC220	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC220	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC222	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC222	CONTROL	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1
JC224	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC224	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC226	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC226	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC228	CONTROL	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC228	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC230	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC230	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1
JC232	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC232	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC234	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC234	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC235	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC235	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC236	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC236	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC237	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC237	CONTROL	0	0	0	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1
JC238	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC238	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC240	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC240	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC241	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC241	CONTROL	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1
JC242	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC242	CONTROL	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	1
JC243	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
JC243	CONTROL	1	1	1	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1

Figure 31 Haplotype analysis of a 1.6 Mb region on ECA5 including the gene *AMPD1* identified a 19 and 21 marker haplotype found exclusively in 6 affected foals (highlighted in orange and demarcated by black boxes). Foal IDs highlighted in dark green are affected foals from the Phenotype Group 2 analysis. Foal IDs highlighted in light green are foals from other phenotype groups who also have at least one copy of this haplotype. The SNP coordinates highlighted in orange are those within or closest to the candidate gene.

Foal ID	Affection Status	55184021	55290030	55293804	55294311	55304751	55307605	55317182	55319162	55503606	55519047	55628374	55728630	55758920	55776139	55836086	55898566	56110634	56110906	56177960	56265761	56348491	56392983	56446872
JC4	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC4	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC32	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC32	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC34	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC34	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC36	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC36	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC38	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC38	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC51	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC51	CASE	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC59	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC59	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC65	CASE	1	1	1	F	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC65	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC86	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC86	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC89	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC89	CASE	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC91	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC91	CASE	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0	1	1	1	0
JC93	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC93	CASE	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC98	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC98	CASE	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC102	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC102	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC106	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC106	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC108	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC108	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC114	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC114	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC118	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC118	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC120	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC120	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC134	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC134	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC136	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1
JC136	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1

Foal ID	Affection Status	55184021	55290030	55293804	55294311	55304751	55307605	55317182	55319162	55503606	55519047	55628374	55728630	55758920	55776139	55836086	55898566	56110634	56110906	56177960	56265761	56348491	56392983	56446872
JC210	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0
JC210	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC212	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC212	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC213	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC213	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC214	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC214	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC215	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC215	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC216	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC216	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC218	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC218	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC220	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC220	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC222	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC222	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC224	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC224	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC226	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
C226	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC228	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC228	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC230	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC230	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC232	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC232	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC234	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC234	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC235	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC235	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC236	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC236	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC237	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC237	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC238	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC238	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC240	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC240	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC241	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC241	CONTROL	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC242	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC242	CONTROL	1	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC243	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC243	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1

Figure 32 Haplotype analysis of a ~1.3 Mb region on ECA5 including the gene *CAPZA1* identified a 23 marker haplotype (highlighted in orange) exclusively found in 5 affected foals. Foal IDs highlighted in dark green are affected foals from the Phenotype Group 2 analysis. Foal IDs highlighted in light green are foals from other phenotype groups who also have at least one copy of this haplotype. The SNP coordinates highlighted in orange are those within or closest to the candidate gene.

LD plots generated in Haploview indicate that there is consistently more pairwise LD across the 11.2 Mb region on ECA 5 (5:49860757-56392983) in the case animals of Phenotype Group 2 than in the control population (Figure 33). It appears that no associated

SNP are in LD with the genes, *TBX15*, *CASQ2 or AMPD1* but 7 SNP (BIEC2-910026, BIEC2-910111, BIEC2-910126, BIEC2-910155, BIEC2-910173, BIEC2-910227, and BIEC2-910232) are in complete LD with *CAPZA1*. The black diamonds represent SNP pairs with an LD of 1.

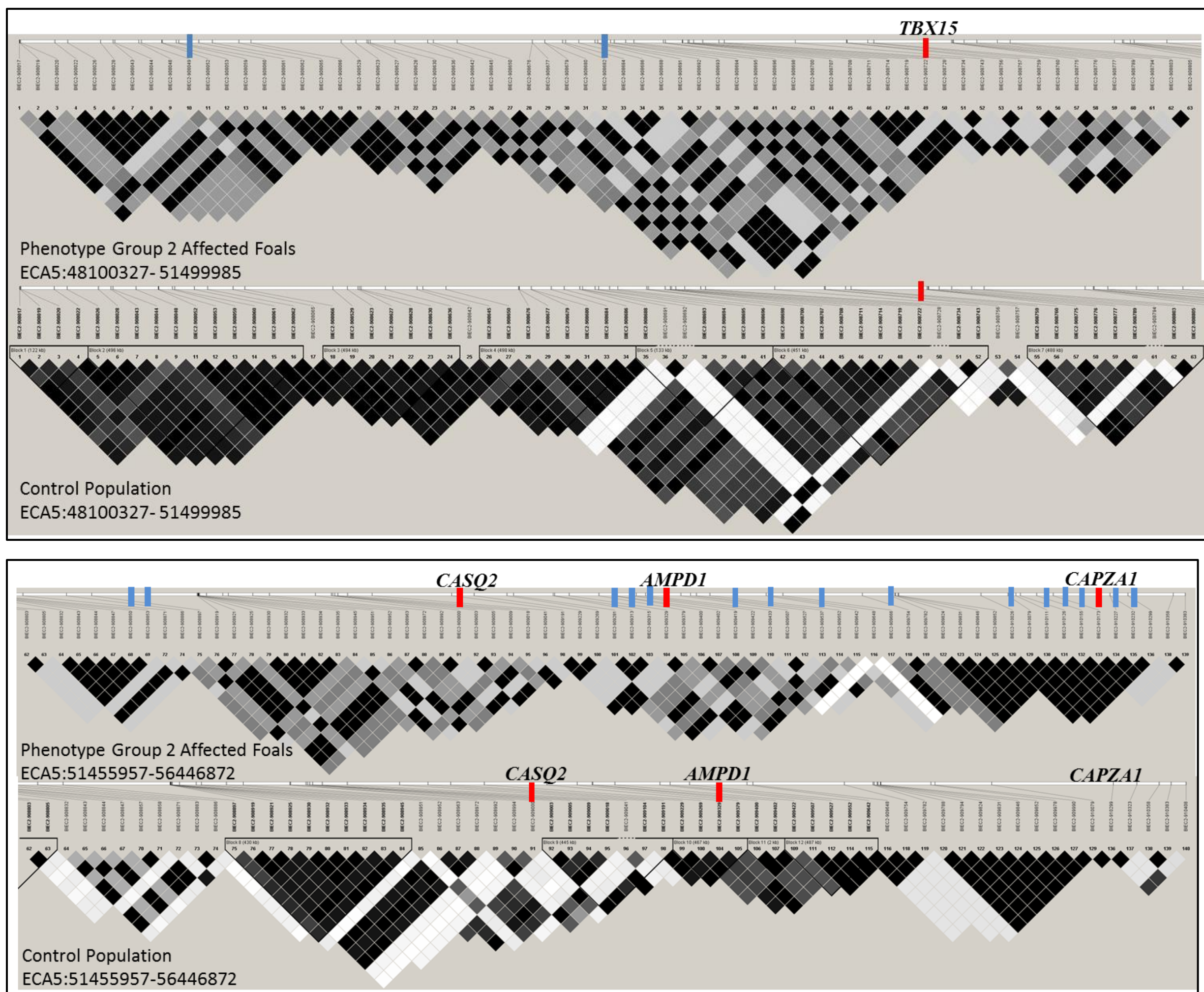


Figure 33 LD structures of affected foals from Phenotype Group1 (top plots) and the control population of 23 unaffected foals (bottom plots). The blue bars indicate associated SNP after Bonferroni correction for multiple testing. The red bars point to the SNP closest to or within the putative candidate genes labeled above each plot.

Phenotype Group 3 and Phenotype Group 4 Analyses

Thus far, our findings support that flexion contractures and CFS could likely be genetically heterogeneous disorders. In our first analysis, associations to SNP on ECA5, ECA13, ECA18, and ECA20 were questionable due to moderately weak *P*-values. However, by phenotypic classification, subsequent analysis provided stronger evidence supporting the existence of disease risk alleles on ECA18 and ECA5. Results from a third analysis based on the phenotype of carpal contractures (Phenotype Group 3), however, were not remarkable after multiple testing corrections even though the basic allelic test identified two SNP on ECA24 that were near the threshold of $P = 1.15 \times 10^{-6}$ (BIEC2-634340, $P=7.74 \times 10^{-6}$; BIEC2-634341, $P=7.74 \times 10^{-6}$). Similarly, Phenotype Group 4 analysis which included 7 foals with carpal *and* fetlock contractures and 23 unaffected foals did not yield a significant result; therefore, no further investigations were carried out for either phenotype group.

Overall, haplotype analysis identified 12 affected foals from the case/control study associated to one or more loci on ECA5 and ECA18 and with some overlap in phenotypes. Table 4 lists the 12 foals, their phenotypes, and their affiliated loci.

Table 4 Affected foals, their respective phenotypes, and the genomic loci each foal is associated with as indicated by haplotype analysis. The boxes highlighted in green denote the affected foals assigned to Phenotype Group 1. The boxes highlighted in yellow indicate affected foals originally assigned to Phenotype Group 2.

Foal ID	Phenotype	*TTN*	*TBX15*	*CASQ2*	*AMPD1*	*CAPZA1*
JC32	Moderate bilateral carpal and fetlock contractures		x	x	x	x
JC34	Severe bilateral carpal contractures; distal limb laxity		x	x	x	x
JC36	Severe bilateral carpal contractures; carpal valgus; distal limb laxity	x	x	x	x	x
JC51	Left Carpal and fetlock contracture	x				
JC59	Mild fetlock contractures x 3	x	x	x	x	x
JC65	Moderate bilateral carpal and fetlock contractures	x				
JC91	Hind fetlock contracture	x	x			
JC98	Bilateral carpal and fetlock contractures				x	
JC108	Severe bilateral front fetlock contractures					
JC114	Moderate to severe bilateral front fetlock contractures		x	x		
JC118	Left front and right hind fetlock contractures	x				
JC136	Bilateral front fetlock contractures	x	x	x	x	x

Family-based GWAS

In addition to utilizing genome-wide genotype data of a kindred to validate findings of the case/control study, we also performed a genome-wide association analysis of the family data with the understanding that low power in terms of sample size may limit results. After quality control filtering of the genotypes from the family cohort, there were 43,467 SNP with an overall call rate of 0.994. Two individuals (JC125 and JC126) were shown to be duplicates and so JC125 was removed from the study.

Three statistical models were used for the test of association including TDT, DFAM, and FBAT. Each model yielded similar results in that there were no SNP that reached the genome-wide significance threshold. A cluster of SNP on ECA 10 in the interval from 10:24251833-25883117 had the smallest *P*-values (TDT $>P_{raw} = 1.8 \times 10^{-4}$ to 4.7×10^{-4};

DFAM >P_{raw} = 2.4 x 10^{-4} to 9.7 x 10^{-4}; and FBAT >P_{raw} = 2.4 x 10^{-4} to 2.8 x 10^{-4}). From a candidate gene search, the collagen 12a (COLXII; chr10:29777861-29883049) gene was of interest considering it modulates the structure of the extracellular matrix of tendons and ligaments and is linked to some myopathies characterized by contractures and muscle weakness (Chiquet *et al.* 2014; Hicks *et al.* 2014). However, haplotype analysis did not reveal any predominance of one haplotype in cases vs. half-sibling controls.

Adaptive permutation was performed on this data set and a cluster of SNP on ECA1 with *P*-values of 0.001 (Figure 34) were of interest due to several SNP in this region residing adjacent to or within the gene, ankyrin repeat domain 1 (*ANKRD1*). This gene is known to act as a nuclear transcription factor and interacts with the *TTN* gene in striated muscle (Miller *et al.* 2003). It also has been reported to co-localize with thrombospondin in adjacent tendon mesenchyme during embryonic development where it is thought to act in a signaling capacity (Baumeister *et al.* 1997).

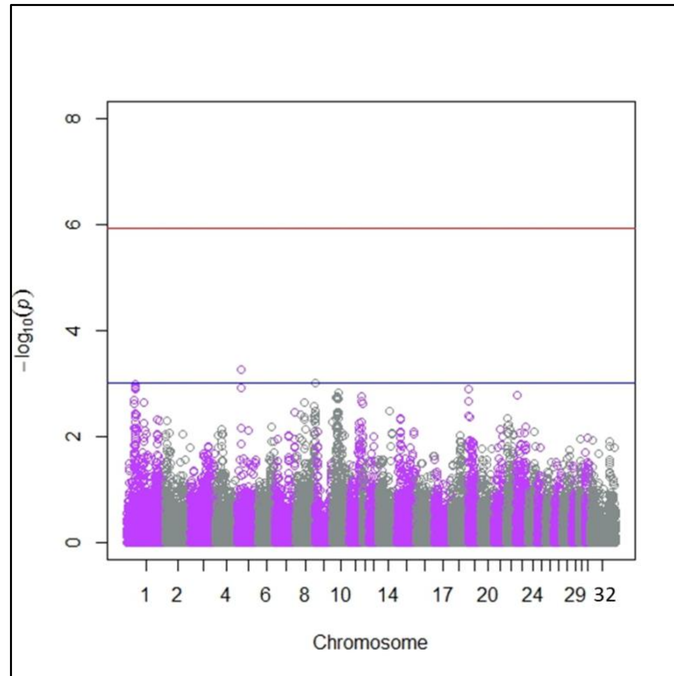


Figure 34 Manhattan plot representing permuted *P*-values ($-\log_{10}$) from the DFAM test of association in a kindred of 60 individuals.

The 3 Mb region (42 markers) containing the weakly associated SNP on ECA1 was phased and one haplotype was identified in much higher frequency in affected foals when compared to control animals. This haplotype was present in two copies in 11 of 15 affected foals with an overall frequency (f) of 0.733 in the case population. Among the related control animals, 3 of the 10 foals carry two copies with an overall $f = 0.3$ in this group.

We hypothesized that, given there is a rare causal variant and this variant occurred recently, it could go undetected in a large haplotype with low recombination using common SNP for association testing. To search for a variant that would differentiate the haplotype, exon sequencing of *ANKRD1* was carried out in 16 case animals and 10 half-sibling controls. In all, 9 SNP were identified. Table 5 describes all oligonucleotides used for exon

sequencing. An A/G SNP (1:37673496) was located in 5′ region of the gene 293 bp upstream from exon 1. Three transversions (A/T, A/C, and C/G) were discovered in intron 1 with genomic locations of 1:37673853, 1:37674385, and 1:37674435, respectively. Three A/G transitions were found in intron 2; 1:37674672, 1:37674686, 1:37675809, respectively. Between exon 7 and exon 8, an A/T transversion and a C/G transition were identified at loci 1:37678910 and 1:37678928. However, no particular SNP genotype was found exclusively in affected animals. See Appendix C for the equine *ANKRD1* sequence and the marked genomic locations of each SNP. From these SNP, a 4 SNP haplotype (ACAA in Figures 35 and 36) was identified in the affected group with f = 0.882 and only f = 0.65 in the half-sibling controls. Figure 35 shows the individual genotypes (and diplotypes) for each SNP and is organized by affection status. Figure 36 also shows the genotypes but is arranged by half-sibling relationships.

Table 5 A summary of oligonucleotide primers and standard conditions used for exon and exon boundary resequencing of *ANKRD1* in normal and affected foals.

Primer Name	Left Primer	Right Primer	Annealing Temp.	Product Size (bp)	Coordinates:EquCab
Ankrd1_Ex1	GTGGTGCAATATCAACAGACAG	TGCAATAGAACTTCCTCCCTAA	64°C	680	chr1:37673422-37674104
Ankrd1_Ex2	GCATCCTCTGTGGAGAGTGAC	GGACCACAAACCACGTTATTG	64°C	609	chr1:37674389-37674998
Ankrd1_Ex3_4	GTCCAAAGCTGACGCTACAAG	TAATGAGCTGGATTTCGCAGT	64°C	651	chr1:37675324-37675974
Ankrd1_Ex5	AGCACTCGAGGTCCTATCTGT	AGCAGACTGCCGTGTATAATG	64°C	590	chr1:37676848-37677438
Ankrd1_Ex6_7	ATTATGCACACACATGCACAC	ATGAAGCTTAAAGAGGCCAAA	64°C	736	chr1:37678360-37679096
Ankrd1_Ex7_8	AAGCTCATGGTGTGCATAGTC	TAGGCACTGAAGTCAAAGCAG	64°C	604	chr1:37678839-37679516
Ankrd1_Ex9	TGGAGATGTATGGAGGCATT	AGGTGCAGCTTTACAACAGG	64°C	554	chr1:37681527-37682031

Affected	1:37673496	1:37673853	1:37674385	1:37674435	1:37674672	1:37674686	1:37675809	1:37678910	1:37678928
JC61	A	A	A	C	A	A	G	A	C
JC69	A	A	A	C	A	A	G	A	C
JC104	A	A	A	C	A	A	G	A	C
JC132	A	A	A	C	A	A	G	A	C
JC134	A	A	A	C	A	A	G	A	C
JC136	A	A	A	C	A	A	G	A	C
JC158	A	A	A	C	A	A	G	A	C
JC160	A	A	A	C	A	A	G	A	C
JC162	A	A	A	C	A	A	G	A	C
JC168	A	A	A	C	A	A	G	A	C
JC172	A	A	A	C	A	A	G	A	C
JC147	A/G	A/T	A	C	A	A	A/G	A	C
JC164	A/G	A/T	A	C	A	A	A/G	A	C
JC170	A/G	A/T	A	C	A	A	G	A	C/T
JC126	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC166	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC 101	G	T					A		
Unaffected									
JC222	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC252	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC254	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC261	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC267	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC273	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC258	G	T	A/C	C/G	A/G	A/G	G	A/T	C/T
JC256	A	A	A	C	A	A	G	A	C
JC269	A	A	A	C	A	A	G	A	C
JC271	A	A	A	C	A	A	G	A	C

Figure 35 Individual genotypes of 9 SNP discovered from exon sequencing of *ANKRD1*. Cells with a single letter are homozygous for that variant. Foal IDs highlighted in red=affected foals and green IDs=unaffected foals. Black boxes demarcate the 4 marker haplotype that is homozygous for all 4 alleles.

