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Fine mapping of quantitative trait loci (QTL) for osteochondrosis in Hanoverian warmblood horses

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Dedicated to my family.

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List of abbreviations

A	adenine
Acc. No	Accession number
APS	ammonium persulphate
ATM	animal threshold model
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary deoxyribonucleic acid
chrM	mitochondrial sequence
chrUn	chromosome unknown
cM	centiMorgan
df	degrees of freedom
DL	Dempster Lerner transformation
DFG	Deutsche Forschungsgemeinschaft (German Research Council)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy nucleoside 5'triphosphates (N is A, C, G or T)
ECA	Equus caballus autosome
EDTA	ethylenediamine tetraacetic acid
EquCab2	Equus caballus assembly 2
EST	expressed sequence tag
F	forward
FISH	fluorescence *in situ* hybridisation
G	guanine
GS	Gibbs sampling
HET	heterozygosity
HSA	Homo sapiens autosome
HW	Hanoverian warmblood

HWE	Hardy Weinberg equilibrium
IBD	identical by descent
IRD	infrared dye
kb	kilobase
LAM	linear animal model
LD	linkage disequilibrium
LOD	logarithm of the odds
LSM	linear sire model
MAF	minor allele frequency
MAS	marker assisted selection
Mb	megabase
n	number
NCBI	National Center for Biotechnology Information
ng	nanogram
NPL	non-parametric linkage
OC	osteochondrosis
OC-F	osteochondrosis in fetlock joints
OC-H	osteochondrosis in hock joints
OCD	osteochondrosis dissecans
OCD-F	osteochondrosis dissecans in fetlock joints
OCD-H	osteochondrosis dissecans in hock joints
OMIM	Online Mendelian Inheritance in Man
P or p	error probability
P_L	error probability of LOD score
P_Z	error probability of Zmean
PCR	polymerase chain reaction
PIC	polymorphism information content
pmol	picomol
POF	palmar/plantar osseous fragment
POS	position
QTL	quantitative trait locus
R	reverse

REML	residual maximum likelihood
RFLP	restriction fragment length polymorphism
RH	radiation hybrid
SAS	statistical analysis system
SGC	South German coldblood
SNP	single nucleotide polymorphism
SSAHA2	Sequence Search and Alignment by Hashing Algorithm combined with the cross-match sequence alignment program developed by Phil Green at the University of Washington
SSC	sus scrofa chromosome
STM	sire threshold model
T	thymine
T_a	annealing temperature
TBE	tris-borate-ethylenediamine tetraacetic acid
TEMED	N,N,N’,N’-tetrametylenediamine
U	unit
UCSC	University of California Santa Cruz
WB	warmblood
WGS	whole genome shotgun sequence
X^2	ChiSquare

CHAPTER 1

Introduction

1 Introduction

"Where ever man has left his footprint in his long ascent from barbarism to civilization, we will find the hoofprint of a horse beside it" (John Trotwood Moore).

The fascination coming up from horses has not let up. Horses are employed in different disciplines and are exposed to miscellaneous challenges. However the use of horses is often delimited because of health problems mainly affecting the locomotory system. One of the most common and clinically important skeletal diseases is osteochondrosis occurring in many different species. As a developmental orthopaedic disorder osteochondrosis (OC) is a frequent cause of lameness in growing horses. Reports on the prevalence indicate that OC is present in warmblood and trotter horse populations at frequencies between 5% and 20%.

Articulations mainly affected are the fetlock, hock and stifle joints. Defects in enchondral ossification, especially abnormal differentiation and maturation of chondrocytes result in a number of different manifestations, including subchondral fractures, cyst-like lesions, wearlines, chondromalacia, cartilage flaps, synovitis and osteochondrosis dissecans (free joint bodies). These lesions can further develop into chronic degenerative joint diseases such as osteoarthrosis or arthropathy.

The specific causes of osteochondrosis are still unknown but OC is generally assumed to be multifactorial in origin. Environmental factors like growth rate, body size, nutrition, mineral imbalance, endocrinological dysfunction and biomechanical trauma are most discussed. Furthermore a hereditary disposition to osteochondrosis has been demonstrated in Trotter, Coldblood and Warmblood horse breeds but no responsible genes have yet been identified. In order to reach this goal a whole genome scan was performed to identify quantitative trait loci for the different traits of osteochondrosis.

The objective of the present study was fine mapping of the identified QTL on horse chromosome 5 and 16, 18 and 21 using microsatellites and single nucleotide polymorphisms (SNPs) in order to develop genetic tests based on gene-associated markers.

The development of new genetic tools in the course of the horse genome sequencing facilitates a more efficient search for associated genetic polymorphisms. We performed whole genome association analyses of SNPs with the aim to further refine the known QTL and to detect new potential QTL for osteochondrosis in Hanoverian warmblood horses.

Overview of chapter contents

The contents of the present thesis are presented in single papers as allowed by §4(4) of the Rules of Graduation (Promotionsordnung) of the University of Veterinary Medicine in Hannover.

Chapter 2 reviews the literature for OC in horses, including pathogenesis, clinical signs and proposed etiologies. Furthermore, the results of whole genome scans for osteochondrosis in Hanoverian warmblood and South German coldblood are presented, as well as new developments in horse genomics and potential candidate genes for osteochondrosis.

Chapter 3 contains refinement of a quantitative trait locus for fetlock osteochondrosis on equine chromosome 5.

In **Chapter 4** the refinement of a quantitative trait locus responsible for osteochondrosis on equine chromosome 16 is described.

Chapter 5 provides a new quantitative trait locus for osteochondrosis on equine chromosome 18.

Chapter 6 comprises fine mapping of a quantitative trait locus on equine chromosome 21.

Chapter 7 describes the confirmation and delimiting of all before mentioned QTL through the performance of a whole genome single nucleotide polymorphism assay.

Chapter 8 contains a whole genome scan with single nucleotide polymorphisms and an association analysis in order to determine new genomic regions responsible for OC in Hanoverian warmblood horses.

Chapter 9 provides a general discussion and conclusions referring to chapters 2-8.

Chapter 10 is a concise English summary of this thesis, while **Chapter 11** is an

expanded, detailed German summary which takes into consideration the overall research context.

CHAPTER 2

Osteochondrosis in horses

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2 Osteochondrosis in horses

2.1 Abstract

Osteochondrosis (OC) is a developmental disease in growing individuals due to disturbed differentiation, maturation and vascularisation of cartilage particularly at predilection sites of fetlock, hock and stifle joints. Hereditary factors play an important role in the aetiology of OC. In recent years, research in genetics of equine osteochondrosis has made great progress as quantitative trait loci (QTL) for OC have been identified. Now the second version of the horse genome assembly has been put online and recently, a single nucleotide polymorphism (SNP) array became available. Besides a brief overview of the pathogenesis and aetiology of OC, this paper provides the current developments in horse genomics, as well as the present results of the molecular genetic analyses of equine osteochondrosis. Furthermore, potential candidate genes are presented, which may help to unravel the genetic basis of OC.

2.2 Introduction

Osteochondrosis (OC) is a common and clinically important joint disorder and has been described in many different domestic species. The disease has been described in pigs (Dewey 1999), dogs (Morgan et al. 1999), horses (McIllwraith 2002), cattle (Jensen et al. 1981), cats (Ralphs 2005) and rats (Kato et al. 1987) where it is often a serious and disabling condition. It is also clinically important in human beings (Bohndorf 1998). Increasing use of radiography in equine medicine has brought to light the great importance of this disease in horses. OC can result in a number of different manifestations including radiologically detectable osteochondrosis dissecans (OCD), cartilage flaps and synovial effusions (Jeffcott and Henson 1998, Trotter and McIlwraith 1981). Articulations affected are primarily fetlock, hock, carpal, stifle, elbow, hip and vertebral joints. Finally, these lesions can further develop into chronic degenerative joint diseases such as osteoarthrosis or arthropathy in the cervical spine, which can lead to vertebral stenosis and wobbler syndrome (Jeffcott 1996).

A hallmark of OC is that lesions almost always occur at certain predilection sites within a joint. In the tarsocrural joint, the most common site is the cranial end of the distal intermediate ridge of the tibia, followed by the distal end of the lateral trochlea of the talus and the medial malleolus of the tibia (McIlwraith et al. 1991). In the femoropatellar joint, the most common predilection site is the lateral trochlear ridge of the femur. Less common sites are the medial trochlear ridge of the femur, the trochlear groove and the distal end of the patella (Watkins 1999). The predilection site in the metacarpo-/metatarsophalangeal joints the dorsal end of the sagittal ridge of the metacarpus and metatarsus. Opinions on the nature of fragments seen at the dorsal margin of the proximal phalanx differ, and the palmar or plantar osteochondral fragments (POFs) that were originally reported as being part of the osteochondrosis complex are considered traumatic in origin by several authors (Dalin et al. 1993, Nixon et al. 1995).

The inheritance of OC has been demonstrated in many studies in different horse breeds. Recent studies provided evidence that many genes are involved in the development of osteochondrosis. However, responsible genes have not been identified in any breed yet, furthermore, detailed gene interaction is still unclear. The capacity to investigate inherited equine diseases was given an incredible boost since the release of the horse genome assembly. A collection of single nucleotide polymorphisms (SNPs), mainly derived from the thoroughbred mare Twilight and further horses from seven breeds built the basis of an equine SNP microarray. This new technology enables investigating osteochondrosis in the horse in a thorough and efficient manner and promises substantial progress in the molecular genetic analysis of osteochondrosis.

The objective of the present paper is to review pathogenesis and etiology of osteochondrosis. Furthermore status quo in research of the genetic basis of OC is presented as well as new developments in horse genomics which can be useful for further research work.

2.3 Definition of the term osteochondrosis

The term osteochondrosis or rather osteochondritis dissecans was introduced by König (1888) and described a pathologic condition in epiphyseal cartilage, which causes a predisposition to the formation of loose or semiloose bodies in the joints of young individuals without the involvement of trauma or primary arthritis. The term osteochondritis has been replaced with osteochondrosis by many scientists because it is generally accepted that inflammation is not primarily involved in the development of lesions.

In veterinary medicine, the terms osteochondritis and osteochondrosis were not used until the 1960s but then introduced as an appropriate name for a developmental orthopaedic disorder with an origin in disturbed enchondral ossification.

2.4 Pathogenesis

Osteochondrosis (OC) develops due to disturbances in enchondral ossification of growing cartilage of the growth plates and/or the articular/epiphyseal complex (Jeffcott 1996, Van De Lest et. al 1999). Enchondral ossification is a sequential process of cell proliferation, extracellular matrix synthesis, cellular hypertrophy, matrix mineralisation and vascular invasion (Lefebvre and Smits 2005). Growth and elongation is achieved by continuous addition of cartilage and subsequent replacement by bone. In osteochondrosis focal areas of growth cartilage fail to undergo matrix calcification or vascular invasion and therefore do not become converted to bone (Ekman and Carlson 1998). The altered process of enchondral ossification leads to retention and irregularities in thickness of the epiphyseal cartilage resulting in the development of a cartilage core as primary lesion histologically characterized by a focal area of necrosis. This focal failure causes further damage and secondary lesions due to the regress of cartilage canals which affects the nutrition of deeper layers and results in necrosis. Thereupon biomechanical forces lead to the formation of fissures, cartilage flaps or loose bodies as well as subchondral bone cysts (Van Weeren 2005b).

Ytrehus et al. (2007) suggested a refinement of the terminology of osteochondrosis and included the modifiers “latens” for lesions confined to the epiphyseal cartilage,

"manifesta" for lesions accompanied by delay in enchondral ossification and thus radiologically visible, and "dissecans" for cartilage flaps or loose bodies. They draw on the theory that the primary lesion of articular osteochondrosis is initiated by necrosis of cartilage canal blood vessels and therefore should be defined as a focal ischemic necrosis of growth cartilage. As the necrotic cartilage does not undergo mineralisation or vascular penetration a secondary focal failure of enchondral ossification occurs when the ossification front approaches the lesion. Necrosis of cartilage canal vessels develop because anastomoses with bone marrow vessels in the growing animal are exposed to considerable mechanical stress and are vulnerable to damage as they cross through a zone of intense remodelling activity.