ID	1:37673496	1:37673853	1:37674385	1:37674435	1:37674672	1:37674686	1:37675809	1:37678910	1:37678928
JC61	A	A	A	C	A	A	G	A	C
JC104	A	A	A	C	A	A	G	A	C
JC132	A	A	A	C	A	A	G	A	C
JC136	A	A	A	C	A	A	G	A	C
JC252	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC258	G	T	A/C	C/G	A/G	A/G	G	A/T	C/T
JC261	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC162	A	A	A	C	A	A	G	A	C
JC170	A/G	A/T	A	C	A	A	G	A	C/T
JC166	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC273	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC134	A	A	A	C	A	A	G	A	C
JC256	A	A	A	C	A	A	G	A	C
JC254	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC172	A	A	A	C	A	A	G	A	C
JC168	A	A	A	C	A	A	G	A	C
JC267	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC222	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C
JC160	A	A	A	C	A	A	G	A	C
JC164	A/G	A/T	A	C	A	A	A/G	A	C
JC269	A	A	A	C	A	A	G	A	C
JC69	A	A	A	C	A	A	G	A	C
JC271	A	A	A	C	A	A	G	A	C
JC158	A	A	A	C	A	A	G	A	C
JC147	A/G	A/T	A	C	A	A	A/G	A	C
JC 101	G	T					A		
JC126	A/G	A/T	A/C	C/G	A/G	A/G	G	A/T	C

Figure 36 Individual genotypes of SNP discovered from exon sequencing of *ANKRD1* arranged by half-sibling relationships except where singletons are described. Foal IDs highlighted in red=affected foals and green IDs=unaffected foals. Genomic locations are described in the column headings.

Discussion

The biological and/or environmental mechanisms underlying flexion contractures in neonatal foals are poorly understood. The range of phenotypes and varying degrees of joint contracture make elucidating those mechanisms challenging. However, the prevalence and mortality risk associated with these conditions warranted an initiative to begin investigating their etiology. To our knowledge, this study represents the first reported attempt to identify a genetic factor for congenital flexion contractures in foals using a molecular-based approach. Herein, we describe the results of a case/control GWAS, a family based validation study, and a family based GWAS that has led to the identification of at least 2 putative candidate genes associated with the condition, and possibly a third DSL segregating within the Thoroughbred population.

The initial GWAS was conducted based upon the premise that congenital flexion contractures in Thoroughbred foals are caused by a genetic factor; specifically, a DSL of large effect. This proposition was based on (1) the observation of a familial predisposition in several cases and (2) that congenital contractures in other species are caused by mutations in major genes (Bamshad *et al.* 2009; Beck *et al.* 2013a; McMillin *et al.* 2013). An initial power analysis supported our ability to detect a DSL with large effect with the parameters: affected (n=24) and unaffected (n=24), an $h^2 \geq 0.6$ and an estimated r^2 of 0.4 for the Thoroughbred breed (Table 3). However, the genetic (locus) heterogeneity that we now believe exists in the population would likely diminish our power to achieve statistical significance under these parameters, especially when implementing a conservative Bonferroni adjusted significance threshold. This may explain the modest signals detected on

ECA5, ECA13, ECA18 and ECA20 from the first analysis of 23 case animals and 23 controls (Figure 23) (one affected foal and 1 unaffected foal were removed from the original study group).

When we subgrouped individuals by phenotype and performed subsequent analyses, this reduced our sample size of case animals significantly, which may have inadvertently increased the likelihood of false positive associations. The respective QQ plots (Figures 23 and 28) may be reflective of this due to some early departure of the test statistics from the expected distribution that was observed. We believe however, that SNP on ECA5 and ECA18 passing multiple testing corrections are likely true positive associations to flexural deformities in some cases. First, the same genomic region on ECA18 has consistently shown some degree of association to the condition in almost all of the analyses performed including the original case/control GWAS, the Phenotype Group 1 analysis, the family validation study, and from the homozygosity mapping approach. Secondly, SNP on both ECA5 and ECA18 passed multiple testing corrections by Bonferroni standards with minimal evidence of genomic inflation. And lastly, phasing of these regions identified unique haplotypes found exclusively in select case animals from the case/control study group (Figures 24, 29-32).

Congenital joint contractures in human neonates are a clinical feature found in over 300 disorders (Bamshad *et al.* 2009; Hall 2014). Mutations in at least 7 major genes have been identified in various subtypes of the distal arthrogryposes (DA) (Beck *et al.* 2013a), a set of musculoskeletal disorders similar to flexural deformities and CFS in foals. When reporting on the characterization of DA10, Bamshad and colleagues (2006) emphasize how phenotypic classification of the DA disorders facilitated the discovery of several genes linked

to the various subtypes of DA. Following this basis, we performed GWAS on subgroups of affected foals classified by specific phenotypic criteria. The results, a 2 fold decrease in –log10 *P*-values of SNP on ECA5 and ECA18, indicated that a different major locus was associated with at least two types of clinical descriptions; specifically, fetlock contractures (ECA18) and then, carpal contractures accompanied by carpal valgus deformities and distal limb ligament laxity (ECA5). Haplotype analyses of these regions, however, suggest that there can be some overlap of phenotypes but this finding is not surprising. Beck *et al.* (2013a) describe similar findings with subtypes of DA in human neonates caused by major genes and propose that in some cases, two overlapping phenotypes may actually be extremes of the same disorder rather than two separate ones. Moreover, if there are indeed causal mutations, there may be varying degrees of penetrance in the population as well as variable expressivity among individuals which also could explain the overlapping phenotypes. Overall, the outcomes of the case/control GWAS by phenotypic classification (Phenotype Group 1 and Phenotype Group 2) and 23 control animals, suggest that instead of one major locus in the general population causing these deformities, locus heterogeneity in the population is more plausible.

Family models are robust against locus heterogeneity and a recommended method for validating results of a case/control GWAS when more than one locus is suspected to be segregating in a population (Laird & Lange 2009). We took this approach and although there were select SNP from the ECA5 and ECA18 regions that approached significance in the family, haplotype analysis did not support an association. However, this may be due to variation in LD structure or SNP filtering during the quality control process. Homozygosity

mapping of the family genotype data, however, did identify a significant region of autozygosity on ECA18 (Figure 25) that bridges the region between associated SNP from case/control Phenotype Group 1 analysis and the candidate gene, *TTN*. Autozygosity in this region would support the findings of the haplotype analysis in the case/control population, in that, if a DSL exists, it is likely recessive in nature.

The LD structure of the case population including 7 foals with fetlock contractures (Phenotype Group 1) showed moderately strong pair-wise LD between 4 significant SNP and two SNP in the *TTN* gene (Figure 26), and when evaluating the control population, very little LD was noted between these SNP and the candidate locus. A future potential implication of this finding is that SNP BIEC2-415468, BIEC2-415469, BIEC2-415501, and BIEC2-415509, serving as tag SNP, can possibly be used for predicting risk of distal limb contractures. It should also be noted that *TTN* is known to have developmental isoforms in other species (Guo *et al.* 2010). In humans, the N2A isoform predominates in skeletal muscle and is notably larger during fetal development with a tendency to gradually shorten towards gestation end. The shortest N2A isoform is observed postnatally and at maturity (Kruger *et al.* 2006; Guo *et al.* 2010). This means that there is a possibility that the prenatal isoform of *TTN* in equines may also be larger than the sequence provided by the Ensembl and USCS genomic databases. Thus, our significant SNP could be closer to the gene than what is shown by our data. Isoform characterization of *TTN* will be important work for the future.

The analysis of Phenotype Group 2 foals (presenting with carpal contractures, carpal valgus deformities and/or distal limb laxity) provided evidence of an association to a large, ~11.2 Mb region on ECA5 (5:4851904-59705304) (Figure 27). We turned to haplotype

analysis of this muscle function gene rich region to narrow down the DSL interval. This approach proved to be informative in that unique haplotypes exclusive to case animals in all 4 gene regions were identified but failed to single out any one gene as the best candidate. However, when we evaluated the LD between significant SNP and the candidate loci, we only observed complete LD between 4 of the significant SNP and *CAPZA*1. Little to no LD was noted between significant SNP and *TBX15*, *CASQ2*, or *AMPD1* among the affected foals.

Titin is an extraordinarily good candidate for flexion contractures. It is a giant protein that spans half the length of sarcomeres and plays strategic roles in myofibrillogenesis of striated muscle, maintenance of sarcomere integrity, and most notable, its role in the control of passive tension properties of skeletal muscle (Hackman *et al.* 2003; Neagoe *et al.* 2003; Granzier & Labeit 2004; Selcen & Bushby 2005; Kruger *et al.* 2006; Linke 2008; Luther 2009; Ottenheijm *et al.* 2009a; Ottenheijm *et al.* 2009b; Guo *et al.* 2010; Linke & Kruger 2010). What is even more significant is that *TTN* is thought to indirectly interact with *CAPZA1* at the Z-disk of myofibers (Papa *et al.* 1999; Pappas *et al.* 2008). And interestingly, *TTN* also bears binding sites for ankyrin-repeat domain 1 (*ANKRD1*), a nuclear transcription factor preferentially found in striated muscle (Miller *et al.* 2003; Linke 2008) and identified to be weakly associated to flexion contractures in the family GWAS. If causal variants are found in more than one of these loci, and they are segregating independently in the Thoroughbred population, these findings could provide much insight as to how disrupted members of a subsarcomere protein network can autonomously lead to prenatal myodysgenesis.

A third and fourth test of association of Phenotype Group 3 (carpal contractures) and Phenotype Group 4 (carpal and fetlock contractures) did not result in significant outcomes after multiple testing corrections. In all, 12 of 23 affected foals were found to be associated with a chromosomal region on either ECA5 or ECA18 (Table 5). Possible explanations for why 11 affected foals did not show any genomic associations include: (1) there is a third DSL segregating in this study population but was not detected due to a loss of power from locus heterogeneity or smaller sample sizes, or (2) there are non-genetic factors at work causing contractures in these foals.

A GWAS of the family genotype data did not yield an association to ECA18 or ECA5 but low power due to sample size could have been a confounding factor. However, a weak association to ECA1 (Figure 34) was of interest due to SNP with relatively low *P*-values being in close proximity to or within the gene *ANKRD1*, a nuclear transcription factor found in the sarcomeres of striated muscle (Miller *et al.* 2003; Tee & Peppelenbosch 2010). Sequencing of the exons and exon boundaries of *ANKRD1* (9 exons) was within the scope of this project and resulted in the identification of 9 SNP within the gene (Figures 35 and 36). From these SNP, a 4 marker haplotype was found in higher frequency in case animals (f=0.882) when compared to unaffected foals (f=0.65) and was found to approach significance when 3 and 4 marker sliding windows of this haplotype were tested for association (P = 0.03 with α = 0.02 and α = 0.0125, respectively). Although no SNP was found exclusively in affected foals, it is clear that there is a genetic disparity between the case and control animals. Deeper sequencing into the 5' and 3' regions of the gene may result

in the identification of a variant in a regulatory region altering the expression of the gene and may be the focus of future studies.

In summary, we conclude that there are susceptibility loci on ECA5 and ECA18 that predispose foals to congenital contractures in some cases. There may be a degree of consistency in phenotypes associated with these DSL but variations of those phenotypes may also be observed which may imply variability in penetrance and expressivity of the alleles. The LD structures of both Phenotype Group 1 and Phenotype Group 2 case animals support the association of significant SNP to candidate genes in the two regions. Titin (ECA18) and *CAPZA1* (ECA5) are strong candidates for flexion contractures considering the crucial role each play in sarcomere signaling, musculoskeletal development, striated muscle contractility, or sarcomere structure.

The validation of these loci in the family data did not provide overwhelming evidence that loci on ECA5 and ECA18 are associated with congenital contractures in this particular lineage. However, the family-based GWAS did guide us to a region on ECA1 harboring a gene (*ANKRD1*) that directly interacts with *TTN* and although no variants were found exclusively in affected foals, a significant discrepancy was noted in a 4 SNP haplotype within *ANKRD1* when comparing case and control animals. The gene, *ANKRD1,* is known as a transcription co-inhibitor, is a member of the titin N2A complex and translocates to the nucleus in response to stretch in striated muscle. Upregulation of *ANKRD1* has been associated with certain pathological conditions such as dilated cardiomyopathy (Wei *et al.* 2009) by repressing expression of sarcomeric proteins or by disruption of normal stretch-based signaling ultimately causing contractile dysfunction (Moulik *et al.* 2009). Therefore,

mutations in promoters and other cis-acting regulatory elements should not be ruled out considering these can lead to perturbations in gene expression. The complexity of gene regulatory elements is underscored by de Vooght *et al.* (2009) but these investigators also emphasize the importance of promoter mutation analysis in terms of disease diagnosis. They encourage routine labs and research groups to pursue gene promoter mutation research, an area of great importance for understanding genetic diseases. The potential relevancy of *ANKRD1* to flexion contractures and CFS is too great not to pursue further investigations and should include the identification of promoters and other regulatory elements.

Overall, these finding support our initial hypothesis of a DSL of large effect causing congenital flexion contractures in Thoroughbred foals. Yet, instead of one major gene, there are likely 2 or more genes of large effect segregating independently in the population that lead to similar phenotypes. However, not all cases may have a genetic basis and other extrinsic mechanisms that could be causing flexural deformities should be evaluated for in future studies.

CHAPTER IV

CLASSIFICATION AND DELINEATION OF CONGENITAL JOINT CONTRACTURES AND CONTRACTED FOAL SYNDROME IN THE NEONATAL FOAL

Introduction

Contracted foal syndrome (CFS), characterized by flexion contractures and other congenital anomalies, is a condition that is challenging to investigate for a pathogenesis due to the inconsistency of clinical features that may be observed from case to case. By clearly defining and classifying a clinically variable disorder such as CFS, we can achieve several things in terms of clinical relevancy. First, clinical descriptions are often vague, ambiguous, and inconsistent; therefore, a classification system should enable clinicians and researchers to more effectively communicate the disorder(s) through clinical documentation. Secondly, distinct phenotypes can be identified and associated with optimal treatment protocols and more accurate prognoses. And lastly, accurate phenotypic characterization is essential for designing appropriate experiments when investigating the pathoetiology. After reviewing 242 case documents on contracted foals, it is apparent that more clearly defined clinical descriptions are needed before a classification system can be implemented with sufficient utility; however, we have developed a *preliminary* classification system for CFS that will be tested and improved upon as more clinical and research data is collected. Over time, we intend to use these data to serve as the infrastructure for molecular-based studies, which will inevitably lead to a more sophisticated classification system by incorporating biological networks and pathways.

Methods

Data Collection

The first task in developing our classification system was to gather data on foals diagnosed with flexion contractures or CFS. Medical records (2003-2011) were provided by Hagyard Equine Medical Institute in Lexington, Kentucky, 4 Kentucky breeding farms, Peterson and Smith Equine Hospital in Ocala, Florida, and necropsy reports from Texas A&M University College of Veterinary Medicine and Biomedical Sciences.

Breed, gender, foal size, gestation length, number of foals euthanized, and number of dystocia births were also recorded when the information was available. Sizes of the foals was documented as small, average, and large and gestation length as pre-term, term, and post-term as this descriptive terminology was most often used.

A category or subcategory was only given to phenotypes occurring 3 or more times. Until more data are collected, the occurrence of singular phenotypes will be considered sporadic and potentially isolated events.

Classifying Congenital Flexion Contractures and CFS

Cases selected for inclusion in this study were foals born with at least one contracted joint requiring medical intervention. From this cohort, we placed foals into two groups. The first group (Group I) included individuals with joint contractures only and the second group (Group II) consisted of foals with joint contractures and other physical abnormalities.

Next, Group I foals were divided into 3 subgroups based on proximal or distal joint involvement. From Group II foals, categories were only created for phenotypes occurring at least 3 times and were based on the nature of the clinical features. For instance, groups CFS IIA-IIC all describe abnormalities of the limbs (in addition to flexion contractures) but are considered different clinical findings.

Results

After reviewing all records, 242 cases were selected for participation in this retrospective study. Of these, 6 breeds of equids were identified: 216 Thoroughbreds, 5 Standardbreds, 4 Quarter Horses, 2 appendix (TB/QH), 1 Hanoverian, and 1 mule. Gender descriptions were only available for 131 cases. Of these, males comprised 55% (72/131) of cases and females, 45% (59/131). Size of foal was described in only 59 records in which small foals constituted 19% (11/59), average 49% (29/59), and large foals 32% (19/59). In 35 records, gestation length was documented. Pre-term foals represented 14% (5/35), term, 74% (26/35), and post-term, 11% (4/35). Out of 242 cases, 76 foals were reported to have been euthanized and 34 were dystocia births.

Preliminary Classification System

From Group I foals, the following categories were established:

- Flexion contractures only (170)
    - CFS IA: distal limb contractures affecting the distal interphalangeals and metacarpophalangeals (clubfoot and/or fetlock contractures) (22%)
    - CFS IB: proximal limb contractures affecting the carpi or tarsi (73%)
    - CFS IC: distal and proximal joint contractures (5%)

From Group II foals (72):

- Flexion contractures and other abnormalities of the limbs.
    - CFS IIA: angular limb deformities (valgus and varus) (16%)
    - CFS IIB: ligament/tendon laxity (dropped fetlock phenotype) (16%)
    - CFS IIC: limb bone malformations (bony protuberances, polydactyly, and other bone deformities) (4%)
- Flexion contractures and malformations of soft tissue structures not associated with the appendicular skeleton.
    - CFS IIIA: entropion (18%)
    - CFS IIIB: patent urachi (15%)
    - CFS IIIC: entropion and patent urachi (7%)

CFS IV: abdominal wall defects (herniations) (6%)

- Flexion contractures and malformations of the axial skeleton.
    - CFS V: scoliosis, spina bifida, roach back, wry nose, prognathism (14%).
- Other
    - CFS VI: overlap of clinical features from CFS II, III, V, or VI (4%)

Grading Ucale

The grading scale is used to describe the degree of contracture from a normal anatomical position (Figures 37 and 38). The number “1” represents mild contractures up to 15° towards flexion. Treatment duration is normally 1-3 days and the outcome is very favorable. A severity assignment of “2” indicates the degree of flexion is between 15° and 30°. Treatment duration can be up to 2 weeks. Prognosis is generally favorable but is often contingent on secondary complications. Severe contractures are represented by the number “3” and the degree towards flexion is > 30°. Treatment duration can be one week to several weeks but in the most severe cases, treatment is often terminated within two weeks due to a poor prognosis for an athlete or pasture soundness (Hunt, 1997).