Articular lesions due to OC can develop very early in life. Abnormal radiographic findings and growth irregularities are commonly detected in warmblood horses at the age of one month (Dik et al. 1999), but most of these abnormalities disappear during the first months of life. The acknowledgement of a dynamic character of OC has led to a sophisticated approach. Extracellular matrix of the articular cartilage goes through a phase of rapid remodelling in the neonatal animal. Whereas bone retains its capacity to remodel throughout the whole life, cartilage metabolism decreases quickly in the juvenile period. Osteochondrotic lesions may develop and regress spontaneously as long as the metabolic level of the extracellular matrix is still high enough to permit such repair (Van Weeren 2005a). The point of no return may be determined by the metabolic status of the chondrocytes. This time-frame differs between the different joints and thus, the windows of susceptibility of horses for OC vary between the different types of joints (Barneveld and Van Weeren 1999). In warmblood horses, hock OC was considered as permanent with the age of five months as no resolutions of abnormal radiological findings were detected and shifts from normal to abnormal changes became rare (Dik et al. 1999). Stifle OC appeared as permanent with the age of eight months.

Ytrehus et al. (2007) constitute this time-frame with the narrowed period when epiphyseal cartilage is supplied by vulnerable blood vessels. Osteochondrosis only develops during skeletal growth because epiphyseal cartilage becomes avascular prior to adulthood.

2.5 Proposed aetiologies of osteochondrosis

Osteochondrosis is a complex disease and regarded as multifactorial in origin. Dietary factors, growth rate, anatomic characteristics, trauma and exercise are the main environmental factors discussed to influence the formation of dyschondroplastic alterations in the growing individual.

The majority of the published literature fails to support a direct role of increased growth rate. There is little evidence that the body weight affects the prevalence of early lesions of osteochondrosis, but it is plausible that increased body weight promotes the progression from osteochondrosis to osteoarthritis.

While trauma seems to facilitate the development of osteochondrosis into osteochondrosis dissecans it does not play an important role in the development of primar lesions. Nevertheless trauma may alter blood supply and cause rare cases of focal ischemic necrosis of growth cartilage in any site, including other locations than the typical predilection sites.

The most important aetiologic contributors seem to be hereditary and anatomic factors. Inherited faulty joint conformation and joint motion can increase mechanical stress to vessels during a limited period of time which leads to failure of cartilage canal blood supply, the possible key to the development of osteochondrosis. Heritability estimates for OC vary widely, possibly due to differences in materials and methods used between studies (Table 1). Linear models underestimate heritabilities and sire models do not use all available information. A part of the differences in the size of heritability estimates may be explained by these methodological aspects. Heritabilities were highest for hock OCD and lowest for stifle OC or OCD. For fetlock OC and OCD heritability estimates were in between.

Other generalized factors, such as dietary imbalance or hemodynamic disorders may be involved in the pathogenesis of osteochondrosis only in the case when they affect cartilage canal function (Ytrehus et al. 2007).

2.6 Current developments in equine genetics

Many traits of economic interest in animals are of quantitative genetic nature, which means that many genes each with a small effect contribute to a particular phenotype.

Genetic traits can be analyzed using genomic markers like single nucleotide polymorphisms (SNPs) and microsatellites.

Helpful tools for molecular genetic analyses in horses are on the one hand several linkage and RH maps (Swinburne et al. 2000, Chowdhary et al. 2003 Perrocheau et al. 2006, Penedo et al. 2005), which provide microsatellites, and on the other hand the horse genome assembly. In recent years much effort has been made on the equine genome sequencing. This project was performed mostly at the Broad Institute, BAC end reads for the project were generated by the University of Veterinary Medicine in Hannover, and the Helmholtz Centre for Infection Research in Braunschweig, Germany. The outcome was a high-quality draft sequence of a female thoroughbred horse, which has been sequenced to 6.8X coverage. Approximately 84% of the sequence has been anchored to chromosomes, which include autosomes 1-31 and sex chromosome X. Unanchored contigs that could not be localized to a chromosome have been concatenated into the virtual chromosome "chrUn", separated by gaps of 1,000 bp. The mitochondrial sequence is also available in the Genome Browser as the virtual chromosome "chrM". Gene annotation and analysis is currently ongoing in collaboration with the Equine genome research community.

To date the second horse genome assembly (EquCab2) is available (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=11760), which allows the development of new microsatellites by searching the horse genome for variations of repeat motifs.

In the course of the horse genome sequencing a large collection of single nucleotide polymorphisms (SNPs) was generated. SNPs were identified from a variety of modern and ancestral breeds, including the Akal-teke, Andalusian, Arabian, Icelandic, Quarterhorse, Standardbred, and Thoroughbred.

Microsatellites differ from SNPs in two important respects. First, individual microsatellites tend to be more polymorphic, and thus, more informative than SNPs. Consequently it is easier to detect genotyping errors in microsatellites and fewer microsatellites provide the same information. Second, SNPs are far more common than microsatellites, which means that SNP maps can be far denser and therefore potentially more informative than microsatellite maps.

A classical approach in molecular biology is to perform linkage analysis based on pedigrees in order to detect quantitative trait loci (QTL). For further refinement and for the analysis of candidate genes, association analyses with a large data set of unrelated individuals in case-control groups can be applied.

Derived from the EquCab2.0 SNP collection compiled by the Broad Institute the EquineSNP50 Genotyping BeadChip was developed by Illumina in collaboration with the International Equine Genome Mapping Workshop and the Morris Animal Foundation's Equine Genome Consortium. This BeadChip features 54,602 highly informative SNPs evenly distributed across the entire genome. This new technology is available since 2008 and enables horse researchers to conduct a broad range of genome-wide genotyping applications, such as whole-genome association studies and quantitative trait loci identification. Designed to enable identification of genes and mutations that contribute to traits of interest in all major horse breeds, this BeadChip offers a powerful platform for improving horse breeding programs.

2.7 Quantitative trait loci for osteochondrosis

Following the classical approach, a whole genome scan was performed in Hanoverian warmblood horses to detect QTL for the OC phenotypes (Dierks et al. 2007). Traits used were OC (fetlock and/or hock joints affected), OCD (fetlock and/or hock joints affected), fetlock OC, fetlock OCD, hock OC and hock OCD. The approach was based on 260 highly informative microsatellites, evenly spread over all autosomes and the X-chromosome with an average distance of 16.9 cM and an increased marker distance of about 5 cM in the genome-wide significant QTL regions. Significant QTL were located on eight different equine chromosomes: 2, 3, 4, 5, 15, 16, 19 and 21. QTL for fetlock OC and hock OC were mostly mapped on different chromosomes, indicating that these traits may be inherited independently. The QTL located on equine chromosomes 2, 4, 5 and 16 reached the genome-wide significance level.

A whole genome scan in South German Coldblood horses was performed for the same traits as for Hanoverian warmblood horses and additionally for palmar/plantar osseous fragments (POFs) as heritability estimates implicate a genetic component in

the variation of the development of osteochondrosis in South German Coldblood horses (Wittwer et al. 2007a). The genome scan included 250 polymorphic microsatellites equally spaced over all chromosomes with a mean distance of 17.7 cM with a higher coverage of microsatellites in putative QTL regions. QTL with chromosome-wide significant linkage was found on 10 chromosomes, including seven QTL for fetlock OC and one QTL on ECA18 for hock OC and fetlock OC. Significant QTL for POF in fetlock joints were located on equine chromosomes 1, 4, 8, 12 and 18. The QTL for POFs on equine chromosome 4 reached the genome-wide significance level (Wittwer et al. 2007b) (Table 2).

Based on this whole genome scan Wittwer et al. (2008) were able to identify SNPs in the *acyloxyacyl hydrolase* (*AOAH*) gene on equine chromosome 4 significantly associated with OCD in fetlock joints which could serve as a suitable marker for fetlock OCD in South German Coldblood horses. Furthermore intronic SNPs in the *xin actin-binding repeat containing 2* (*XIRP2*) gene on ECA18 were significantly associated with fetlock OC, fetlock OCD and hock OC resulting in the suggestion that dominant variants of *XIRP2* may be involved in pathogenesis of equine osteochondrosis (Wittwer et al. 2009).

2.8 Candidate genes for osteochondrosis

The whole genome scans were the first step towards the identification of genomic regions harbouring genes responsible for equine OC.

To select potential candidate genes, different information can be used.

Candidate genes are genes which code for hormones, enzymes, metabolic factors and/or their receptors involved in the complex of cartilage differentiation and maturation during enchondral ossification, in growth processes, or vascularisation. It can be helpful to use the Equine Articular Cartilage cDNA Library to select candidate genes which are at least expressed in cartilage. At the moment a total of 13,964 equine articular ESTs (expressed sequence tag) can be found at the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez).

Genes causing osteoarthritis in other species can also be used as candidate genes for the molecular genetic analysis of OC in horses.

Andersson-Eklund et al. (2000) identified three QTL for OC in pigs on *Sus scrofa* chromosomes SSC5, 13 and 15. Possible candidate genes derived from these QTL might be *pituitary specific transcription factor 1* (*POU1F1*), *insulin-like growth factor* (*IGF-I*), *cartilage homeoprotein 1* (*CART1*) because of their indicated role in the development of OC and their location in the homologous region of the human genome.

For man, these genes can be taken from the Online Mendelian Inheritance in Man (OMIM) database (Table 3). This database is a catalog of human genes and genetic disorders developed by NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim). Some of these genes, for example a part of the collagen genes or *MMP13* could be confirmed by various expression studies in horses with osteochondrosis.

Studies on the variation in gene expression of key chondrogenic genes and genes associated with cartilage pathology between normal and OC chondrocytes may also help to identify candidate genes and their potential role in the pathogenesis of osteochondrosis.

The endocrinological procedures of skeletal development and growth are controlled by hormones that are most likely to participate in enchondral ossification, such as insulin, thyroxine, growth hormone, parathyroid hormone and calcitonin (Glade 1986, Jeffcott 1997). Of the regulating proteins involved in enchondral ossification, the *transforming growth factor ß (TGF-ß*) plays an important role in growth cartilage metabolism, particulary in the control of chondrocyte differentiation and hypertrophy (Glade 1986, Henson et al. 1997, Jeffcott and Henson 1998). Henson et al. (1997) described a reduced expression and immunoreactivity in focal lesions compared to normal cartilage but strong expression of *TGFß1* in the chondrocyte clusters immediately surrounding a lesion and therefore a possible involvement in the pathogenesis of OC. Semevolos et al. 2001 found a higher expression of *TGF-ß* in affected tissue, but not significantly so, and suggested a healing response to the OC lesion. Hypertrophic differentiation and enchondral ossification of growth cartilage are regulated by a complex array of signaling peptides, including *parathyroid hormone related protein (PTHrP)*, *Indian hedgehog (Ihh*) and *bone morphogenetic proteins* (*BMPs*). A negative feedback loop between *PTHr*P and *Ihh*, controlling the rate of

hypertrophic differentiation has been well described (Chung et al. 1998, Juppner et al. 2000, Vortkamp et al. 1996). Hedgehog signaling occurs through the transmembrane receptor, *Patched (Ptc*), which upon binding of *Ihh*, releases its inhibition of a second transmembrane recteptor, *Smoothened (Smo*). *Smo* activation then results in stimulation of transcription factors, *Gli1, Gli2* and *Gli3*, which translocate into the nucleus to bind the DNA. While a significant increase of *PTHrP* and *Ihh* expression in chondrocytes from OC-affected cartilage and a decrease of *Gli1* expression could be observed, no different expression patterns were identified for *BMP, Gli2, Gli3, Ptc* and *Smo* (Semevolos et al. 2002, 2004, 2005).

Insulin like growth factors (IGFs) play an important role in cartilage metabolism and growth, including the introduction of increasing cellular proliferation and the synthesis of cartilage aggrecan and collagen (Semevolos et al. 2001). There has been ascertained an interdependecy of OC in hock joints and plasma IGF-I levels (Sloet van Oldruitenborgh-Oosterbaan 1999), Foals with osteochondrotic findings showed significantly lower IGF-I levels than unaffected foals. It is suggested that reduction in chondrocyte differentiation, as caused by lower plasma IGF-I concentrations, may contribute to the development of osteochondrosis. The significantly higher expression of *IGF-I* in cartilage obtained from osteochondrotic lesions (Semevolos et al. 2001) in eight to twelve month old horses, reflects a healing response to injured tissue rather than a primary alteration.