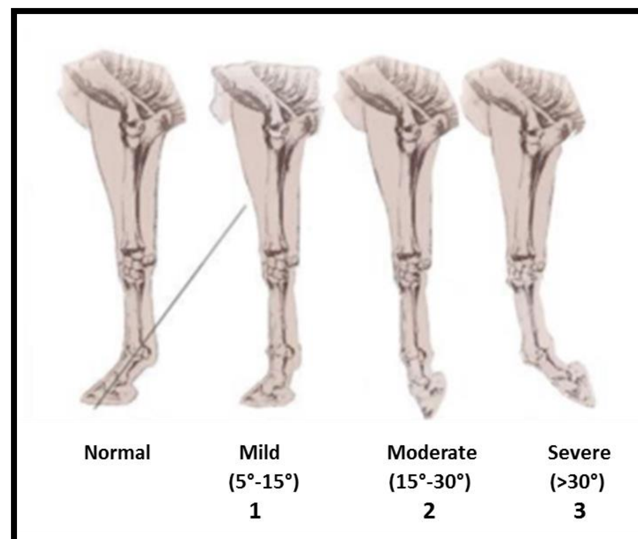


Figure 37 Illustrations depict examples of mild, moderate, and severe contractures of the metacarpophalangeal joint measured in degrees from the zero position (assuming normal anatomical position). Illustrations by J. Eisenrich

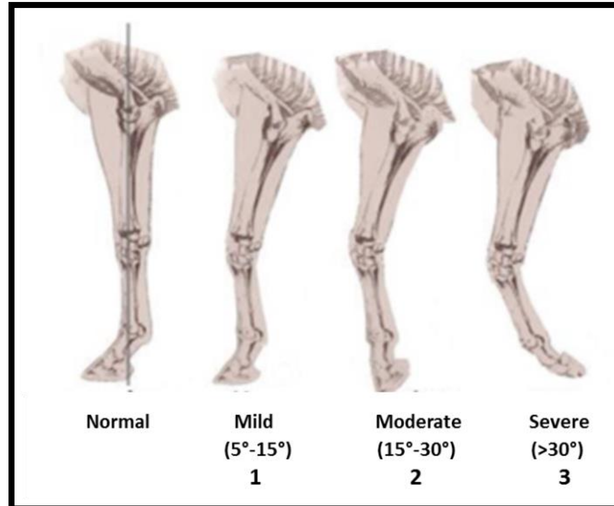


Figure 38 Illustrations depict examples of mild, moderate, and severe contractures of the carpus measured in degrees from the zero position (assuming normal anatomical position). Illustrations by J. Eisenrich.

Discussion

For over a century, classification models have been proposed with the attempt to characterize various congenital anomalies. Many of the bases for classification include; (1) morphological similarities, (2) severity, (3) complexity, (4) familial or sporadic occurrences, (5) etiological origin (genetic, neurological, or environmental), (6) types of defects (failure of formation, failure of differentiation, or duplication), or (7) patterns of occurrence (Bamshad *et al.* 2009; Manske & Oberg 2009). These methods are often criticized for being too simplistic and fall short in some aspect of describing the conditions or providing insight into etiology. However, the nature of deformities will generally indicate the logical approach to take. For example, the International Federation of Societies for Surgery of the Hand (IFSSH) established a classification system on hand and arm deformities in humans based on specific

types of developmental malformations such as failure of formation, failure of differentiation, and duplication. Currently, attempts are being made to incorporate molecular mechanisms. Manske and Oberg (2009) discuss the classification and developmental biology of congenital anomalies of the hand and upper extremity in human neonates. They report that targeted disruption of organizing tissues (ectoderm, mesoderm, and endoderm, respectively) and their associated molecular pathways have provided much insight into causative mechanisms of congenital limb defects. Using animal models, they demonstrated that transverse deficiencies can be generated by removing the ectodermal ridge or by disrupting the fibroblast growth-factor signaling pathway. However, classifying deformities based on these criteria is challenging due to the complex nature of signaling pathways within and amongst germ line tissues and would require years of continued research in equines before it could be accurately implemented.

In 2009, Bamshad and colleagues provided an update on the current status of arthrogryposis and the classification of the distal arthrogryposis (DA) disorders in humans. Included were discussions of diagnostic criteria that could be applicable during clinical assessment of foals with CFS. It was first suggested to rule out a neurological basis. If the patient has normal neurological function, then the contractures are likely due to amyoplasia, a distal arthrogryposis, a connective tissue disorder, or fetal crowding. Because contractures due to amyoplasia are generally associated with the replacement of limb muscle with fibrous and fatty tissue (Hall *et al.* 1983), histological examination may facilitate diagnosing the condition.

Classification of F istal Crthrogryposis F isorders in J umans

The DAs are a group of genetic disorders with contractures of the distal limbs, limited proximal limb involvement, and variable expressivity and has served as a model disorder when investigating a genetic basis for CFS in Chapter 3. Presently, there have been 10 distal arthrogryposes described. Classification was established according to a hierarchical system and based on the proportions of clinical features they share. For example, DA1 and DA2 share more of an overlap of clinical signs than do DA1 and DA3. Major diagnostic criteria have been established to distinguish the DAs. For example, DA1 includes individuals with distal contractures and other abnormalities of the limbs and is the mildest form of DA. Distal arthrogryposis Type 5 describes individuals with congenital contractures and ocular abnormalities suggesting a common factor among skeletal muscle of the limbs and extraocular muscles. As discussed in detail elsewhere, entropions were not uncommon in cases of contracted foals. These are inversions of the lower eyelid and are thought to be caused by retractor muscle dysgenesis (Pereira *et al.* 2010).

Because the nature of defects associated with CFS are somewhat congruent with those observed with the DAs, we used the DA system as a guide for classifying CFS. In addition to the DA system, we considered the preliminary data from our *by phenotype* genome-wide association analyses. Particular phenotypes had stronger associations to certain genomic regions. For example, distal contractures were strongly affiliated with a region on ECA18 and foals presenting with distal and proximal contractures, ligament laxity, and angular limb deformities were associated with a region on ECA5. These data suggest limb contractures in foals, in some cases, are major gene disorders with genetic heterogeneity

existing in the Thoroughbred population, that is, more than one major gene can cause the same or similar pathological trait. Furthermore, there are different modalities for the spatial development of limb tendons. It is reported that *distal* tendon formation occurs in sync with limb skeleton formation, whereas *proximal* tendons form in accordance with their respective muscles (Edom-Vovard & Duprez 2004). This supports that a classification system should allow for the distinction of distal or proximal joint involvement.

CFS IA, IB, and IC

We gave foals born with just flexion contractures exclusive classification categories primarily because these are the simplest phenotypes observed among the case histories. The 3 subclassifications, IA, IB, and IC, were created on the basis of distal or proximal (or a combination of the two) joint involvement. The rationale is based on evidence that there may be a familial predisposition, in some cases, to contractures of a particular joint type. For instance, we documented one mare producing foals with bilateral carpal contractures in years 2005, 2007, 2008, and 2009. Another mare was reported having foals born in 2004 and 2006 with moderate to severe left hind fetlock contractures. We have at least 3 other records in which mares produced foals consecutively with fetlock contractures. Moreover, preliminary data from a genetic study indicate that distal joint contractures may be a major gene disorder (Chapter 3). The outcome of that study emphasizes the need to make careful distinctions between phenotypes as each may warrant a different experimental design and analysis model (as opposed to more complex phenotypes) when investigated for an etiology.

CFS IIA, IIB, and IIC

From the case records, angular limb deformities (ALD) such as valgus and varus deviations of the distal and proximal joints and joint hypermobility were often associated with flexion contractures. Angular limb deformities are lateral or medial deviations in the frontal plane. This type of deformity is difficult to classify because the causes can be soft-tissue related (dysgenesis of muscle, tendon, or ligament) or bone-related (disturbances in the ossification process) (Caron 1988) and therefore, potentially differing pathogeneses. Musculoskeletal abnormalities that may lead to ALDs in foals include (1) immature periarticular soft tissues, (2) dysplasia of the metaphyseal growth plate and epiphyseal ossification center, (3) incomplete ossification of the carpal and tarsal cuboidal bones, (4) dysplasia of the metaphyseal growth plate and epiphyseal ossification center sepsis (Caron 1988). Although periarticular ligament laxity may cause ALDs, we refer to joint hypermobility as lax or loose tendons or ligaments and/or associated muscles that cause a "dropped" fetlock appearance (flexural deformity in the sagittal plane) in which the palmar aspect of the fetlock is abnormally low to the ground. We gave the two types of limb deformities (ALDs and ligament/tendon laxity) separate but related categories because they are both abnormalities of the limbs but medical records describe them as clinically different conditions. A third subcategory within this classification group (CFS IIC) is reserved for cases in which foals are born with flexion contractures and anomalies of the bony tissue other than at the joint surfaces such as protuberances and polydactyly.

A molecular perspective of osteodysgenesis

A multitude of genes such as Sonic hedgehog, fibroblast growth factors, *WNT*, and *SOX* genes play key roles in intra and intercellular communication directing skeletal limb development. Mutations in these genes have been linked to various osteodysplasias in other species (Gilbert 2010). Furthermore, it is now known that physeal growth is directed by a sophisticated network of interacting biomolecules including parathyroid hormone related protein, its receptor (parathyroid hormone related protein receptor), and Indian hedgehog, a regulator of bone growth and differentiation as well as a myriad of growth factors and other signaling molecules (Auer 2006).

Incomplete ossification

The limb skeleton develops predominately by endochondral ossification, the replacement of cartilage with bone (Vega *et al.* 2004). Chondrocytes produce cartilage and during the ossification process, these cells undergo hypertrophy and apoptosis as the matrix they secrete changes and causes mineralization. The transcription factor, *RUNX2* mediates this process and is regulated itself by histone deacetylase 4 (*HDAC4*). When *HDAC4* is overexpressed in the cartilage of the ribs or limbs, ossification can be delayed (Vega *et al.* 2004). Incomplete ossification of cuboidal bones can be seen in neonatal foals and has been observed in foals with contractures.

Limb skeleton development is an important process in equines to understand in a biological sense considering good conformation is associated with soundness and long term athletic durability (Anderson *et al.* 2004; Weller *et al.* 2006). Hence, continued research into

the identification of the molecular pathways and networks leading to angular limb deformities and joint hypermobility in equines is much needed. The ability to carry out molecular fingerprinting for the various phenotypes will facilitate the development of the highest resolution classification system and greatly aid in the development of novel therapies.

CFS IIIA, IIIB, and IIIC

Bilateral or unilateral entropions and patent urachi were often documented in contracted foal cases and it was not uncommon to see the two clinical features co-occurring. Because of this co-occurrence, we gave them related subcategories. Entropions are inversions of the eyelid margins and generally require surgical correction (Brooks 2002). Factors thought to cause entropions in humans include (1) lower eyelid retractor dysgenesis, (2) structural defects in the tarsal plate, and (3) shortening of the posterior lamella. Abnormalities of the tarsal plate are usually associated with upper eyelid entropions (Pereira *et al.* 2010). In placental mammals, the bladder is connected to the allantois by the urachus during fetal development and does not generally persist postnatally (Peyton 1981; Robinson 2009). However, the urachus can fail to obliterate perinatally and leads to a leakage of urine from the umbilicus (Peyton 1981; Robinson and Sprayberry 2009). Although both structures involve smooth and skeletal muscle (Gilbert 2010), it is not known if these seemingly unrelated defects actually share a common early developmental pathway and therefore share in their pathogeneses. However, there are many reports describing how mutations in one gene expressed during early stages of developmental can lead to complex phenotypes. One example includes Piebald syndrome which is characterized by deafness, anemia, underpigmentation, sterility, and gut malformations. It is caused by a mutation in the KIT

gene which is expressed in neural crest cells during embryonic development (Giebel & Spritz 1991).

CFS IV

The co-occurrence of axial skeleton defects with flexion contractures is less common than the phenotypes previously described and are more often associated with severe cases of CFS. Overall, they represent 14% of cases involving flexion contractures and other physical anomalies. Because these cases specifically involve structures of the head, neck, and vertebral column, we created an exclusive category to represent these types of malformations. From all the clinical manifestations documented fitting this category, vertebral defects were the most common. Molecular and environmental factors have been reported to cause these conditions, therefore it is critical that these cases are well-documented and that other potentially contributing variables are included in the record.

Potential causes and mechanisms of vertebral anomalies, wry nose, and prognathism

Scoliosis and other vertebral defects have been associated with similar disorders to CFS in other species (Abbott *et al.* 1986; Agerholm *et al.* 2001; Bamshad *et al.* 2009; Tejedor *et al.* 2010; Kimber *et al.* 2012). In early vertebrate embryogenesis, the mesoderm segments into pairs of somites that surround what will eventually become the spinal cord. As the somites mature, they further segment into the sclerotome, myotome, and dermamyotome. Sclerotomal cells migrate to the developing spinal cord and eventually form the vertebral column (Erol 2002). The proper patterning and segmentation of somites during somitogenesis is essential for normal vertebrae formation. Studies in animal models have

identified environmental and genetic factors that can disrupt somitogenesis resulting in abnormally formed vertebrae as seen with congenital scoliosis in human neonates (Erol *et al.* 2002). Moreover, the study by Erol and colleagues (2002) indicate that renal, cardiac, and *lower limb anomalies* are not uncommon when this disruption occurs. The "notch" family of genes regulates cell-fate determination and embryonic patterning in animals and disruption of the notch pathway in mice has led to segmentation defects and vertebral anomalies (Erol *et al.* 2002). It is now believed that in some cases of congenital defects, environmental factors interfere with the delivery of genetic instructions during embryogenesis and the magnitude of their effects is dependent upon the time and extent of exposure (Erol *et al.* 2002). For example, induced transient hypoxia resulted in malformations of the axial skeleton in mice. The location of the vertebral defects was correlated with the time during development and arose from the somites being formed at that time (Erol *et al.* 2002). Non-molecular mechanisms have also been proposed. A study on dystocia births in horses suggested scoliosis of the head, in combination with limb deformities and torticollis, were the result of a space constraint on the cranial half of the fetus due to the narrow tip of the uterine horn (Vandeplassche *et al.* 1984).

Wry nose is the most common congenital defect of the nasal passages in horses. The etiology is unknown but uterine conditions and genetic factors are suspected (Tremaine & Dixon 2002).

In other species, disease susceptibility loci have been associated with mandibular prognathism (Cruz *et al.* 2008) but little is known about its pathogenesis in equines. It is a rare occurrence but has been described in cases of CFS. In foals, this condition has been

associated with low thyroid hormone levels and is often accompanied by other severe developmental defects, primarily flexural and angular limb deformities, immature carpal and tarsal bones, and common digital extensor rupture (McLaughlin *et al.* 1986).

CFS V

We documented 3 foals as having flexion contractures with inguinal, umbilical, or abdominal wall hernias. CFSV was established to distinguish these cases from other phenotypes. In one study, Hall *et al.* (1983) reported that 10% of cases with flexion contractures due to amyoplasia in human neonates presented with hernias. This suggests that there may be an association between the two clinical findings. Knock-out experiments in mice that led to abdominal wall anomalies suggest that regulatory and developmental genes, Msx1/Msx2, Ap-2, Hox, Alx4 and Pax3 are associated. However, the molecular mechanisms are still obscure (Ogi *et al.* 2005). In human neonates, fetal distress, induced by dystocia births, has been associated with occlusion failure resulting in hernias (Askar 2001) and in some animal models, teratogens have been known to induce congenital abdominal wall defects (Van Dorp *et al.* 2010).

CFS VI

Albeit rare, neonatal foals can present with various combinations of clinical features from the previously described subtypes. As the phenotypes become more complex, it may be that the pathogeneses are also more complex or alternatively, the insult may have occurred early in development and was caused by a major factor. Potential contributing variables,

whether of genetic or environmental origin, should always be considered in these cases and thoroughly documented in the medical records.

Because this is the first attempt to classify CFS and considering that little is known about the etiology or pathogeneses, we do not expect this system to be fully implemented into clinical practice but rather tested for a period of time. It is also imperative that we provide a means to collect more accurate data through consistent documentation so necessary revisions can be made during the trial period. We therefore created a proof of concept for a data entry interface by which clinicians globally can input demographic and clinical data on contracted foals. This may also serve as a model for characterizing other complex diseases and disorders.

Electronic Data Entry System

The next step for further delineating CFS is to recruit participants from equine hospitals to assist in the collection of more clinical data. We have established a proof of concept for a user-friendly data entry system. The participating veterinarian or staff member will be able to log on to a site with username and password and enter demographic and clinical data. The data can then be analyzed to find commonalities and patterns among the various criteria catalogued.

In conclusion, due to the mortality risk associated with both the mare and foal, it is critical that an instrument is put in place that will empower veterinarians and researchers to accurately characterize CFS. A classification system that will provide uniformity in clinical documentation is a critical step to achieving a better understanding of the mechanisms

underlying the condition. Communicating the condition in a consistent manner will reduce the inherent ambiguity that comes with the subjective nature of clinical documentation. This should allow for improved diagnostic ability, treatment approaches, and greater insight into prognosis. As for researchers, current techniques in molecular biology have given us a "tool kit" for understanding the molecular mechanisms underlying complex diseases and this should be the focus for CFS and other equine congenital disorders. However, for now, we propose the first system for classification of CFS and ask that it be tested while more information is gathered. The input of data we expect from our proposed system will greatly facilitate implementing appropriate experimental approaches for discovering the pathogeneses underlying CFS. We anticipate that the system will require continuous modification as more data are collected and etiological mechanisms are discovered.

CHAPTER V

CONCLUSION AND FUTURE WORK

Flexion contractures and CFS in neonatal Thoroughbred foals are complex musculoskeletal conditions and we have only begun to understand the etiology and pathogeneses underlying them. We took a molecular genetics approach for our initial investigation based on the familial predisposition observed in numerous cases and because of the similarities in phenotypes of a predominately genetic model disorder in humans, the distal arthrogryposes.

Sequencing of the *TPM2* gene exons and intron boundary regions identified 5 SNP, 1 of which was located in exon 2 but did not cause an alteration in the amino acid sequence. The remaining SNP were in non-coding sequence including the 5' and 3' flanking sequence of exon 2, the 5' boundary region of exon 4, and 3' UTR. Statistical analysis did not identify any of the variants to be significantly associated with the disease condition. However, when we conducted a 3, 4, and 5 marker sliding window haplotype analysis, one haplotype approached significance indicating there may be variants in the region not yet discovered that are linked to flexion contractures. Future work here includes promoter and putative alternative promoter identification as well as deeper sequencing into regulatory regions for additional variant discovery. Additionally, expression studies of this gene may provide insight as to how SNP identified in this study influence the efficacy of transcription and translation.

Results from the case-control population, family validation study, and family based GWAS led us to the identification of 2 putative candidate genes (*TTN* and *CAPZA1)* and potentially a third DSL (*ANKRD1*). The significant SNP markers, BIEC2-415468, BIEC2-415469, BIEC2-415501, and BIEC2-415509 on ECA18, were found to be in high LD with markers in the *TTN* gene and therefore could potentially serve as tag SNP and genotyped to predict the risk of flexion contractures. A 26 marker, 11.2 Mb region of ECA5 was found to be associated with the phenotype characterized by carpal contractures, carpal valgus deformities, and distal hind limb laxity (Phenotype Group 2). With the current annotation of the equine genome, we were able to identify 4 genes in this interval exclusively related to muscle function and physiology. These genes included *TBX15*, *CASQ2*, *ADMP1*, and *CAPZA1*. Phasing led to the discovery of haplotypes in all 4 candidate regions in select affected foals. These haplotypes were not observed in any control animal. To narrow down the DSL interval, we looked at the LD structure of both the case animal and control populations. Of the 26 significant SNP, markers BIEC2-910111, BIEC2-910126, BIEC2-910155, BIEC2-910173, BIEC2-910227, and BIEC2-232, were in complete LD with *CAPZA1*. This could be significant considering *TTN* and *CAPZA1* are both skeletal muscle sarcomere Z-disk proteins and are thought to indirectly interact during myofibrillogenesis (Papa *et al.* 1999).

As putative candidate genes, we propose that sequence variants in equine *TTN* and/or *CAPZA1* (ECA18) may cause a protein structural change resulting in increased passive tension of the muscle fibers. Consequently, this change may cause increased resistance to movement during fetal development thus leading to flexion contractures. Therefore, our first

objective for a future study is to sequence *TTN* using a custom exon array in the 7 affected foals identified by haplotype analysis to be homozygous for the tag SNP mentioned above and 23 unaffected foals to investigate for variants causing a structurally altered *TTN* protein. Next, we plan to RNAseq *TTN* transcripts using embryonic and neonatal skeletal muscle tissue for the identification of fetal and perinatal isoforms. Additionally, *CAPZA1* would be considered for sequencing in those affected foals with unique haplotypes associated with that gene region. Because muscle composition, function, and physiology are such important attributes in racehorses, *TTN* and *CAPZA1* are very important genes to characterize considering their diverse role in skeletal muscle development, structure, signaling, and mechanosensory properties.

The family-based GWAS identified a cluster of SNP on ECA1 that were weakly associated with flexion contractures in a kindred of 60 animals. We investigated this region further only because SNP BIEC2-17038 was located in the gene, *ANKRD1*, which acts as a molecular messenger between *TTN* and the nucleus (Miller *et al.* 2003). Sequencing of the exons and exon boundary regions of *ANKRD1* did not reveal a variant exclusive to affected foals; however, one haplotype was found to be overrepresented in affected foals. Additional studies would include sequencing the remaining introns segments as well as the 5' promoter and 3'UTR regions to search for variants that may alter the expression of this gene in affected foals.

If pathogenic mutations in the *TTN* gene are discovered in the future, it would be interesting to take a look into the selection pressures that are maintaining these alleles in the Thoroughbred population. It has been reported that SNP in the myostatin gene dictate the

sprinting or long distance capabilities of Thoroughbred racehorses (Hill *et al.* 2012). Myostatin and *TTN* reside only 9.5 Mb apart on ECA18. If LD is significant between these two loci, selecting for particular genotypes in myostatin may cause selection for disease susceptibility variants in *TTN*. Therefore, future work could include the evaluation of positive artificial selection for myostatin genotypes and how it may inadvertently perpetuate musculoskeletal disorders through mutations in *TTN*.

Overall, our data strongly point to genetic (locus) heterogeneity but still supports the initial hypothesis that flexion contractures are major gene disorders. From these data, it appears that there are potentially 3 major genes belonging to a cooperative of subsarcomeric proteins that, if harboring causal variants, may autonomously cause flexion contractures in neonatal foals. This would be an unprecedented finding in equine genomic research. As it is, our research has provided a solid framework for many future projects.

By creating a model for global data collection, we can continue to accurately characterize these conditions in the neonatal foal. Consistent and precise clinical documentation is imperative for implementing the most effective experimental design in future studies. Collecting demographic and clinical data on foals with limb deformities and CFS will be ongoing. We anticipate our user-friendly data entry interface system will be implemented for this purpose and may serve as a model for collecting information on other diseases and conditions. This system will provide a mechanism for the accurate design of countless future studies and greatly aide our efforts to further classify and delineate these variable conditions.

REFERENCES

Abbott L.C., Finnell R.H., Chernoff G.F., Parish S.M. & Gay C.C. (1986) Crooked calf disease: a histological and histochemical examination of eight affected calves. *Vet Pathol* **23**, 734-40.

Adams S.B.a.S., E.M. (2000) Management of Congenital and Acquired Flexural Limb Deformities. *Proceedings of the Annual Convention of the American Association of Equine Practitioners* **46**.

Agerholm J.S., Bendixen C., Andersen O. & Arnbjerg J. (2001) Complex vertebral malformation in holstein calves. *J Vet Diagn Invest* **13**, 283-9.

Aldred J. (1998) Developmental orthopaedic disease in the horse. *Rural Industries Research and Development Corporation Report, Project No. US-45A*.

Alkuraya F.S. (2010) Homozygosity mapping: one more tool in the clinical geneticist's toolbox. *Genet Med* **12**, 236-9.

Anderson T.M., McIlwraith C.W. & Douay P. (2004) The role of conformation in musculoskeletal problems in the racing Thoroughbred. *Equine Vet J* **36**, 571-5.

Askar O.M. (2001) *Abdominal Wall Hernias: Principles and Management, X Edn.* Springer-Verlag, New York, Berlin, Heidleberg.

Ball R.D. (2005) Experimental designs for reliable detection of linkage disequilibrium in unstructured random population association studies. *Genetics* **170**, 859-73.

Bamshad M., Jorde L.B. & Carey J.C. (1996) A revised and extended classification of the distal arthrogryposes. *Am J Med Genet* **65**, 277-81.

Bamshad M., Van Heest A.E. & Pleasure D. (2009) Arthrogryposis: a review and update. *J Bone Joint Surg Am* **91 Suppl 4**, 40-6.

Baumeister A., Arber S. & Caroni P. (1997) Accumulation of muscle ankyrin repeat protein transcript reveals local activation of primary myotube endcompartments during muscle morphogenesis. *J Cell Biol* **139**, 1231-42.

Beck A.E., McMillin M.J., Gildersleeve H.I., Kezele P.R., Shively K.M., Carey J.C., Regnier M. & Bamshad M.J. (2013a) Spectrum of mutations that cause distal arthrogryposis types 1 and 2B. *Am J Med Genet A* **161a**, 550-5.

Benjamini Y. & Hochberg Y. (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. . *Journal of the Royal Statistical Society* **57**, 289-300.

Binanti D., Zani D.D., De Zani D., Turci T., Zavaglia G. & Riccaboni P. (2013) Contracted Foal Syndrome Associated with Multiple Malformations in Two Foals. *Anat Histol Embryol*. 43 (1), 71-74.

Brooks D.E. (2002) Equine Ophthalmology. *Proceedings of the Annual Convention of the American Association of Equine Practitioners* **48**, 300-13.

Brooks S.A., Gabreski N., Miller D., Brisbin A., Brown H.E., Streeter C., Mezey J., Cook D. & Antczak D.F. (2010) Whole-genome SNP association in the horse: identification of a deletion in myosin Va responsible for Lavender Foal Syndrome. *PLoS Genet* **6**, e1000909.

Caron J.P. (1988) Angular limb deformities in foals. *Equine Vet J* **20**, 225-8.

Chiquet M., Birk D.E., Bonnemann C.G. & Koch M. (2014) Collagen XII: Protecting bone and muscle integrity by organizing collagen fibrils. *Int J Biochem Cell Biol* **53c**, 51-4.

Clarke N.F., Waddell L.B., Sie L.T., van Bon B.W., McLean C., Clark D., Kornberg A., Lammens M. & North K.N. (2012) Mutations in TPM2 and congenital fibre type disproportion. *Neuromuscul Disord* **22**, 955-8.

Clayton L., Reinach F.C., Chumbley G.M. & MacLeod A.R. (1988) Organization of the hTMnm gene. Implications for the evolution of muscle and non-muscle tropomyosins. *J Mol Biol* **201**, 507-15.

Coleman S.J., Zeng Z., Hestand M.S., Liu J. & Macleod J.N. (2013) Analysis of unannotated equine transcripts identified by mRNA sequencing. *PLoS One* **8**, e70125.

Corbin L.J., Blott S.C., Swinburne J.E., Vaudin M., Bishop S.C. & Woolliams J.A. (2010) Linkage disequilibrium and historical effective population size in the Thoroughbred horse. *Anim Genet* **41 Suppl 2**, 8-15.

Corbin L.J., Liu A.Y., Bishop S.C. & Woolliams J.A. (2012) Estimation of historical effective population size using linkage disequilibria with marker data. *J Anim Breed Genet* **129**, 257-70.

Crowe M.W. & Swerczek T.W. (1985) Equine congenital defects. *Am J Vet Res* **46**, 353-8.

Cruz R.M., Krieger H., Ferreira R., Mah J., Hartsfield J., Jr. & Oliveira S. (2008) Major gene and multifactorial inheritance of mandibular prognathism. *Am J Med Genet A* **146A**, 71-7.

Dastur D.K., Razzak Z.A. & Bharucha E.P. (1972) Arthrogryposis multiplex congenita. 2. Muscle pathology and pathogenesis. *J Neurol Neurosurg Psychiatry* **35**, 435-50.

de Vooght K.M., van Wijk R. & van Solinge W.W. (2009) Management of gene promoter mutations in molecular diagnostics. *Clin Chem* **55**, 698-708.

Doherty M.L., Kelley E.P., Healy A.M., Callanan J.J., Crosby T.F., Skelly C. & Boland M.P. (2000) Congenital arthrogryposis: an inherited limb deformity in pedigree Suffolk lambs. *Vet Rec* **146**, 748-53.

Drera B., Zoppi N., Barlati S. & Colombi M. (2006) Recurrence of the p.R156X TNNI2 mutation in distal arthrogryposis type 2B. *Clin Genet* **70**, 532-4.

Edom-Vovard F. & Duprez D. (2004) Signals regulating tendon formation during chick embryonic development. *Dev Dyn* **229**, 449-57.

Epstein D.J. (2009) Cis-regulatory mutations in human disease. *Brief Funct Genomic Proteomic* **8**, 310-6.

Erol B.e.a. (2002) Etiology of Congenital Scoliosis. *The University of Pennsylvania Orthopaedic Journal* **15**, 37-42.

Fassier A., Wicart P., Dubousset J. & Seringe R. (2009) Arthrogryposis multiplex congenita. Long-term follow-up from birth until skeletal maturity. *J Child Orthop* **3**, 383-90
.

Feng S., Wang S., Chen C.C. & Lan L. (2011) GWAPower: a statistical power calculation software for genome-wide association studies with quantitative traits. *BMC Genet* **12**, 12.

Finno C.J., Spier S.J. & Valberg S.J. (2009) Equine diseases caused by known genetic mutations. *Vet J* **179**, 336-47.

Genini S., Malek M., Spilar S., Nguyen T.T., Menetrey F., Gebert S., Hagger C., Neuenschwander S., Kadarmideen H.N., Stranzinger G. & Vogeli P. (2004) Arthrogryposis multiplex congenita (AMC), a hereditary disease in swine, maps to chromosome 5 by linkage analysis. *Mamm Genome* **15**, 935-41.

Giebel L.B. & Spritz R.A. (1991) Mutation of the KIT (mast/stem cell growth factor receptor) protooncogene in human piebaldism. *Proc Natl Acad Sci U S A* **88**, 8696-9.

Gilbert S.F. (2010) *Developmental Biology, 9th Ed.* . Sinauer and Associates.

Giles R.C., Donahue J.M., Hong C.B., Tuttle P.A., Petrites-Murphy M.B., Poonacha K.B., Roberts A.W., Tramontin R.R., Smith B. & Swerczek T.W. (1993) Causes of abortion, stillbirth, and perinatal death in horses: 3,527 cases (1986-1991). *J Am Vet Med Assoc* **203**, 1170-5.

Goebel H.H., Anderson J.R., Hubner C., Oexle K. & Warlo I. (1997) Congenital myopathy with excess of thin myofilaments. *Neuromuscul Disord* **7**, 160-8.

Goebel H.H. & Bonnemann C.G. (2011) 169th ENMC International Workshop Rare Structural Congenital Myopathies 6-8 November 2009, Naarden, The Netherlands. *Neuromuscul Disord* **21**, 363-74.

Granzier H.L. & Labeit S. (2004) The giant protein titin: a major player in myocardial mechanics, signaling, and disease. *Circ Res* **94**, 284-95.

Greet T.R.C. (2000) Managing flexural and angular limb deformities. The Newmarket Perspective. *Proceedings of the Annual Convention of the American Association of Equine Practitioners*, 130-6.

Guo W., Bharmal S.J., Esbona K. & Greaser M.L. (2010) Titin diversity--alternative splicing gone wild. *J Biomed Biotechnol* **2010**, 753675.

Gurnett C.A., Desruisseau D.M., McCall K., Choi R., Meyer Z.I., Talerico M., Miller S.E., Ju J.S., Pestronk A., Connolly A.M., Druley T.E., Weihl C.C. & Dobbs M.B. (2010) Myosin binding protein C1: a novel gene for autosomal dominant distal arthrogryposis type 1. *Hum Mol Genet* **19**, 1165-73.

Gustafson A.L., Tallmadge R.L., Ramlachan N., Miller D., Bird H., Antczak D.F., Raudsepp T., Chowdhary B.P. & Skow L.C. (2003) An ordered BAC contig map of the equine major histocompatibility complex. *Cytogenet Genome Res* **102**, 189-95.

Ha K., Buchan J.G., Alvarado D.M., McCall K., Vydyanath A., Luther P.K., Goldsmith M.I., Dobbs M.B. & Gurnett C.A. (2013) MYBPC1 mutations impair skeletal muscle function in zebrafish models of arthrogryposis. *Hum Mol Genet*. 22(24):4967-77.

Hackman J.P., Vihola A.K. & Udd A.B. (2003) The role of titin in muscular disorders. *Ann Med* **35**, 434-41.

Hall J.G. (1997) Arthrogryposis multiplex congenita: etiology, genetics, classification, diagnostic approach, and general aspects. *J Pediatr Orthop B* **6**, 159-66.

Hall J.G. (2014) Arthrogryposis (multiple congenital contractures): Diagnostic approach to etiology, classification, genetics, and general principles. *Eur J Med Genet*. 57 (8), 464-472.

Hall J.G. & Reed S.D. (1982) Teratogens associated with congenital contractures in humans and in animals. *Teratology* **25**, 173-91.

Hall J.G., Reed S.D. & Driscoll E.P. (1983) Part I. Amyoplasia: a common, sporadic condition with congenital contractures. *Am J Med Genet* **15**, 571-90.

Hall J.G., Reed S.D. & Greene G. (1982) The distal arthrogryposes: delineation of new entities--review and nosologic discussion. *Am J Med Genet* **11**, 185-239.

Haubitz M., Neuenschwander S. & Vogeli P. (2012) Porcine arthrogryposis multiplex congenita (AMC): new diagnostic test and narrowed candidate region. *Mol Cell Probes* **26**, 248-52.

Hicks D., Farsani G.T., Laval S., Collins J., Sarkozy A., Martoni E., Shah A., Zou Y., Koch M., Bonnemann C.G., Roberts M., Lochmuller H., Bushby K. & Straub V. (2014) Mutations in the collagen XII gene define a new form of extracellular matrix-related myopathy. *Hum Mol Genet* **23**, 2353-63.

Hill E.W., Fonseca R.G., McGivney B.A., Gu J., MacHugh D.E. & Katz L.M. (2012) MSTN genotype (g.66493737C/T) association with speed indices in Thoroughbred racehorses. *J Appl Physiol* **112**, 86-90.

Hong C.B., Donahue J.M., Giles R.C., Jr., Petrites-Murphy M.B., Poonacha K.B., Roberts A.W., Smith B.J., Tramontin R.R., Tuttle P.A. & Swerczek T.W. (1993) Equine abortion and stillbirth in central Kentucky during 1988 and 1989 foaling seasons. *J Vet Diagn Invest* **5**, 560-6.