The composition of the extracellular matrix has been target as another molecular mechanism involved in the development of OC. Various collagen types that are represented in the extracellular cartilage matrix are known to play a role in the development and maturation of cartilage. It is well known that the extracellular matrix of the articular cartilage goes through a phase of rapid remodelling in the neonatal animal (Van Weeren 2005b). Additional evidence for the crucial role of collagen was provided by the demonstration of differences in post-translational modifications of collagen type II in samples from early osteochondrotic lesions (Van de Lest et al. 2004). The expression of *Coll-I, -II* and *–X* in chondrocytes from OC cartilage was significantly higher than in normal cartilage *(Garvican et al. 2008).* These results could partly be confirmed by Mirams et al. (2008) who found a significantly higher

expression of *Coll-I* and *–X* in the lesions, but no differences in the expression patterns of *Coll-II.* Also Semevolos et al. (2001) could not find any significant differences in expression of *Coll-I, -II and -X* between OC and normal joints.

The *ADAM metallopeptidase with thrombospondin type 1 motif, 4* (*ADAMTS4*) gene encodes for an enzyme, which is responsible for the degradation of aggrecan, a major proteoglycan of cartilage. Aggrecan degradation is an important factor in the erosion of articular cartilage in arthritic diseases, which is also reflected in a significantly higher expression of *ADAMTS-4* in OC cartilage, than in chondrocytes from normal cartilage (Garvican et al. 2008). However, aggrecan itself was not differently expressed (Garvican et al. 2008, Mirams et al. 2008, Semevolos et al. 2001).

Metalloproteinases are considered to be a key feature in the loss of articular cartilage seen in many joint diseases. Different studies on the expression of *matrix metalloproteinases MMP-1, -3* and *-13* revealed the same results as there was no significant difference in the expression of either *MMP-1* or *MMP-3* but a significant upregulation of *MMP-13* in OC-chondrocytes (Garvican et al. 2008, Kuroki et al. 2005, Mirams et al. 2008). Brama et al. (2000) investigated the role of *MMP-3* activity in synovial fluid in common joint disorders in the horse and concluded that *MMP-3* activity in OC joints appears not to be different from normal joints but was four times higher in osteoarthritis joints.

The proteins encoded by the *TIMP (tissue inhibitor of metalloproteinase)* gene family are natural inhibitors of the matrix metalloproteinases (MMPs), and therefore vindicate further observation. While *TIMP-1* showed a significant increase of expression in chondrocytes from OC cartilage in comparison to normal cartilage, the expression of *TIMP-2* and *TIMP-3 in* OC chondrocytes was significantly less (Garvican et al. 2008).

The fact that nearly all mentioned genes are not located in the identified QTL regions leads to the assumption that the hitherto definition of a candidate gene for osteochondrosis leaves a lot to be desired. Maybe one has to detach from the cascade of ossification, maturation and vascularisation, but rather for example focus on secondary responses or repair processes. The unsatisfying knowledge of the

aetiopathogenesis of osteochondrosis further complicates the identification of candidate genes but leaves an ample scope. For this reason it is so much the better to delineate the QTL, so the number of potential candidate genes might be limited just by delimited genomic regions.

2.9 Conclusions

Osteochondrosis (OC) is widespread in many breeds of horses. The disease is important for horse keeping, breeding and performance. Besides environmental factors hereditary dispositions play an important role in the aetiology of the osteochondrosis syndrome. Genetic traits can be analyzed using genomic markers like single nucleotide polymorphisms (SNPs) and microsatellites. Whole genome scans were performed in order to detect quantitative trait loci for the OC phenotype. Remarkable progress has been made in the past few years in clarification of the horse genome. The release of the horse genome assembly made it possible to develop new microsatellites for further refinement of the OC-QTL. With the development of new and efficient tools such as marker sets including more than 50,000 SNPs, it is now possible to verify and delineate QTL for equine osteochondrosis. The identification of causal gene mutations will allow breeding strategies to reduce the incidence in affected horse breeds.

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Table 1 Prevalence and heritabilities (h^2) of osteochondrosis (OC) and osteochondrosis dissecans (OCD) in different limb joints by horse breeds

Population	Number of animals	Radiographic finding	Prevalence	Heritability estimate	Method of analysis	Reference
Dutch WB	n=811	OC (stifle) OC (hock) OC (fetlock) OCD (stifle) OCD (hock) OCD (fetlock)		0.07±0.06 0.15±0.08 0.08±0.10 0.02±0.04 0.26±0.09 0.06±0.07	LAM[5] (REML[2])	Van Grevenhof et al. 2009
Dutch WB stallions	n=1965	OC (stifle) OC (hock)	11.5% 16.0%	0.09 0.11	ATM[1] (REML[2], DL[3])	Der Kinderen 2005
Dutch WB mares	n=590	OC (hock)	13.7%	0.01±0.06 0.14±0.17	LSM[4] (REML[2]) LAM[5](REML[2])	KWPN 1994
Italian WB	n=350	OCD (stifle) OCD (all)	 16.6%	0.09±0.24 0.14±0.23	ATM[1] (AIREML)	Pieramati et al. 2003
Hanoverian WB	n=624	OC (hock) OC (fetlock) OCD (hock) OCD (fetlock)	5.9% 9.1% 7.2% 11.9%	0.08±0.05 0.07±0.08 0.10±0.05 0.15±0.07	LAM[5] (REML[2])	Schober 2003
Hanoverian WB	n=3725	OCD (hock) OCD (fetlock)	9.6% 20.8%	0.37±0.06 0.19±0.03	LAM[5] (REML[2], DL[3])	Stock et al. 2005
Hanoverian WB	n=5231	OCD (hock) OCD (fetlock) OCD (hock) OCD (fetlock) OCD (hock) OCD (fetlock)	9.2% 23.5%	0.28±0.04 0.17±0.03 0.27±0.04 0.17±0.03 0.17±0.07 0.12±0.05	LAM[5] (REML[2], DL[3]) LSM[4] (REML[2], DL[3]) STM[6] (GS[7])	Stock and Distl 2006
Danish trotters	n=325	OCD (hock)	12.0%	0.26±0.14	STM[6]	Schougaard et al. 1990
South German Coldblood	n=167	OC (hock) OC (fetlock) OCD (fetlock)	40.1% 53.9% 26.4%	0.04±0.07 0.16±0.16 0.08±0.09	LAM[5] (REML[2], DL[3])	Wittwer et al. 2007b

WB=warmblood
[1] Animal threshold model
[2] Residual Maximum Likelihood
[3] Dempster Lerner transformation onto the liability model
[4] Linear sire model
[5] Linear animal model
[6] Sire threshold model
[7] Gibbs sampling

Table 2 Quantitative trait loci (QTL) for the different traits of osteochondrosis in Hanoverian warmblood (HW) and South German coldblood (SGC) horses. Positions in Mb only result from a simple transformation of the limitative microsatellites, rearrangements of markers in EquCab2 are not considered.