Hudgson P. (1970) The value of electron microscopy in muscle biopsies. *Proc R Soc Med* **63**, 470-4.

Hunt R.J. (1997) In: *Current Therapy in Equine Medicine* (pp. 623-4. W.B. Saunders, Philadelphia.

Ilkovski B., Cooper S.T., Nowak K., Ryan M.M., Yang N., Schnell C., Durling H.J., Roddick L.G., Wilkinson I., Kornberg A.J., Collins K.J., Wallace G., Gunning P., Hardeman E.C., Laing N.G. & North K.N. (2001) Nemaline myopathy caused by mutations in the muscle alpha-skeletal-actin gene. *Am J Hum Genet* **68**, 1333-43.

Jackson C.E. (2008) A clinical approach to muscle diseases. *Semin Neurol* **28**, 228-40.

Katoh H., Watanabe Y., Ebukuro M., Muguruma K., Takabayashi S. & Shiroishi T. (2003) Chromosomal mapping of the peroneal muscular atrophy (pma) gene in the mouse. *Exp Anim* **52**, 433-6.

Kee A.J. & Hardeman E.C. (2008) Tropomyosins in skeletal muscle diseases. *Adv Exp Med Biol* **644**, 143-57.

Kidd J.A. & Barr A.R.S. (2002) Flexural deformities in foals. *Equine Veterinary Education* **14**, 311-21.

Kimber E., Tajsharghi H., Kroksmark A.K., Oldfors A. & Tulinius M. (2006) A mutation in the fast skeletal muscle troponin I gene causes myopathy and distal arthrogryposis. *Neurology* **67**, 597-601.

Kimber E., Tajsharghi H., Kroksmark A.K., Oldfors A. & Tulinius M. (2012) Distal arthrogryposis: clinical and genetic findings. *Acta Paediatr* **101**, 877-87.

Klein R.J. (2007) Power analysis for genome-wide association studies. *BMC Genet* **8**, 58.

Knottenbelt D.C., Holdstock N. & Madigan J.E. (2004) *Equine Neonatal Medicine and Surgery: Medicine and Surgery*. Elsevier Health Sciences UK.

Kruger M., Kohl T. & Linke W.A. (2006) Developmental changes in passive stiffness and myofilament Ca2+ sensitivity due to titin and troponin-I isoform switching are not critically triggered by birth. *Am J Physiol Heart Circ Physiol* **291**, H496-506.

Laing N.G. & Nowak K.J. (2005) When contractile proteins go bad: the sarcomere and skeletal muscle disease. *Bioessays* **27**, 809-22.

Laird N.M. & Lange C. (2009) The Role of Family-Based Designs in Genome-Wide Association Studies. Statistical Science 24 (4), 388-397.

Lamont P.J., Udd B., Mastaglia F.L., de Visser M., Hedera P., Voit T., Bridges L.R., Fabian V., Rozemuller A. & Laing N.G. (2006) Laing early onset distal myopathy: slow myosin defect with variable abnormalities on muscle biopsy. *J Neurol Neurosurg Psychiatry* **77**, 208-15.

Laurila K. & Lahdesmaki H. (2009) Systematic analysis of disease-related regulatory mutation classes reveals distinct effects on transcription factor binding. *In Silico Biol* **9**, 209-24.

Libri D., Lemonnier M., Meinnel T. & Fiszman M.Y. (1989) A single gene codes for the beta subunits of smooth and skeletal muscle tropomyosin in the chicken. *J Biol Chem* **264**, 2935-44.

Linke W.A. (2008) Sense and stretchability: the role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction. *Cardiovasc Res* **77**, 637-48.

Linke W.A. & Kruger M. (2010) The giant protein titin as an integrator of myocyte signaling pathways. *Physiology (Bethesda)* **25**, 186-98.

Luo Z.W. (1998) Detecting linkage disequilibrium between a polymorphic marker locus and a trait locus in natural populations. *Heredity (Edinb)* **80 (Pt 2)**, 198-208.

Luther P.K. (2009) The vertebrate muscle Z-disc: sarcomere anchor for structure and signalling. *J Muscle Res Cell Motil* **30**, 171-85.

Lykkjen S., Dolvik N.I., McCue M.E., Rendahl A.K., Mickelson J.R. & Roed K.H. (2010) Genome-wide association analysis of osteochondrosis of the tibiotarsal joint in Norwegian Standardbred trotters. *Anim Genet* **41 Suppl 2**, 111-20.

Manske P.R. & Oberg K.C. (2009) Classification and developmental biology of congenital anomalies of the hand and upper extremity. *J Bone Joint Surg Am* **91 Suppl 4**, 3-18.

Marttila M., Lehtokari V.L., Marston S., Nyman T.A., Barnerias C., Beggs A.H., Bertini E., Ceyhan-Birsoy O., Cintas P., Gerard M., Gilbert-Dussardier B., Hogue J.S., Longman C., Eymard B., Frydman M., Kang P.B., Klinge L., Kolski H., Lochmuller H., Magy L., Manel V., Mayer M., Mercuri E., North K.N., Peudenier-Robert S., Pihko H., Probst F.J., Reisin R., Stewart W., Taratuto A.L., de Visser M., Wilichowski E., Winer J., Nowak K., Laing N.G., Winder T.L., Monnier N., Clarke N.F., Pelin K., Gronholm M. & Wallgren-Pettersson C. (2014) Mutation Update and Genotype-Phenotype Correlations of Novel and Previously Described Mutations in TPM2 and TPM3 Causing Congenital Myopathies. *Hum Mutat* **35**, 779-90.

Mayhew I.G. (1984) Neuromuscular arthrogryposis multiplex congenita in a thoroughbred foal. *Vet Pathol* **21**, 187-92.

McKinnon A.O. (2011) *Equine reproduction*. Wiley-Blackwell, Chichester, West Sussex, U.K.

McLaughlin B.G., Doige C.E. & McLaughlin P.S. (1986) Thyroid hormone levels in foals with congenital musculoskeletal lesions. *Can. Vet. J.* **27**, 264-7.

McMillin M.J., Below J.E., Shively K.M., Beck A.E., Gildersleeve H.I., Pinner J., Gogola G.R., Hecht J.T., Grange D.K., Harris D.J., Earl D.L., Jagadeesh S., Mehta S.G., Robertson S.P., Swanson J.M., Faustman E.M., Mefford H.C., Shendure J., Nickerson D.A., Bamshad M.J. & University of Washington Center for Mendelian G. (2013) Mutations in ECEL1 cause distal arthrogryposis type 5D. *Am J Hum Genet* **92**, 150-6.

Metzger J., Ohnesorge B. & Distl O. (2012) Genome-wide linkage and association analysis identifies major gene loci for guttural pouch tympany in Arabian and German warmblood horses. *PLoS One* **7**, e41640.

Miller M.K., Bang M.L., Witt C.C., Labeit D., Trombitas C., Watanabe K., Granzier H., McElhinny A.S., Gregorio C.C. & Labeit S. (2003) The muscle ankyrin repeat proteins: CARP, ankrd2/Arpp and DARP as a family of titin filament-based stress response molecules. *J Mol Biol* **333**, 951-64.

Mokbel N., Ilkovski B., Kreissl M., Memo M., Jeffries C.M., Marttila M., Lehtokari V.L., Lemola E., Gronholm M., Yang N., Menard D., Marcorelles P., Echaniz-Laguna A., Reimann J., Vainzof M., Monnier N., Ravenscroft G., McNamara E., Nowak K.J., Laing N.G., Wallgren-Pettersson C., Trewhella J., Marston S., Ottenheijm C., North K.N. & Clarke N.F. (2013) K7del is a common TPM2 gene mutation associated with nemaline myopathy and raised myofibre calcium sensitivity. *Brain* **136**, 494-507.

Moulik M., Vatta M., Witt S.H., Arola A.M., Murphy R.T., McKenna W.J., Boriek A.M., Oka K., Labeit S., Bowles N.E., Arimura T., Kimura A. & Towbin J.A. (2009) ANKRD1, the gene encoding cardiac ankyrin repeat protein, is a novel dilated cardiomyopathy gene. *J Am Coll Cardiol* **54**, 325-33.

Munroe G.A.a.C., C.C.H. (1996) Congenital flexural deformities of the foal. *Equine Veterinary Education* 8 (2), 92-96.

Neagoe C., Opitz C.A., Makarenko I. & Linke W.A. (2003) Gigantic variety: expression patterns of titin isoforms in striated muscles and consequences for myofibrillar passive stiffness. *J Muscle Res Cell Motil* **24**, 175-89.

Nes N., Lomo O.M. & Bjerkas I. (1982) Hereditary lethal arthrogryposis ("muscle contracture") in horses. *Nord Vet Med* **34**, 425-30.

Nonaka I., Kikuchi A., Suzuki T. & Esaki K. (1986) Hereditary peroneal muscular atrophy in the mouse: an experimental model for congenital contractures (arthrogryposis). *Exp Neurol* **91**, 571-9.

O'Flaherty P. (2001) Arthrogryposis multiplex congenita. *Neonatal Netw* **20**, 13-20.

Ogi H., Suzuki K., Ogino Y., Kamimura M., Miyado M., Ying X., Zhang Z., Shinohara M., Chen Y. & Yamada G. (2005) Ventral abdominal wall dysmorphogenesis of Msx1/Msx2 double-mutant mice. *Anat Rec A Discov Mol Cell Evol Biol* **284**, 424-30.

Orr N., Back W., Gu J., Leegwater P., Govindarajan P., Conroy J., Ducro B., Van Arendonk J.A., MacHugh D.E., Ennis S., Hill E.W. & Brama P.A. (2010) Genome-wide SNP association-based localization of a dwarfism gene in Friesian dwarf horses. *Anim Genet* **41 Suppl 2**, 2-7.

Ottenheijm C.A., Hidalgo C., Rost K., Gotthardt M. & Granzier H. (2009a) Altered contractility of skeletal muscle in mice deficient in titin's M-band region. *J Mol Biol* **393**, 10-26.

Ottenheijm C.A., Knottnerus A.M., Buck D., Luo X., Greer K., Hoying A., Labeit S. & Granzier H. (2009b) Tuning passive mechanics through differential splicing of titin during skeletal muscle development. *Biophys J* **97**, 2277-86.

Owen J.M. (1975) Abnormal flexion of the corono-pedal joint or "contracted tendons" in unweaned foals. *Equine Vet J* **7**, 40-5.

Owji M., Modarressi F., Geramizadeh B., Borhani Haghighi A., Alizadeh A. & Heidari T. (2010) Diagnosis of Myopathies Using Histology, Histochemistry, Immunohistochemistry and Electron Microscopy: A Single Center Experience. *Iran Red Cres Med J*, 434-40.

Paciello O. & Papparella S. (2009) Histochemical and immunohistological approach to comparative neuromuscular diseases. *Folia Histochem Cytobiol* **47**, 143-52.

Panter K.E., Gardner D.R. & Molyneux R.J. (1998) Teratogenic and fetotoxic effects of two piperidine alkaloid-containing lupines (L. formosus and L. arbustus) in cows. *J Nat Toxins* **7**, 131-40.

Papa I., Astier C., Kwiatek O., Raynaud F., Bonnal C., Lebart M.C., Roustan C. & Benyamin Y. (1999) Alpha actinin-CapZ, an anchoring complex for thin filaments in Z-line. *J Muscle Res Cell Motil* **20**, 187-97.

Pappas C.T., Bhattacharya N., Cooper J.A. & Gregorio C.C. (2008) Nebulin interacts with CapZ and regulates thin filament architecture within the Z-disc. *Mol Biol Cell* **19**, 1837-47.

Pereira M.G., Rodrigues M.A. & Rodrigues S.A. (2010) Eyelid entropion. *Semin Ophthalmol* **25**, 52-8.

Petersen J.L., Mickelson J.R., Cothran E.G., Andersson L.S., Axelsson J., Bailey E., Bannasch D., Binns M.M., Borges A.S., Brama P., da Camara Machado A., Distl O., Felicetti M., Fox-Clipsham L., Graves K.T., Guerin G., Haase B., Hasegawa T., Hemmann K., Hill E.W., Leeb T., Lindgren G., Lohi H., Lopes M.S., McGivney B.A., Mikko S., Orr N., Penedo M.C., Piercy R.J., Raekallio M., Rieder S., Roed K.H., Silvestrelli M., Swinburne J., Tozaki T., Vaudin M., C M.W. & McCue M.E. (2013) Genetic diversity in the modern horse illustrated from genome-wide SNP data. *PLoS One* **8**, e54997.

Peyton L.C. (1981) Surgical Repair of a Patent Urachus and Ruptured Bladder in a Newborn Foal. *Journal of Equine Veterinary Science* **1**, 146-9.

Polizzi A., Huson S.M. & Vincent A. (2000) Teratogen update: maternal myasthenia gravis as a cause of congenital arthrogryposis. *Teratology* **62**, 332-41.

Purcell S., Neale B., Todd-Brown K., Thomas L., Ferreira M.A., Bender D., Maller J., Sklar P., de Bakker P.I., Daly M.J. & Sham P.C. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81**, 559-75.

Raudsepp T. & Chowdhary B.P. (2008) FISH for mapping single copy genes. *Methods Mol Biol* **422**, 31-49.

Raudsepp T., McCue M.E., Das P.J., Dobson L., Vishnoi M., Fritz K.L., Schaefer R., Rendahl A.K., Derr J.N., Love C.C., Varner D.D. & Chowdhary B.P. (2012) Genome-wide association study implicates testis-sperm specific FKBP6 as a susceptibility locus for impaired acrosome reaction in stallions. *PLoS Genet* **8**, e1003139.

Reid C.O., Hall J.G., Anderson C., Bocian M., Carey J., Costa T., Curry C., Greenberg F., Horton W., Jones M. & et al. (1986) Association of amyoplasia with gastroschisis, bowel atresia, and defects of the muscular layer of the trunk. *Am J Med Genet* **24**, 701-10.

Robinson N.E.a.S., K.A. (2009) *Current Therapy in Equine Medicine*. Linda Duncan and Penny Rudolph.

Robinson P., Lipscomb S., Preston L.C., Altin E., Watkins H., Ashley C.C. & Redwood C.S. (2007) Mutations in fast skeletal troponin I, troponin T, and beta-tropomyosin that cause distal arthrogryposis all increase contractile function. *FASEB J* **21**, 896-905.

Rodrigues A., Gates L., Payne H.R., Kiupel M. & Mansell J. (2010) Multicentric squamous cell carcinoma in situ associated with papillomavirus in a ferret. *Vet Pathol* **47**, 964-8.

Rudolph J.A., Spier S.J., Byrns G., Rojas C.V., Bernoco D. & Hoffman E.P. (1992) Periodic paralysis in quarter horses: a sodium channel mutation disseminated by selective breeding. *Nat Genet* **2**, 144-7.

Sambrook J., E. F. Fritsch and T. Maniatis (1998) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schevzov G., Whittaker S.P., Fath T., Lin J.J. & Gunning P.W. (2011) Tropomyosin isoforms and reagents. *Bioarchitecture* **1**, 135-64.

Schild A.L., M.P.S., M.C.D., , Portianski E.L. & Riet-Correa a.F. (2003) Arthrogryposis in Murrah buffaloes in southern Brazil. *Pesquisa Veterinária Brasileira* **23**, 13-6.

Seelow D., Schuelke M., Hildebrandt F. & Nurnberg P. (2009) HomozygosityMapper--an interactive approach to homozygosity mapping. *Nucleic Acids Res* **37**, W593-9.

Selcen D. & Bushby K. (2005) Titinopathies: what happens when a big gene mutates in a big family? *Neurology* **64**, 596-7.

Spencer C.C., Su Z., Donnelly P. & Marchini J. (2009) Designing genome-wide association studies: sample size, power, imputation, and the choice of genotyping chip. *PLoS Genet* **5**, e1000477.

Stephens M. & Donnelly P. (2003) A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am J Hum Genet* **73**, 1162-9.

Sturgill T.a.C., C. (2008) Causes of Foal Mortality. *Equine Disease Quarterly*, 1.

Sung S.S., Brassington A.M., Grannatt K., Rutherford A., Whitby F.G., Krakowiak P.A., Jorde L.B., Carey J.C. & Bamshad M. (2003) Mutations in genes encoding fast-twitch contractile proteins cause distal arthrogryposis syndromes. *Am J Hum Genet* **72**, 681-90.

Tajsharghi H., Kimber E., Kroksmark A.K., Jerre R., Tulinius M. & Oldfors A. (2008) Embryonic myosin heavy-chain mutations cause distal arthrogryposis and developmental myosin myopathy that persists postnatally. *Arch Neurol* **65**, 1083-90.

Tee J.M. & Peppelenbosch M.P. (2010) Anchoring skeletal muscle development and disease: the role of ankyrin repeat domain containing proteins in muscle physiology. *Crit Rev Biochem Mol Biol* **45**, 318-30.

Tejedor M.T., Ferrer L.M., Monteagudo L.V., Ramos J.J., Lacasta D. & Climent S. (2010) Ovine heritable arthrogryposis multiplex congenita with probable lethal autosomal recessive mutation. *J Comp Pathol* **143**, 14-9.

Tetens J., Widmann P., Kuhn C. & Thaller G. (2013) A genome-wide association study indicates LCORL/NCAPG as a candidate locus for withers height in German Warmblood horses. *Anim Genet* **44**, 467-71.

Teyssedre S., Dupuis M.C., Guerin G., Schibler L., Denoix J.M., Elsen J.M. & Ricard A. (2012) Genome-wide association studies for osteochondrosis in French Trotter horses. *J Anim Sci* **90**, 45-53.

Toydemir R.M., Rutherford A., Whitby F.G., Jorde L.B., Carey J.C. & Bamshad M.J. (2006) Mutations in embryonic myosin heavy chain (MYH3) cause Freeman-Sheldon syndrome and Sheldon-Hall syndrome. *Nat Genet* **38**, 561-5.

Tremaine W.H. & Dixon P.M. (2002) Diseases of the nasal cavities and paranasal sinuses. In: *Equine respiratory diseases* (ed. by Lekeux P). International Veterinary Information Service (www.ivis.org), Ithaca, New York, USA.

Tsuda T., Yoshida K., Yanase T., Ohashi S. & Yamakawa M. (2004) Competitive enzyme-linked immunosorbent assay for the detection of the antibodies specific to akabane virus. *J Vet Diagn Invest* **16**, 571-6.

Van Dorp D.R., Malleis J.M., Sullivan B.P. & Klein M.D. (2010) Teratogens inducing congenital abdominal wall defects in animal models. *Pediatr Surg Int* **26**, 127-39.

Van Eenennaam A. (2009) Curly Calf Syndrome (Arthrogryposis Multiplex) Test Available. URL http://animalscience.ucdavis.edu/animalbiotech/Outreach/Curly_Calf_test.pdf.

Van Vleck L.D. & Boldman K.G. (1993) Sequential transformation for multiple traits for estimation of (co)variance components with a derivative-free algorithm for restricted maximum likelihood. *J Anim Sci* **71**, 836-44.

Vandeplassche M., Simoens P., Bouters R., De Vos N. & Verschooten F. (1984) Aetiology and pathogenesis of congenital torticollis and head scoliosis in the equine foetus. *Equine Vet J* **16**, 419-24.

Vanpaemel L., Schoenmakers M., van Nesselrooij B., Pruijs H. & Helders P. (1997) Multiple congenital contractures. *J Pediatr Orthop B* **6**, 172-8.

Vega R.B., Matsuda K., Oh J., Barbosa A.C., Yang X., Meadows E., McAnally J., Pomajzl C., Shelton J.M., Richardson J.A., Karsenty G. & Olson E.N. (2004) Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* **119**, 555-66.

Veugelers M., Bressan M., McDermott D.A., Weremowicz S., Morton C.C., Mabry C.C., Lefaivre J.F., Zunamon A., Destree A., Chaudron J.M. & Basson C.T. (2004) Mutation of perinatal myosin heavy chain associated with a Carney complex variant. *N Engl J Med* **351**, 460-9.

Wang Z., Gerstein M. & Snyder M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**, 57-63.

Wei Y.J., Cui C.J., Huang Y.X., Zhang X.L., Zhang H. & Hu S.S. (2009) Upregulated expression of cardiac ankyrin repeat protein in human failing hearts due to arrhythmogenic right ventricular cardiomyopathy. *Eur J Heart Fail* **11**, 559-66.

Weller R., Pfau T., Verheyen K., May S.A. & Wilson A.M. (2006) The effect of conformation on orthopaedic health and performance in a cohort of National Hunt racehorses: preliminary results. *Equine Vet J* **38**, 622-7.

APPENDIX A

Contracted foals used in the sequencing of TPM2 and their respective clinical descriptions.

	Clinical Description
JC4	Bilateral carpal contractures; left front fetlock contracture; bilateral hindlimb laxity; bilateral carpal valgus
JC32	Contracted foal
JC34	Severe bilateral carpal contractures and bilateral hindlimb laxity
JC36	Severe bilateral carpal contractures; bilateral hindlimb laxity; 128ilateral carpal valgus
JC38	Severe bilateral carpal contractures
JC40	Contracted foal
JC42	Contracted foal
JC45	Bilateral carpal and left hind fetlock contractures
JC46	Bilateral carpal contractures
JC47	Bilateral carpal contractures
JC48	Bilateral carpal contractures
JC51	Left carpal and left front fetlock contracture
JC53	Contracted left hind fetlock
JC55	Left hind fetlock contracture
JC57	Contracted foal
JC59	Bilateral front fetlock contractures and right hind fetlock contracture
JC61	Bilateral front fetlock and left hind fetlock contractures
JC63	Left hind fetlock contracture

JC65	Bilateral carpal and front fetlock contractures
JC67	Bilateral carpal contractures
JC69	Moderate bilateral fetlock contractures
JC70	Moderate bilateral fetlock contractures
JC71	Moderate bilateral carpal contractures
JC73	Bilateral front fetlock contractures
JC74	Contracted foal
JC75	Contracted right front fetlock
JC76	Severe bilateral fetlock contractures
JC77	Mildly contracted front limbs
JC79	Contracted right limb
JC80	Bilateral carpal fetlock contractures
JC82	Contracted foal
JC84	Contracted foal
JC86	Contracted foal
JC87	Contracted foal
JC88	Contracted foal
JC89	Moderate bilateral carpal contractures
JC91	Contracted hind fetlock contracture
JC93	Bilateral carpal and bilateral front fetlock contractures; mild hindlimb contracture
JC96	Moderate bilateral carpal and front fetlock contracturs
JC98	Bilateral carpal and front fetlock contractures

JC101	Bilateral carpal contractures (moderate)
JC102	Severe bilateral carpal contractures
JC104	Mild bilateral carpal contractures
JC106	Bilateral carpal and left hind fetlock contractures
JC108	Severe bilateral front fetlock contractures
JC112	Mild to moderate bilateral carpal contractures
JC114	Bilateral fetlock contractures (one mild, one severe)
JC116	Moderate bilateral carpal and bilateral front fetlock contractures
JC118	Left front fetlock and right hind fetlock contracture
JC120	Severe (90°) bilateral carpal contractures
JC124	Bilateral front fetlock contractures
JC126	Forelimb contractures
JC128	Severely contracted foal, bilateral carpi
JC130	Bilateral front fetlock contractures
JC132	Bilateral front and bilateral hind fetlock contractures
JC134	Bilateral front fetlock contractures
JC136	Moderate bilateral front fetlock contractures

APPENDIX B

Tropomyosin Beta 2 (TPM2) EquCab2:25:926112-917080 (reverse strand). Sequence in red font and highlighted in pink denote exons and single nucleotide polymorphisms discovered by resequencing of TPM2 are highlighted in yellow.

TGCGGGCGCGTGGGGGCGGGGCGGCGGCCCGAGCTGGGCCCCGAGGCCGGGCGAC
CAGAAGAGGGGCCCTGGCTCCAGGGGCAGGGGCTGGAAGAGGAAGCGAGGGGAAG
TGAGGTCAGGCACGCCCTCGCTCCGCACTGCGACCGGGGCCTTGGGCTCCCTTCCTG
CCTCAGTTTCCCCAAGTCACTGGTGAGACTGCGTTATCACTACCACGTCAATAACTC
AAACCCAGAGTCTGGGACTCAGGGGAAACTGAGGCACTCGAACCGCGAAGGAGCC
CCGCCTCCGAAGAGACATTTAATCCGGGGGGATTTACAGGAAACTTCTAAATTAAG
GGCAGCGGCTGCTGCAGCTGAGGGGGGGCACGCCGGTCCCTTCTCGCGGGCAGCTG
CCGTGAGCTCACGCCCCGAAATAGCCCCAGGGGCCCCAGCCACAGCTGCCATGGGG
CCAGGCTGTCACTCAGAGGAAGCCCGGAGCCCCCCGTCCGAGGGACCCCTCCCCGC
CGCAGCGCTAGTGTTTTTTCAGCGGACTGCCCCGGCCACTTTCCCTTCCGACGGCTC
GAGGGAGGGGGAGGCGATCCCGAAGGGGTGGGTGCTGGGG**GCCGCCGCGCCCCC**
GTCCGGGCTGTGCTTCTGCCCCTAAAAGGTTTGGGCCGCGGTGGGGGAGGGTC
CCGGTCCCCGGCTCCGCCCGGTCCCTCCCCGCCTTTTAGGCGCCCGCGCGGCT
GGGACATCCCAGTCCCGCTTGGTCCTCCTCGCCCGCCACCGGTGCGTCCAGTC
CACGCAGCCAGCCCAGTTCACCCGGTCCATACCGCCCGCCGGCCGGCCCGTCC
CCCACCGCAGCCATGGACGCCATCAAGAAGAAGATGCAGATGCTAAAGTTGGA
CAAGGAGAATGCCATCGACCGCGCTGAGCAGGCCGAGGCCGACAAGAAGCAA
GCTGAGGACCGCTGCAAGCAGGTGGGGGCCCCGGACCCGGTGCAACCCTCTCCCC
CTCAACGAGGTCGAGTAGGCCCTGCCCTGAGGGCCCTAGCCGCGGGCCTGCCCTAG
CCGCGGGCCTGCCGTGCAAGGCTCCTTCCCATCCAGTGTGGGGGCTGGGCCTTCACA
GTACCCCCAGGGCCCTAGCCAGAGCCACGTTTCATCTCACCCACCCTAGCCTGGGAA
TCCCCAGCTCCCTACTGTCCACTCCTGCCCATCTGGAACAATGATGGGCCCTACCCC
TGGGGACTGGGAGCGTTCTCTCTACCCCCACCAGGTCCCTCCAACACCCTTGGCTGC
ACGGCCCCCTCTGCTATCCCTCTTCCTCCCCAAGAGCCTCTAAGTATGTCCTAGTGCC
CCTCACACAGCCCTGCCTC(C/T)AGCTGGCCGGGCCTGGCTAACCGAGACCTCTTTCT
CCCAG**CTGGAGGAGGAGCAGCAGGCCCTCCAGAAGAAGCTGAAGGGGACGGA**
GGA(C/T)GAGGTGGAGAAGTATTCTGAGTCAGTGAAGGATGCCCAGGAAAACT
GGAGCAGGCCGAGAAGAAGGCCACCGACGTGAGTGTGCACCTGGGCAACTGTGG
GACAGTGAGCACACTGGGAGGGGTCCTGGACGTGGGGATGAAGGAGAGGGGACCC
AGATATCCCCAGCACCAGGACAGAAGGGGTTTCCTCTTAGGAACCAGGCTCAGGCA
AGCTTGCACAAATCCTGGAGGCAGTAGGTAAATCAAAACCACCTCCCACTTACCCC
GAGGACAAGGAGTCTTATAATTC(C/T)GTTTGGTGCCAGCTCTTAGAATGTACGGTGA
TCAAGCTGTGAGTCATCCTGGGGGTGGGTGGAACAGGAATTTACCATCCACCTCAAT
TCATCCTCCAGCTGAAAATGGAGAGGAGACTGCAGCTACATTTGCGGGGTGTGGGT
GTAGATGCTGTAGGCAGGAGGAGGAAGAGGAATCCAGAGATGGGGCCCGATTTGA

ATTACATCCCTGGGGGGCCATTAGAAATGCCCTGGCCTTGGCCTCTGGCCCCAGCGG
GCTTGCTGTGCACTAGGCCCAGCTCAGGTTCTAACAGCCCCTCATTTACCCTCATTA
AGCTGTCAGCCTTGAGACCATGCCAGGCCAAAGGGCAGAGGGACAGGGCCACAAA
CGCCAAGGCACCACCTTGGCAGGTCCTACTCCCTCTGAGATTTCTCTGCCCACCCTA
CACCCCCTAACCAGACTTCTCCACCTACTGCCATGCCTAACCCCCACCTTGAGGTTA
GAGAATGGCCATATGACTTTGGACATGGTACTTCTACTATTCTTTGCCTCAGTTTCCC
CATTTGCCTTCCACCCAGGATTCTCTTAAGTTTAAGATGAGCCTATGGTTGGATGGAT
GAGGGGATTAGCAGAAGTATGATCTGAACATCCAAGATCAGAGATGACATTACTGT
TTGTGTTGACCATTGTCCATGTTCCCCAACCCGCCCCCCCAAGGTTGGTTTCTCTGAC
CTCCAGCCTTTGGTGTTGGTTCTCCACCAGCCCTGGCACTCGGGAGGGAACTGGCCT
GCAGCTGCTAGTGAAGACAACCCCGGGCATCTGACACTCTATAAATAACCAAGGCC
CAGTGCAGCTGGAGGCAGGACTGGAAACCCAAGGAGCTGGATGGGGGCTGGAGGG
GGGAGCAGGGGCCAGGGACAGAGCTGGAGAAGCAGGGCTGCAGTGCCAGGCCAGA
TGGGAAACACTGCCCAAACTTACTGTGTGACCTTGAACGCAGCGCTTAACTTACACA
CTGCCTCAGTTTCTCCCTTCTCTCTTGCTTCCCATTTGTCCAGTGGGGACAGTAATGC
CTGCCCTGGGGTCAGAGAGCTGCATCCATAACTGGGAAGAGCTTGAGAAGCAGAAA
ATGGCAAATGTCGGTCCTGTGATGAGGACCCCAGGACTTCCCAGATTCTCCAGTCAT
TTGTCACCCCACTACCATGAATATCATGAAGAGAGTTTTCCTTTAAAGTCTCATCTCT
CTCTAGAGGAGACCCCCAATGTCCCCCAGAGTCATTCTTCCCAACCCCCAGTGCCGT
CCCCAGAATCCTTCCACACTGCGTCATCTGGCTCTGTGCCCACAATGGGCTCTTGTC
ACTTGTCCAAGTGGCCTTCAGCACCTGGAGGAGAAGCTACAACTACTTTCTATGGAG
GGTGCTGTTTTCCCTGCCTGGTAGTCCAAGCCAGGACCTGCTTTCCACCCCCTCGGCC
CCCAGTGCTTCCACCCCTCTCTTCCCTCTCACTCACTGCTTCTCCGGCTGCTCCTCTCT
GCTTTCCAGTCTTAAATCTCAGGCTCTCTCAGTCTTTGTCTTTGAGAGAGCGCAGGGG
TAGCCTAACACTTAGGAAGTGTGGACTCTGTGCTGGCAAGGGGCTCAAGTGCTCTCT
GCTTATACTCTTTCCCAAACTTGGCTTATCAGAATTGCCTCGGAGGTTTAAAGAAAA
AGACACAAAGATTTCCGAGGCCCTGCCCTGCAGAGCCCAGTTCGGTGGGTCTAGTGT
GGAGCTGAGAACCCGATTATTTTAGAAGCCTCCCAGGTGGCTCTGTTGATTAATCGA
GTGTTAGAAGAACTGCCTTATCACAGAACAGCCTATGAAGGCGGAACTGCTGTTGTC
AACTCTGAGGCTTAGAGAGATTCAGTAAGTTGCTCAAGGTCACACAAGTGTTGAGTG
GCTGAGCTGTGGCTTAAAGACAGCGGTCTGATTCAGAGCCGATGCTCTTAACACCAA
GCTGCTCTGCCGCTCCCGTTTTCCTTTATTGGGGCCCTTTTTTTTTTGAGGAAGACTG
GCCCTGAGCTAACATCTGTGCCCATCTTCCTCTACTTATATGTGGAGCGCCCTCCACA
GCTTGCTTGATAAGCAGAGCTAGGTCCGCTCCTGGGATCCGAACCGGCGAACCCCA
GGCTGCCGAGGCAGAGTACAGGAGCTTACTACTGCGCCCCCGGGCCGGCTGCTGGG
GCCCTTCTTGCTCTGCCTCTTTTCCTCAAGGTGTCCTCAGTTTCCATGGACCTTCTCCA
AAACCTCCATGGTTTTGCCTCTATGTTGCAGGATCAGGGGCTGAGTATCCATCACTC
ACACTGGCCTTGCACCACCCCCAGTTGCCTCCCAGCCTTGGCAGGGAGCCCCATGGC
ACAGCCAAGGAGGTCCCATATAGGCCTGGCTTGCCTCCTGAGCCCCTCGCTGGGAGT
CTGCCTGGCTTCTAGTGACATCTCAGAGCTTGAGTCTGTGACTGGCTATTTTTAGCAG
CAGAGAGGTCTGGTTGGCCTCATTCTTCCCCACCCCCTTACCGGCCAGGCCATGCTG
TGGAGGAGTCGGGGCTGCCTCAGACCTGGTCCCAGCTCTCCAGGGGACAGAGGGAC
CTGCATGCAGATTACCACTGGGAGACTAACCATGCACCGTGCCAGAAGAGGCCCCG
TGGCAGGGGAGTGGAGGCTGTCAAGGAATGCGTACTTTGGGCTGGAGCACAGTCTG