ECA	POS in cM	POS in Mb	Trait	Population
1	150.0-194.2	161.5-181.4	OC-F	SGC
	150.0-194.2	161.5-181.4	OCD-F	SGC
	110.0-138.0	99.8-136.9	POF	SGC
2	26.9-43.0	21.6-30.9	OC	HW
	26.9-43.0	21.6-30.9	OCD	HW
	22.0-49.0	19.2-37.2	OC-F	HW
	26.9-43.41	21.6-31.9	OCD-F	HW
	41.7-49.0	28.5-37.2	OC-H	HW
3	20.7-30.2	11.1-16.4	OCD	HW
	20.7-30.2	11.1-16.4	OCD-F	HW
4	7.7-46.0	6.2-36.6	OC	HW
	24.3	13.3	OC-F	HW
	66.0-66.7	58.8-59.8	OC-F	HW
	0.0	2.9	OC-H	HW
	7.8-38.0	6.3-19.6	POF	SGC
	70.0-73.3	59.8-71.1	POF	SGC
5	73.0-100.1	83.7-98.4	OC-F	HW
	65.5-100.1	78.3-98.4	OCD-F	HW
	44.30-52.00	44.3-53.4	OCD-H	HW
	44.31	56.7	OC	SGC
	40.0	53.4	OC-F	SGC
15	63.5	61.8	OC-H	HW
	37.0	39.4	OC	SGC
	24.0-35.2	18.7-35.2	OC-H	SGC
	37.0	39.4	OC-H	SGC

Table 2 continued

ECA	POS in cM	POS in Mb	Trait	Population
16	33.0	23.1	OCD	HW
	3.0-33.0	6.6-23.1	OC-F	HW
	0.0-3.0	3.9-6.6	OCD-H	HW
	33.0-59.0	23.1-54.2	OC-H	HW
	33.0-59.0	23.1-54.2	OCD-H	HW
	87.0-89.0	70.4-75.4	OC-H	HW
	33.0-39.0	23.1-28.0	OC-F	SGC
18	45.9-54.0	37.3-51.6	OCD-F	SGC
	78.2-87.6	66.8-75.3	OC-H	SGC
	78.2	66.8	POF	SGC
19	0.0-2.0	0.8	OCD	HW
21	16.0-24.5	11.5-17.1	OC-H	HW
	0.0-24.5	1.9-17.1	OCD-H	HW

ECA: *Equus caballus* autosome
POS in cM: position in centiMorgan on the horse maps (Swinburne et al. 2006, Penedo et al. 2005, Chowdhary et al. 2003)
POS in Mb: position in Megabases on EquCab2
OC: osteochondrosis in fetlock and/or hock joints
OCD: osteochondrosis dissecans in fetlock and/or hock joints
OC-F: osteochondrosis in fetlock joints
OCD-F: osteochondrosis dissecans in fetlock joints
OC-H: osteochondrosis in hock joints
OCD-H: osteochondrosis dissecans in hock joints
POF: palmar/plantar osseous fragment

Table 3 Description, their cytogenetic locations and their start of the genomic sequence in bp on the human chromosome (Homo sapiens genome view, Build 36.3) and equine chromosome (EquCab2) for genes responsible for osteoarthritis in man

Gene name	Gene description	HSA	Start of sequence in bp on the respective human chromosome	ECA	Start of sequence in bp on the respective equine chromosome
ADAM17	ADAM metallopeptidase domain 17	2p25	9,613,368	15	83,835,465
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	21q21.3	26,760,399	26	24,396,759
ACAN	aggrecan	15q26.1	86,965,593	1	94,381,944
ANKH	ankylosis, progressive homolog (mouse)	5p15.1	14,716,208	21	45,329,159
AR	androgen receptor	Xq11.2-q12	66,680,599	X	49,635,250
ASPN	asporin	9q22	94,284,609	23	55,293,084
ATP7B	ATPase, Cu++ transporting, beta polypeptide	13q14.3	51,334,118	17	19,261,909
CALM1	calmodulin 1 (phosphorylase kinase, delta)	14q24-q31	89,933,126	24	33,773,237
CASR	calcium-sensing receptor	3q13	123,385,220	19	37,184,938
CCL2	chemokine (C-C motif) ligand 2	17q11.2-q12	29,606,409	11	38,592,359
CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)	16q22.1	63,715,420	3	15,767,893
CLCN7	chloride channel 7	16p13	1,465,013	13	41,283,372
COL1A1	collagen, type I, alpha 1	17q21.33	45,633,999	11	25,912,301