GGCAGAAGATAGTTGGCTGGGGGAAAACATGGGTTGACAGTCTGGAAGCCAGGAA
GCCTAGGTTCTTTCTGACAAGAATCTCACTGTTCTCTGGAGCCTCTCTGATCCTCATC
CGAGGCAGACAAAGCCTTCGTGACCTCTGACCTTTGACCCACAG**GCTGAAGCAGAT
GTGGCCTCTCTGAACCGCCGTATTCAGCTGGTAGAGGAGGAGCTGGACCGGGC
ACAGGAGCGCCTGGCTACAGCCCTGCAGAAGCTGGAGGAGGCTGAGAAGGCA
GCCGATGAGAGTGAGAG**GTGCGCAGGGGGCCCTCGGGGTGGGTCAGAGAGGGGG
TGCAGAAACTGTCTCTGCTGATCCTGAACCCCACCCC(C/A)ACCCCTCTCCCTGCCTC
TCAG**AGGAATGAAGGTCATCGAAAACCGAGCCATGAAGGATGAGGAAAAGATG
GAGCTGCAGGAGATGCAGCTGAAGGAGGCCAAGCACATCGCTGAGGATTCAGA
CCGCAAATATGAGGAG**GTGACCGCCCTGCCCCACTTCCTTCTCATTCCCAAGCCCC
AGACACCCCCAGTCATTCCCCATGGATCTGCTCCTCCTCTCCCCGCAG**GTGGCCAG
GAAGCTGGTGATCCTGGAAGGAGAGCTGGAGCGCTCAGAAGAGAGAGCTGAG
GTGGCTGAGAG**GTGAGGATGCCCTGGGGCGGCAGTGGAACTTGGCCTGCGCCTGT
CGCTGAGGGGCAGTCTGCGCAGTGGGCAGGCAGTTTCTCAGGCTCGGGCCGTAGGG
ACAGGTCCTGTCCTCTGACCCCTACCCACTGCCCCCAG**CCGAGCCAGGCAGCTGGA
GGAGGAACTTCGGACCATGGACCAGGCCCTCAAGTCCCTGATGGCCTCAGAGG
AGGAG**GTAGTGGCCTCTCTGGACCTTTCTGGGCAATGGCACCTTCTCTCAGCTCACC
TCCCTCCATCTCAGGCTGCTGTCACCTGCTGGGGTCGGGGAGGGCCGAGACCTCCAA
GCTCTGCCTGCTCTCACTCTCACAACTTTGCTCTTCTCTTCTCTCCTCCATCCCTCCCC
CACTGTGCCTTCTCTGCTGTCTCCCCACGGTGCCCTCACACCCCACCCTGCCACACAC
CCCCTGCAGTAAATGTGGGGACCTAGAGGAGGAGCTGAAAATTGTTACCAACAACT
TGAAATCCCTGGAAGCCCAGGCGGACAAGGTAGAGGGGGGCAGAGGGGCAGTGAG
GCTGGGGTCCTCCAGGGGAGGGAGGCGCTGGGCCAGGCAGGGTGGGGCTGGGTGTT
CACTGGAGACAAGCTGCTGAGTGGCCTGTGGGGCTCAGTGGAGAGGTGAAGGGTCT
GTGGAGGCGATCTGCTGACGGCATGTGTGTGTCCCCGCAAG**TACTCCACCAAAGAG
GATAAATACGAAGAGGAGATCAAACTGCTGGAGGAGAAGCTGAAGGAG**GTGAG
AGGCCTTTACCTCCTCCCCAGCCCCCCTTCCCCGCCAGAGACTGCCTGCGGCCGGCC
GGGAGGCCAGGGCAGGGATGGAAGAGAAGGGGAACTTGAGTGACAGTGTCTGGGA
GGGGCTAACTTTTTGTCATTTCCCCCGTCTTGCCCTTACTCCCAG**GCTGAGACCCGA
GCAGAGTTTGCCGAAAGGTCTGTGGCGAAGTTGGAGAAAACCATCGATGACCT
GGAAG**GTAAAAGGACTACTCCCCAGTACCCGAAACCTTTTTAAATAGATCCACACT
CCCTGCCACTGTTGCTCTGTCCCTCATCTTCCCATCCCATTATATTTGCCACAGTCAG
CTAGGGTGGGCCCACCTGGGGCTCCGCAAACTCTGTGCCTGCCGTTCAAGTTGTAGC
GGCTCTGGCACATGAACGCCGGTGTTTCTCACACTTGGGAGTGCATGAGCACCCCCA
GAGAGTTTAAAACACAGATTCCTGGGCCTGTTCCGAAGCGATCGCTGACTTGGGCTG
GAGAATTTGAATGTTTCCCCAGCTCCCTGGTGGCCAGCGCCATGGTCCTCAGACCAC
ACTTTGAGTAGCACTGAGCTGGACTGTTCTGTCTCCAAGCGCAACACAGCAGAGCCG
CACCACAGCGGCTTCACTGCAGAGCTATCTGCTGTCATGTCTAGGCTTTCAACATTA
ATGGAAAATACTACTTTAAGCTAGATTTAGTGAAAACAAATACCTAGGTATTTCCTA
GTCTGTGGCTATTCCTCACTATTTTCAACAATGTTGTGCCCGAAACTGTCCTTCCTCT
TCACTCTGCGGTGACCCTTGCACCTTCGTAATGGCCACTTCTCTCGCTTCCAGCCTTC
TCAGGCAGGCCTCAGCTCTATCTCCATCTTTAGGACTTTAGACCTTTCTGGCTCGAGC
ACCCTACCTCCACCCTGAAGCAAGCACTCCTGCTTCTCCAAAGCACTGTGCCCCCGG
GTCCCAGGGGAGACAGGAGCTTGGGGGAAAGAACAATTTAGGGGGACCGGGAGGG

GAGGAGGGAGCCCTGACAGCTGTGCAGCCTGTTCTCCAGCCTCTCTGGCACTCCATC
TTTGTCCACCTCTCTCTGATTCTTGTTCTCTCTTTCCATTACCCTCTCTCCTTCCTTTCC
GCTTTCCCTCTGTTCCCTCTCCCCACACTCCCACCTGGTCTTCCTGCCCCGGGCCCCC
CCCCCAGACGAAGTCTATGCACAGAAGATGAAGTACAAGGCCATCAGCGAGGAGCT
GGACAACGCACTCAATGACATCACCTCCCTCTGAGCCCCCACCAGTGTGGCCCCTCG
GCCCCTTCTCCCCTCACCAGTGTGGCCCTTCGGTCCCTTTTCCCCCTGCCGATGTGGC
CCCTTGGCCCTTCTCCCCTCTCCTTTCCATTCTCTCTGTGGGGAGGGGGCAGGCAGAA
GGAGCAGAAATTGCCGGCATCGCATAGCCAGCCTGGGCGCAGCCTAGGAGAGCCCC
CATCGCACCTCCTGCCCACCCTGGCGCTTGCTTCTTCCTTCTCTCCGCCCACTCCCTG
CTCTGCCCACTGGCCTCTACCTCTCTCTGCTGCTTAATAAACTCAACTTGGTCTCCAT
GCTGTTTTCCTGCCCTCCGGAGAGCACTTTCATCACCCCCAGAGCACTGGTTCACTG
CTGAGACTCTGGCTCTTACAGACACCCCCAATCTTGTGACCCCACCACTGAGCTCAC
CCACAAAACACTGTGGCCCACACAAAAACCCCCTGCACACCGGCACTCCCACTCTG
ACCTTCACTGCTGCATGTGGGCATGTGTGACCCCATTAGTCACCGAGCCCTCTGGCG
CCTCTGAGAGACACACCCATCTGCAGACCCAGGCCTGTCACTGACCTCAGGGTGCTC
GGCCGGGTTCCCCGGACTGGAAGGAATGACTGGAAGGGAAGTGACTGGAAGGGAA
ACAGGGCGGTGAGGTGCAGGGGAGGGGCAAGGAGCTGTGAAAGGTCTGGGATGAA
GTTTGGGAAAGTGATGGAGGCCAGCAGCGTGGGGTTTTCACTGCAGGTCTGTTTTCT
GCCTCAAGTCTGTCTGTCTATCTGGCCCTCCCTTTCCTTGCTCAGTCTTTGTCTTTCTT
TCAGTTTCCAACTCAAACCTTCTAAAGAAGGAGCATAAATTAGTTTATGACTTAGGC
CTTCCCATCCCACCTCCATGACCCCGAAGCCTCGAGTGAGGCATCCATGTCTGTCCT
GGTCAATCCGTCCATTCTCCCTCCGGGGTGACTTCATGGGGTCCTCAGATCCCTGAA
TGCTGTGTCCTCCTGGGCCTCCAACCACTCCATGTCTATTTGTCGCATCCCCCCTCGT
GCCTCCCCGTGTCTCTCCCCTCCCCCTCCTCATGTGCTGCTTCCCCACACAG**AGACCT
TGGCCAGTGCCAAGGAGGAGAACGTGGAGATTCACCAGACCCTCGACCAGACC
CTGCTGGAACTCAACAACCTGTGAGGGCTGGCCCTGCCCCGAGCCAGGCTACA
GTTGCCGCCCCAACCCAATAAAACTGATGTTACCAGCATCACAGGGCCCTTTAA
ACTTT**TCTTTAGTGTTGGCGATGGTGATGGTAGGAGAAGGCTGAGAGGATGCCTGA
GAGGAGCAATGGAGTTGGGAGACCTTTGAGTGAGCAGGGCCTGGGTCCATGCTCTG
CAATGGGAGCCAAGAGGTGGGGGCACTGGGCTCATTAAGGCGGGGTTGTATTGGGG
CCGGCCCAGTGCTGCAGCGGTTAAGTTTGCATGTTCCGCTTCGGCAGCCCCGGGTTT
GCCA(G/A)TTTGGATCCAGGGTGCGGACAGGGCACCACTTGGCAAGCCATGCTGAGG
CAGGTGTCCCACATATAAAAAGTAGAGGAAGGTGGGTACCGACGTTAGCTCAGGGC
CAGTCTTCCTCAGCAAAAAGAGGATTGGCAGATGTTAGCTCAGGGCTAATCTTCCTC
AAAAAATTAATTAAATTAAATAAATAAGGAGGGGTTGTGAGCAGGCCACGTGAAGC
AGACAGCCCAACAGGGCCGGGGGGACATCCTGGCCCCTACTTCTACAGGGCAGAGG
AGCTGCTGTCACAGCATTTCCCCATTCCGGCAGGCGGCTGGGGCACACAACACAATC
TGAAGACAACATTGCACAGCTACACAGGTTGTACTTGAGCAGGG

APPENDIX C

Haplotypes of affected and unaffected foals from the family-based GWAS. The SNP marker coordinates in blue are those residing approximate to or within the titin gene and the SNP coordinates highlighted in green are those approaching when the associated region on ECA18 from the case/control GWAS was tested in the kindred of 60 animals.

Foal ID	Affection Status	56567378	56735837	56779448	56785443	56843481	56859971	56861571	56863586	56863692	56889413	56889525	56990492	57022464	57022573	57087413	57117832	57122419	57125689	57139212	57246288	57299129	57318584	57318586	57434860	57503825	57523800	57529010	57532286	57578847	57579230	57703985	57943621	58026623	58029997	58186973	58268935
JC61	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC61	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC69	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC69	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC70	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC70	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC132	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC132	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC104	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC104	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
JC134	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC134	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC136	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC136	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC158	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC158	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC160	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC160	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC162	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC162	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC168	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC168	CASE	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC172	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC172	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC147	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC147	CASE	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
JC164	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC164	CASE	0	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	0	0	1	0	1	0	0	1	1	0	1	1	1	0	0
JC170	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC170	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC126	CASE	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1
JC126	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC166	CASE	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
JC166	CASE	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC101	CASE	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC101	CASE	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC252	CONTROLS	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1
JC252	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC254	CONTROLS	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC254	CONTROLS	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC261	CONTROLS	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC261	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC267	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC267	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC273	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC273	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC258	CONTROLS	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC258	CONTROLS	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	1
JC256	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC256	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC269	CONTROLS	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
JC269	CONTROLS	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
JC271	CONTROLS	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
JC271	CONTROLS	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0

TBX15

Haplotypes of affected and unaffected foals from the kindred of 60 animals for the candidate region on ECA5 identified in the case/control GWAS. This region includes the gene *TBX15*. The SNP coordinate highlighted in red is the most approximate to *TBX15* and the coordinates in orange indicate SNP approaching significance when tested in the family.

Foal ID	Affection Status	49204670	49255753	49292647	49298811	49304134	49389482	49501930	49521450	49603095	49860412	49860757	49903532	49903574	49964648	49974954	50020228	50075387	50085089	50085351	50129380	50141498	50180676	50209292	50310551	50316210	50375328	50376311	50393996	50397789	50398104	50413870	50444342	50500963	50508657	50761956	50938041	50940234	51007116	51011558	51031692	51065448	51151332	51299348	51302555	51304825	51341691	51385651	51455957	51499985
JC61	CASE	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC61	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC69	CASE	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC69	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC132	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC132	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC104	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC104	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC134	CASE	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC134	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC136	CASE	1	0	1	1	1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	1	0	0	1	1	1	1	0	1	0	0	0
JC136	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC158	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC158	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC162	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC162	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC168	CASE	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC168	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC172	CASE	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0
JC172	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC147	CASE	1	0	1	1	1	0	1	0	1	1	0	1	0	1	0	0	0	0	0	1	0	1	1	1	0	1	1	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC147	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC164	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC164	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC170	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC170	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC252	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC252	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC254	CONTROL	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC254	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC267	CONTROL	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	0	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0
JC267	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC261	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC261	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC258	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC258	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC256	CONTROL	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC256	CONTROL	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC269	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC269	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC271	CONTROL	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0
JC271	CONTROL	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0	0

CASQ2

Haplotypes of affected and unaffected foals from the kindred of 60 animals for the candidate region on ECA5 identified in the case/control GWAS. This region includes the gene *CASQ2*. The SNP coordinate highlighted in green is the most approximate to *CASQ2*.

Foal ID	Affection Status	52690753	52690883	52692481	52694367	52696088	52696139	52696540	52699832	52725393	52786942	52806991	52899664	52961430	53133920	53134165	53192988	53198662	53206673	53218124	53253842	53346217	53365624	53538898	53644387	53728115
JC147	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC147	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC164	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC164	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC172	CASE	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0
JC172	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC61	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC61	CASE	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC104	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC104	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC132	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC132	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC158	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC158	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC166	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC166	CASE	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC69	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC69	CASE	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC70	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC70	CASE	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC134	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC134	CASE	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC168	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC168	CASE	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC136	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC136	CASE	1	1	0	1	1	1	1	1	0	0	0	1	1	0	1	0	0	0	1	0	0	1	1	0	0
JC162	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC162	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC170	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC170	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC252	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC252	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC254	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC254	CONTROL	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC256	CONTROL	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC256	CONTROL	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
JC258	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC258	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC261	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC261	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC267	CONTROL	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0
JC267	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC269	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC269	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC271	CONTROL	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC271	CONTROL	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	1	1	1	0	0
JC273	CONTROL	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0
JC273	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

AMPD1

Haplotypes of affected and unaffected foals from the kindred of 60 animals for the candidate region on ECA5 identified in the case/control GWAS. This region includes the gene *AMPD1*. The SNP coordinate highlighted in green is the most approximate to *AMPD1*.

Affection Status	53133920	53134165	53192988	53198662	53206673	53218124	53253842	53346217	53365624	53538898	53644387	53728115	53851924	53875750	54025556	54029966	54030212	54084267	54195545	54251377	54253387	54312432	54335720	54370558	54502557	54539683	54561516	54611785	54823158	54885914
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	0	0	1	0	0	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	0	0	1	1	0	1	0	0	0	0	1	0	0
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	0	0	1	0	0	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CONTROL	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CONTROL	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1
CONTROL	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	0	0	1	0	0	1
CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

CAPZA1

Haplotypes of affected and unaffected foals from the kindred of 60 animals for the candidate region on ECA5 identified in the case/control GWAS. This region includes the gene *CAPZA1*. The SNP coordinate highlighted in red is the most approximate to *CAPZA1* and the coordinates in orange indicate SNP approaching significance when tested in the family.

Foal ID	Affection Status	55184021	55290030	55293804	55294311	55304751	55307605	55317182	55319162	55503606	55519047	55628374	55728630	55758920	55776139	55836086	55898566	56110634	56110906	56177960	56265761	56348491	56392983	56446872
JC147	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC147	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC164	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC164	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC172	CASE	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC172	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC61	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC61	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC104	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC104	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC126	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC132	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC132	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC158	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC158	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC166	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC166	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC69	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC69	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC70	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC70	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC134	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC134	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC160	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC168	CASE	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC168	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC101	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC136	CASE	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1
JC136	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC162	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC162	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC170	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC170	CASE	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC252	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC252	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC254	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC254	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC256	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC256	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC258	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC258	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC261	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC261	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC267	CONTROL	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC267	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC269	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC269	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
JC271	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC271	CONTROL	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
JC273	CONTROL	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0
JC273	CONTROL	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

APPENDIX D

Ankyrin Repeat Domain 1 (*ANKRD1*) EquCab2:1:37682532-37673188 (reverse strand). Sequence in red font and highlighted in pink denote exons and single nucleotide polymorphisms discovered by resequencing of *ANKRD1* are highlighted in yellow.

CTCTAGGGAATTCCAAACAGATATAGACAAGGGCCTTTAGGACCCGGATCCTTCCCT
CTCAGGCTGTTTGCCCACAGGAATAGGATGTCCTGAAGCAACACTTCCCCCCAGTGA
AGTGTTGATAAGTCTGGTTATCAGAAAGATATTATTGGGAGTGTGATATGCAGGGCA
TTTACATTTTCTTGATAGGGTTAGTCATATGAAAGCTGACAAAGAAGGAAAAAGAG
CAGTGCTGTGGTGCAATATCAACAGACAGCTGTCCCCTGGCTTCTCGATAAATAGGA
TGACTTGCATTGCTGAGCGGTGT(A/G)GTCACTGCCAAAGGCATGGCCCTCTCACATT
TCTTCCTGATTCACATATTCAGTGGGGTTACTTGTCATCCCCTCCCTCTTCTGCTTCCC
AGACATTGCGTCTGGAATGAAAATTCACCTGCCTCTGAGTTGGCCGCGGGTGGGGGC
AGGGGTGTTACTTGGGTTCCCAGGTTGGAAGATTATCTCACCCAGCCCTGGCTATAT
AAGCCGACCGGTGTGGAGCGGCTCACAAGGGCCGACTCAAGGGAGTCATTCCACAG
GAGAAGAACATACAGACGAACTTCAGGCTGAC**ATGAAGATGCTGAAAGTAGAAG**
AGCTGGTATGTAAGACATTATTTTTATGCAATGAATACTGG(A/T)TCAGTCTTAATT
CTTATTTGAAAATCGAAATACCCCTTTCTTTCCGGCATAATGCACAGGTACTCTGAA
AATGTCCAATATGGAAAATGGTTGGTTTAGGTGATGAAATAAGTGAAATTGGAAAA
ATGGAGCTGGCAGTAACAGATTCTCTACATAGTTCCTATTTATTATCTTCTCCTTTGG
TTTTATTACTAAACAGTGGAATCGCATGCAGTTACCAAAATGCCTTAGGGAGGAAGT
TCTATTGCAAGAAATGTATGTCTGGAAACTTTACAACTCAAGGCCAAGGGTCATCAA
AAGTATCTAAAATGGAAGAATTTTAAATAATCAGAGAGGGGTTGGGATTTTCCTAG
GAGAGATGTGATGAAGCAGATTGAAAGTAGATTTAACTATATCAACCTAACCTAAG
ACCTGATTTAACTGAGCAGAACAAGGTAATCCTGGAATAGTGCAGCAACACGGCAC
AGCCCAAGATCTGACACTGTGCTGCTCGGAGGTGTCCATCACCAGGGAGGGAGGAC
CGGGGGCCTCATGCAGCATCCTCTGTGGAGAGTGACCCCAGGACCCCACTACTTCTG
ACC(C/A)TGCTATCTT(C/G)TTCCCTCTTAG**GTCACAGGGAAGAAGAATGGCAGCGG**
GAACACAGGAGAGTTCCTTCCTGAGGATTTCAGAGATGGAGAGTATGAAGCTG
CTGTTACTTTAGAGAAGCAAGAGGATCTGAAAACACTTCCAGCCCACTCTGTGA
GCCTGGGGGAGCAACAATGGAAAATTGAGAAGCAACGAGAGGCAGAGGTAAGA
ACCTTCCATGGAATCCATAGTGGGGGGGG(A/G)(A/G)GGGGGTGACCAC(A/G)GAGTA
GGGATGGAGGTGCCAGGCAGGAAGGAGAAAAGAGAGAAACAAGATAACCACAACC
ATTGCAAAGATGTTTCTTTACCAAACTCAAACCCATAAAGAGGCAACTTCCATGGGT
CAGGTAGTGGGAAATAATTGTCTGGCAAGACTATGACCTTTGGATTCTGAATCAATG
ATTCAATGTTCTATGACCATGTTCTGATTGAAAGTCTTGGAAAGACAAACATGTCCA
GTGAATTTCTGATGTCATAGGCCGTCTAGTTAATCCCAAGCAGAGGAAACCTCTGTT
TCTCAATAACGTGGTTTGTGGTCCACTGATCATTGTTTGACTGTGTCCGCTATCTGTG
TTGACAAGATCTGTGTTGCTGGGCAAATTTTCATTACTTACTAACCACAGATAAAAC
ACGCATTAGTTAGCTGTAGTCCCAAGACAGTCTGTTTCATGGGCCTTTCTTAGTGGG

CCACCTGAAGGAGTTTGGCTTTGATATTTCGGAGCTTAATGCTTATTATTGGGTTCTT
TGGGGTAAAAGAAAAAAAAAATTCTTGAGCATGATTTAAAAATCCTACAGCAATT
GGGGGTTTGTTTGTGGAAGTGAATACAAAATCCTCTCTTAGAGACCAGAACCTATCT
CTAAGTAGTCCAAAGCTGACGCTACAAGCAAAAGTGAAGATGAAGGCAATTAGCTG
AAAAACCAGACCCCCAAATACTTAAATCAGAATTCACTGAATCAGAATCAAAGACT
TATCTTAAATGTTTTTATTTTTTTGTTCAAACTGCAACAAACTTATCTCAGACCTTGC
AGATTTTGCTTAGACACAACCAGAATGCTTATCAGTAGAACTCACCCTAGTTTGAAA
TACGGATTTCTTTTTTAG**CTCAAGAAGAAAAAACTAGAACAAAGATCAAAGCTTG**
AAAATTTAGAAGACCTTGAAATAATCATTCAACTGAAGAAAAGGAAAAAAATACA
GGAAAACCAAAGTTCCAGTTGCCAAGGAGCCTGAACCTGAAATCATTGTAAGGA
CCGTTTGGTTTTGTCCTATGACACGTCCGCCGTTGCGCTCCAGGGGAATGAATATCT
GTGGAGGGTTTGACTGATGCTGGAAAGCTCACGGTAACTCTCACGCTATTTTTCTGG
CTGTAG**ACAGAACCTGTGGATGTGCCTCGGTTTCTGAAGGCTGCCCTGGAGAAT**
AAACTGCCAGTAGTAGAAAAATTCTTGTCAGACAAGAACAATCCAGATGTCTGT
GACGAGGTAAGGCTTATACAGAGCACTGCGAAATCCAGCTCATTAATTTTGTGTTTC
TTATGCTTTACTGCAGCCATGCTAATATGGTGCCAAAAATCCATCCACCTGAAACAG
TTTCCCCAGCTGTTGCCACATATGCCAGTAGCATAATAGGCGAGTGCTGTGGTCCAA
AAAGAGGAAAAATGCTGGATTGGTCCTATGGATTGCATCATTTTTTGCACTCATACA
CTTTCAGCTGATTTTTCAGTTTGCCTTTAACTTTTTAGAACCTTGGGATAGACTTTTA
ATGAGGCAGGACCAACAAGTTACTTTTGGAGAAATTATTAGCATTGACTTTGATTGG
ACCCAAACACATCAGCTCTAGACAGGGAATCTGGAGAATGGAGTTCAAGTACCAGC
TCTATCACTACTTGACTATGTGATTTTTAGCAGCTAATTTGCATTTTCTGAACCTCAG
TTTCCTTATCTGTAAATAAAGGGCATTGGGGTGAAATCACGCCCAAAGTTGTTCCAG
CTCTAATAGTCTATTCATCCATTCATATGAAGTCGATTTATCAGAAATCACTAATCCT
TTTTGTATTCTTTCCCCTATGAACTGAAATGGGGATTCATTTCTGAAGTTAAACTAAA
AATGAGTCTCCCATTCTCTTACAAATGAATTGAATACTTTTTGATATTCAACCTAGCA
ACAGTTCCAGACAGAAGAAAACTATCTGATCTGCATGGCAAATACTTCCCAAACAA
GGAAGGTGCAAAATACAAGAAAGGAAAGTGTCAGCATTTTGGTAGCTTTGAACCTT
TAAGTTCACATCCCTTGATTTAGACCTCTGAGCACATTGTGGGAAGCTCAAATCTCA
GTTGCAGGATAATTGTTCTAGTTCTGTAATTTTAGTAAACTTGACATTTCTAATTCAT
AAAGAAGCACTCGAGGTCCTATCTGTTAGGTCATCAGAGTCCCCTGCTTCATACCTC
TTAGAAAGGATTTATACATATTTTTATATGCATTGCTATCTCTCTCTTTTTTTAAAATC
TCTATTCTGACAG**TATAAAGGACAGCTCTTCATAGGGCGTGCTTGGAAGGACA**
TTTGGCAATCGTGGAGAAGTTAATGGAAGCTGGAGCCCAGATCGAATTCCGTG
ATATGGTAAATATATTTCTTTGCTTGGAAATGAGCCAAAATAAGTAGACTATATGAA
GCTTGAGAAAACCTACAATGAAATACTGGGTTGATTGGATTGCAAATTTATCTGGGA
GAACTAGGCAAAAGCTCCTTTCTCCATACAAAGGAAAGCTGGGAGATGGGACCATG
TCATGTGGAAAGTATTTTCCATACACACCAAAAAGGAGAGGAGTGTTCCAGGGAAA
ATAAGGAAGTGAAAGCTGTTGAACAGAATTCAATAACCTCATCAGAGGGCTGGGTC
ATTATCAAGTCAGTAACGTTACTGAGAAGTAATACTGTCAACACTTGCCTTTCAACA
TCCCTGTCCTCCCATTATACACGGCAGTCTGCTCGTGGCCCCTTGGCTGCCAGCAAC
ACATCAGCATTCTCTAACTGCACACCTTCTATGATAGACGCCCACTGCCATCCATGA
GATTTGGGGCCAGACGGTATACAAAATGAACTGCACGTATGGAAAGTCGCTGTTATT
CAGACCTCAATAATTCAACCACTGTGATTTCTACAAAGACCCGTATGCAGCATATTT

TATGACCTGAAGATTGAAGCTGGGAAAATGCTTTATTTATTTAACTGCAGCTCAATT
TGACTTTACAGCAAAGTGGAAAGGGGATATGAAAAGTCCAAAGGATATGGCTAAAA
CTGAACCCAAAAGACAAACACACAGAAGAACAATTAAAGAAACAACAAAAATAGT
TTCCCTAGAAAGACAGTAAAATTCATGAGTGTGGAATCCGGAAAGCCGGAGATCTA
TCAAACAGAGCAGTCAAGAGGGACTCTCCTCTCAGTAAGCGCAGGATGCAGTGACC
ATACATGAAGATACTGGAGGTACAGAGGCTGTGGTAGCACAAATGACAGCAGAGAA
CCAGCAAGAGAGGAGGAGAAAACAACAGGGATACAAGAAGTGTCAGATCCAGATT
AAAGGCTCCTGGAAGGGACTACTCTTATTCTGCCAGAATTTTTAAATATACTTGTCT
AGAACTTTTGGGTATATTTGGCAAAAGTGGCTAAAAGTTCCAGGCAGATGTGGGTCT
GAATATTTATTCTGCTACTTATTAGCTGTGTTTCCTTGGGGAAGAAGCTTGGATATTT
TGGAGCCTTGTTTTTCTCATCTGTAAATGAGCACAGTAACACCTCATAACGTTGCTG
GGGGGATCAATGTGTCTAGAATGTGTAGCACCATGCTGGCATGAGAAAATTCACAA
TAAATTGTAGTTCTGAATCTTTTTAAAAGAGATTTATTAAGAGTCTCCTAATTATGC
ACACACATGCACACAGATACACACACACGAGTGCATTCACACATGTATAGTGAAAA
CATTTGACAATCTAGATGAGGACAACCCAGCGTTACCACAGATGCCGTAGGAAACC
TGGAATTCCTGGGCCTTAGAAACCAAGTACCAATTCCTTGGACCAGGTTCTTCTTTAT
GTTAAGTCATGTCCATTTATCTTCCTGGTACAG**CTTGAATCCACAGCTATCCACTG**
GGCAAGCCGTGGAGGAAACCTGGATGTCTTAAAACTGTTGCTGAATAAAGGAG
CAAAAATCAGTGCCCGGGATAAGGTATTTCTCCTCCTCCCACCACCGCCCCCCTTC
CTGTCCCTCTCCTCTTCCTCCTCTTCTTTCTCTCTCTTCCCCTTTTCTCCTCCCTTGCCT
CCTCTCCTTCCTCCTTCTTCTCCCTCCTCCTCGTTGCCCTCCACTACCAAATCCACCTC
ACCATTGCTGTCAGCTGGAAGCTCATGGTGTGCATAGTCACCATTGTGCCCACATCC
CCTTCTTGCACATGCTTTGGGGTCTGGGAG(A/T)GCCTCTGTCTTCACGC(C/T)CGGTG
AATCAGTGGTGTTCTTCAATTTCCCAG**TTGCTCAGCACAGCGCTGCATGTGGCAG**
TGAGGACTGGCCATTATGAGTGCGCGGAGCATCTCATCGCCTGCGAAGCTGAT
CTCAACGCCAAAGACCGAGTGAGTAGCTGGACAAAGTTTGGCCTCTTTAAGCTTCA
TTCCTCATTTATTTGGAATCACTTCCCAAGCTGGGTACCCTTTAGGTGCCTACTTCCC
GGCCTCAGGCCCACCTTTAGCCTGTTCACTCTGCTTCTCTAG**GAAGGAGATACTCCC**
CTGCATGACGCTGTGAGACTGAATCGTTACAAGATGATCAGACTCCTCATTATG
TATGGCGCCGACCTCAACGTCAAGAACTGTGTAAGTGCTCCAGACACGGGGCCCC
ACTTGCAGGCTTTCTCATTTACAAAAGCGTATCTTACATACTTGAAAATATTCCCACA
GTGGTAGTATTTCAGGGGTCTTCTGATGATTTAAATGCCCCTGTTAACCCCATAAGG
ACATTGCTAACCTTGGCAAGGACTTGGGTCACCTGGTCAGAGATGGGGGAAGCCAT
AGTGACTGCTTTGACTTCAGTGCCTAATATGGTGTCTAGTAATTGTTTAAAAATTGTC
TCTTGAAAATAAAAGACGGTCAAATTGCTCACCTTCCCAAACACAACCGTAGTCTGC
ATGGGCCACCTCTGGCTGAGCAAGACACCCACCATTCCTGAAGTGGGCTAAAATTCC
ACAGTGGTCAACACAGCCAAGGGCCAATGAACCCAAATTAATCAATCTCTATCTGA
GTAATTCAAATAGGCCTCCCCTCTTTACAACAGCATGGCATGCTACCACTTTCTTCCA
GAGGTAGCATGCTTTTGCCTATATTTTTGAGATAATTTCCATTCATTCTCCACTTTGG
TGTATCTGCACATCCCTCAAAATAATTTCCCTATATTTAGACCTGAAAAATGCTTGTA
TAAGTCCCTTCCACAAGTACATATTAATTTAGCTTCATCTGTAAGCAGGGAATGTAA
CCAACTTTTCCTCATGGTCCCAATTCCCAGACAAACGTCATCTAATATAAGCTTTAA
AAATATCACCCAGAGGAATGAGTGTAAATTACTGAAGAAATAACTTGAAATAAGCT
TTTGGAGAATCACTTTACAAAGAAGGAGAAGAAAACCCCAGTCATCTTAAAATGTG

AATAATTTCTTCCCCTTATTGTGTTACTATGAGACTGAAATATTTTAGCACTAAAAAA
AAAATAAACTGGCTTGGTGATTTAGGAACAAAGAATAAATGCAATAAATAGAGACT
GCTTGGGGTAGGAAAGTGTTAACAGTAGGGTTCCCTCATCCTTGATATTAGGACTGG
TCTTATCTAATTATTTAAATAAACTATCAGAAAAAGAGTAGATTCAATGAAATCTCC
AAAGCTGCAGAAAATACGATTTTTTTCAGACAGTGAGACACTACCAGCAGAGGAAG
CTAGCATATAGATTGGGGGAAACTGTGTAAGCAGCCAGAAAATCAGGAGATAAATT
TCAATAGAGAGGAGTGCAAGGAATTTATGGAATAGCCCAAAATATACAAATAGGGC
TCAAGGCTCTGAATTATTAGACTCAGGCATAAGGATTTTGGAACTATTAATATCATT
GACCCAATGGATTGATGTTCCCCAAAAAGACCTCTAAGCTATTGGACACCATCTTGA
TGGGGACTGGAAACAACACACGATGCGTTATTCTATCGTTGTCCAAAACTCAGTCTT
AATGTCTGCTGATGTGTTTTTCAAGGAAGATGCTAGAGAGGTAAGAAGTTCCAAAAT
GAAGGTTAGAGGGCCACTCAGATACGCACAGGGGCCATGCAGATCACATACATGAC
TTAAATGGGTGGCTGCAGGAGTCTGTGCACGTAACTTGTAAAGGTGGTGGCTCTTTC
CCAGCCCAGTCTGTTGCTGCTCTGTAGAACCAGGGGTACTGTGCTGCCAACTCTTCT
GATTTTTTTCAGAGACCACAAATTCAGCTGGGAAATCTCTTCATTGCCAACTGTTGCC
AATTAATTCCAACTTTAAAAAAAAAATCTAAGCTCTGTTTGGGCCCAAACAAAACCG
GTCTCTAGACTGATTTGCATCTCAAAGCCGCCAGTTTGCAATCTCTGGGTTAAAGAG
ACTTCTCAATGAATAAAAATGGAAAAATTTAGTACTTCTCATATTTCAAGACAAGAA
TTGAGGTGACATAGTTTTAATTTTTGTAAATATTTTGAGACACATGCATAAGGGAAG
AAAATTTCGGTCTCAAATATTTATTCACACTTGTCATTGCTGTCACCTCCAAGAAAA
ATAAATTTCCTGGGAGCCCAATGTAATTCTGAGCAGTTATGAAAAACCTACCCCCTG
GAGCAGGAATAGTCCTGAGCAGTACTTGAGCACTTAAGCCCAGGTGTTGAAGGAGT
TCATTTACCACCAGCTTTTATCAGTCAAGGAATCATCTTCCAAATTGATAGGAACCG
GTTTTTGACCAAGAGATCAAGATTACCACCCCATGCCCATTCTGCAGCATACTGGAG
ATGTATGGAGGCATTTTTTTGAATTCCAAAATGCTTGGCGTGCTCCTGCCATTTAGTG
TCTAGGGACTAGGCATTTTTATCAATACCTGAAATAGTCCCATAGGAAGAAAACCAC
TGCTAATCTCAAATGCCAAAAGCATTCCCTGGGAAACAGTGAAGGACTGGTTTGAAT
ATAAACTGCAAATCACTCATAGTGGAAAGCATAAAACACTTCAGTAACACACCTAT
CCATCACCTCATTAAACACACAGAGAGGGGCTGTGAATTTATAGATGCCTCCCATCT
GGAAGTGAAATTTCTTCATCTATTTTGACTAG**GCTGGGAAGACCCCAATGGATCTG**
GTGCTACATTGGCAGAATGGAACCAAAGCAATATTTGACAGCCTCAAAGAGAA
CTCCTACAAAACCTCCCGCATAGCTACGTTCTAAGAGAAATGACGACAGACTTT
TCAACAGCTGCTCTTCTTCGGTATTTTGAAGGCACGGCCCTAATGAGTAACTAGTCA
TAAAATCCAGCTTCTCTTCATCAACCTGTTGTAAAGCTGCACCTAATGAAGTTTTGAC
AAAGCACAGGGATATAGGTATTGAAATTTCCCTTAGTGAAGCTCACTCTATTTATTT
TTATTTATTCCTGTTTATTTATTTTTTTAAACTTACTTCGATACCACAGATATTCAGAT
CCTTCGCAATCATCCGTTTGGTGAGCAAAGCTTAATTTGTGTGTAACACTTCGGAGT
CTTCTTGTAGACAGTTCTTAAGCCATGTTACAGAGCAATGCTCTCAGGGCCTACTCA
TGTGTCACCTCGCTCATGGAAGGTCCTTCAAGGTGTTTTTAAAAGACGAAGGTGATA
ATGCAGCCCTCTTTTCTCTTGAGTTTTTAATGCTGAAGTTTGAGGCAATGGAAGGCAT
GTGGTGACAGGGAAGGAAACAGGAGAATGAAAGCGACTCTGTGGGAAGGACAGTC
GAGCCAGCCTGGGCCTGGTATTTCTTACCAAGTCAGGCACAGTATCGAGGTAAAGA
GAACGTTGGAGCGTGCGCGAAGAGTAGTGG