Table 3 continued

Gene name	Gene description	HSA	Start of sequence in bp on the respective human chromosome	ECA	Start of sequence in bp on the respective equine chromosome
COL1A2	collagen, type I, alpha 2	7q22.1	93,861,809	4	37,977,963
COL3A1	collagen, type III, alpha 1	2q31	189,547,344	18	65,487,214
COL5A1	collagen, type V, alpha 1	9q34.2-q34.3	136,673,473	25	36,468,610
COL5A2	collagen, type V, alpha 2	2q14-q32	189,752,850	18	65,689,370
COL9A1	collagen, type IX, alpha 1	6q12-q14	71,069,494	20	61,850,036
COL9A2	collagen, type IX, alpha 2	1p33-p32	40,555,526	2	17,778,788
COL9A3	collagen, type IX, alpha 3	20q13.3	60,918,859	22	48,667,448
COL10A1	collagen, type X, alpha 1	6q21-q22	116,583,989	10	64,674,525
COL11A1	collagen, type XI, alpha 1	1p21	103,346,640	5	63,514,194
COL11A2	collagen, type XI, alpha 2	6p21.3	33,268,223	20	33,605,499
COMP	cartilage oligomeric matrix protein	19p13.1	18,763,114	21	3,350,190
CTSK	cathepsin K	1q21	149,047,436	5	46,340,299
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	10q11.1	44,200,548	1	69,830,449
CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9	10q24	96,688,430	1	34,575,893
DKK1	dickkopf homolog 1 (Xenopus laevis)	10q11.2	53,744,047	1	42,863,847
ESR1	estrogen receptor 1	6q25.1	152,170,379	31	15,081,963

Table 3 continued

Gene name	Gene description	HSA	Start of sequence in bp on the respective human chromosome	ECA	Start of sequence in bp on the respective equine chromosome
ESR2	estrogen receptor 2 (ER beta)	14q23.2	63,763,504	24	11,029,341
FLNA	filamin A, alpha (actin binding protein 280)	Xq28	153,230,091	X	122,667,614
FRZB	frizzled-related protein	2qter	183,406,982	18	60,406,339
GDF5	growth differentiation factor 5	20q11.2	33,484,563	22	26,184,189
HTRA1	HtrA serine peptidase 1	10q26.3	124,211,047	1	10,439,901
IL2RB	interleukin 2 receptor, beta	22q13.1	35,851,824	28	34,466,894
IL2RG	interleukin 2 receptor, gamma (severe combined immunodeficiency)	Xq13.1	70,243,984	X	53,007,980
IL17A	interleukin 17A	6p12	52,159,144	20	49,863,206
IL32	interleukin 32	16p13.3	3,055,314	13	39,699,269
ITGA1	integrin, alpha 1	5q11.2	52,119,893	21	19,013,140
LEP	leptin	7q31.3	127,668,567	4	83,427,058
LGALS3	lectin, galactoside-binding, soluble, 3	14q21-q22	54,665,625	24	3,352,360
LTBP3	latent transforming growth factor beta binding protein 3	11q12	65,062,850	12	25,612,610
LUM	lumican	12q21.3-q22	90,021,363	28	16,914,405
LRCH1	leucine-rich repeats and calponin homology (CH) domain containing 1	13q14.13-q14.2	46,025,304	17	24,032,734

Table 3 continued

Gene name	Gene description	HSA	Start of sequence in bp on the respective human chromosome	ECA	Start of sequence in bp on the respective equine chromosome
MAP3K2	mitogen-activated protein kinase kinase kinase 2	2q14.3	127,778,609	18	3,599,240
MATN3	matrilin 3	2p24-p23	20,055,294	15	75,150,371
MEFV	Mediterranean fever	16p13.3	3,232,029	13	39,500,687
MMP13	matrix metallopeptidase 13 (collagenase 3)	11q22.3	102,318,934	7	12,803,635
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	10q23-q24	89,409,456	1	40,701,725
PHEX	phosphate regulating endopeptidase homolog, X-linked	Xp22.2-p22.1	21,960,842	X	16,513,614
PRDX5	peroxiredoxin 5	11q13	63,842,145		not annotated
PRG4	proteoglycan 4	1q25-q31	184,532,041	5	14,157,823
PTH2R	parathyroid hormone 2 receptor	2q33	208,979,801	18	82,272,294
RARRES2	retinoic acid receptor responder (tazarotene induced) 2	7q36.1	149,666,351	4	101,880,013
SLC26A2	solute carrier family 26 (sulfate transporter), member 2	5q31-q34	149,320,493	14	27,988,771
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	2q32	190,133,561		not annotated
TNXB	tenascin XB	6p21.3	32,116,911	20	31,765,818
TP53	tumor protein p53	17p13.1	7,512,445	11	50,611,931

Table 3 continued

Gene name	Gene description	HSA	Start of sequence in bp on the respective human chromosome	ECA	Start of sequence in bp on the respective equine chromosome
TRAPPC2	trafficking protein particle complex 2	Xp22	13,640,282	X	9,414,664
VDR	vitamin D (1,25-dihydroxyvitamin D3) receptor	12q13.11	46,521,587	6	65,533,482
WISP3	WNT1 inducible signaling pathway protein 3	6q21	112,481,971	10	61,348,667

HSA: *Homo sapiens* autosome
ECA: *Equus caballus* autosome
bp: base pairs

CHAPTER 3

Refinement of a quantitative trait locus on equine chromosome 5 responsible for fetlock osteochondrosis in Hanoverian warmblood horses

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3 Refinement of a quantitative trait locus on equine chromosome 5 responsible for fetlock osteochondrosis in Hanoverian warmblood horses

3.1 Summary

In this report, we provide 29 new informative microsatellites distributed over a region of 21 Mb on horse chromosome (ECA) 5 and refine a quantitative trait locus (QTL) for fetlock osteochondrosis dissecans (OCD) to a genome-wide significant interval between 78.03 and 90.23 Mb on ECA5. Genotyping was performed in 211 Hanoverian warmblood horses from 14 paternal half-sib groups. Within this OCD-QTL *collagen type XXIV alpha 1* was identified as a potential functional candidate gene for equine osteochondrosis. This report is a further step towards unravelling the genes causing equine osteochondrosis.

Key Words: horse, horse chromosome 5, quantitative trait locus, osteochondrosis.

3.2 Article

Osteochondrosis (OC) is an inherited developmental orthopaedic disorder in young horses characterized by abnormal chondrocyte differentiation and maturation (Jeffcott & Henson 1998). Articulations most commonly affected in horses are fetlock, hock and stifle joints. Whole genome scans in Hanoverian warmblood and South German Coldblood discovered quantitative trait loci (QTL) for osteochondrosis in fetlock and hock joints (Dierks *et al.* 2007; Wittwer *et al.* 2007). In Hanoverian warmblood horses chromosome-wide significant QTL were identified for OC and OCD in fetlock joints on horse chromosome 5 (ECA5) at 65.5-100.1 cM. The aim of this study was to refine the position of this QTL for fetlock OC and fetlock OCD on ECA5 using newly developed microsatellite markers.

We used 14 paternal half-sib families including a total of 211 horses for genotyping. These horses were identical with the families used in the previous QTL study (Table S1). Diagnosis of osteochondrosis was done following the recording and evaluation

scheme developed for warmblood horses (Dierks *et al.* 2007). Sagittal ridge of the 3rd metacarpal/metatarsal bone of fetlock joints and intermediate ridge of the distal tibia, lateral trochlea of talus of hock joints and the medial malleolus of the tibia were considered as predilection sites for OC. Signs consistent with osteochondrosis were irregular bone trabeculation with variable radiolucency, irregular bone margin, new bone formation or osseous fragments only in the case when these changes were located at these predilection sites. Horses showing radiographic changes of osteochondrosis with or without osseous fragments at the predilection sites were classified as affected by fetlock OC and/or hock OC and those horses exhibiting radiodense bodies as signs for joint mice at the above mentioned predilection sites were treated as affected by osteochondrosis dissecans (OCD). Horses with pathological changes in fetlock or hock joints other than osteochondrosis were not used in our study. Animals without any signs of radiographic changes at all joints examined (fetlock, hock and stifle) were considered as free from OC and only these horses were included as controls.

For the refinement of the QTL for fetlock OCD on ECA5, 29 new microsatellites (*ABGe010–ABGe031, ABGe135-ABGe141*) were developed (Table S2). Genotyping was done according to Dierks *et al.* (2007).

Multipoint non-parametric linkage analysis (NPL) was performed using the Merlin software (multipoint engine for rapid likelihood inference, version 1.1.2) (Abecasis *et al.* 2002) for a total of 49 microsatellites (Table S2). The Zmean and LOD score test statistics were used to test for the proportion of alleles shared by affected individuals identical by descent (IBD) for the considered marker loci (Kong & Cox 1997; Whittemore & Halpern 1994). Chromosome-wide significant linkage was determined using a permutation approach as described by Dierks *et al.* (2007). The maximum (minimum) achievable Zmeans were 9.07 (-2.78) for OC in fetlock joints, and 4.12 (-2.32) for OCD in fetlock joints. The corresponding maximum (minimum) values for LOD scores were 6.30 (-0.59) and 3.05 (-1.00) indicating enough power to detect genome-wide significant linkage. Genome-wide probabilities were obtained by applying a Bonferroni correction: $P_{genome\text{-}wide} = 1 - (1 - P_{chromosome\text{-}wide})^{1/r}$, where r = length of ECA5 (99.7 Mb) divided by the total equine genome length (2680 Mb).

In this study, two different analyses were performed for the following phenotypes: (1) OC present in fetlock joints or absent in these joints of all limbs, and (2) OCD present in fetlock joints or absent in these joints of all limbs.

In addition, the genotypic data was evaluated using the ALLELE and CASECONTROL procedures of SAS/Genetics (Statistical Analysis System, Version 9.2, SAS Institute, Cary, NC, USA 2008) to determine the observed heterozygosity (HET), the polymorphism information content (PIC) and Hardy-Weinberg equilibrium and to evaluate genotypic and allelic associations and the trend of the alleles with fetlock OC and fetlock OCD using χ^2-tests.

The marker positions delimiting the QTL for fetlock OC and fetlock OCD from the previous whole genome scan on ECA5 (Dierks *et al.* 2007) were adjusted to 78.29-98.39 Mb on EquCab2 using BLAST analyses for the microsatellite flanking sequences on ECA15. The non-parametric multipoint linkage analysis showed chromosome-wide significant Zmeans and LOD scores in the region from 76.69 to 92.77 Mb on ECA5 for fetlock OCD and in the region from 79.65 to 89.31 Mb for fetlock OC (Fig. 1).

The highest Zmeans were 3.30 for fetlock OCD with corresponding genome-wide error probabilities of 0.01 at 79.65 to 83.74 Mb and at 86.56 to 89.31 Mb. The highest LOD score for fetlock OCD was 2.41 with a corresponding genome-wide error probability of 0.01 at the microsatellite *ABGe138* at 78.03 Mb.

For the trait fetlock OC, chromosome-wide significant error probabilities were reached for both test statistics at 79.65 to 83.74 Mb and at 85.21 to 89.31 Mb. While the highest Zmean was 2.44 at 85.21 Mb with a chromosome-wide error probability of 0.007, the highest LOD score was 1.59 at 79.65 Mb with a chromosome-wide error probability of 0.003 (Table S3).

Significant genotypic and/or allelic association were found for fetlock OCD at 84.44 Mb (*TKY525*), 85.21 Mb (*ABGe016*), 86.56 Mb (*ABGe018*) and 91.52 Mb (*ABGe025*) (Table S4). This result also corroborates the QTL location in the region between 76.69 Mb and 92.77 Mb.

The approximate consistence of the QTL for fetlock OC and fetlock OCD supports to the assumption that the same genes may play a role in the development of this

disease and that OCD is an aggravated form of OC. The delimitation of the QTL using very dense microsatellite scans also allows clarifying how many positional candidate genes are located in the linked region. For that purpose we used the Equine Articular Cartilage cDNA Library (http://www.ncbi.nlm.nih.gov/sites/entrez) to search for genes which are expressed in equine cartilage and located within the QTL at 76.69 to 92.77 Mb on ECA5. From a total of 13,964 equine articular ESTs (expressed sequence tag), we identified 53 ESTs which correspond to 22 different genes (Table S5). Besides these cartilage expressed genes, *collagen type XXIV, alpha 1 (COL24A1)* was identified as a functional candidate gene at 78.1 Mb. *COL24A1* is a marker for embryonic bone formation and may play a role in regulation of type I collagen fibrillogenesis (Koch *et al.* 2003). Furthermore, Matsuo *et al.* (2008) found out that *COL24A1* is not only expressed in the forming skeleton of the mouse embryo, but also transcribed in the trabecular bone and periosteum of the newborn mouse. Due to its function in bone formation *COL24A1* seems to be a suitable functional candidate gene for osteochondrosis because this disease has its origin in the disturbance of ossification.

3.3 Acknowledgements

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3.4 References

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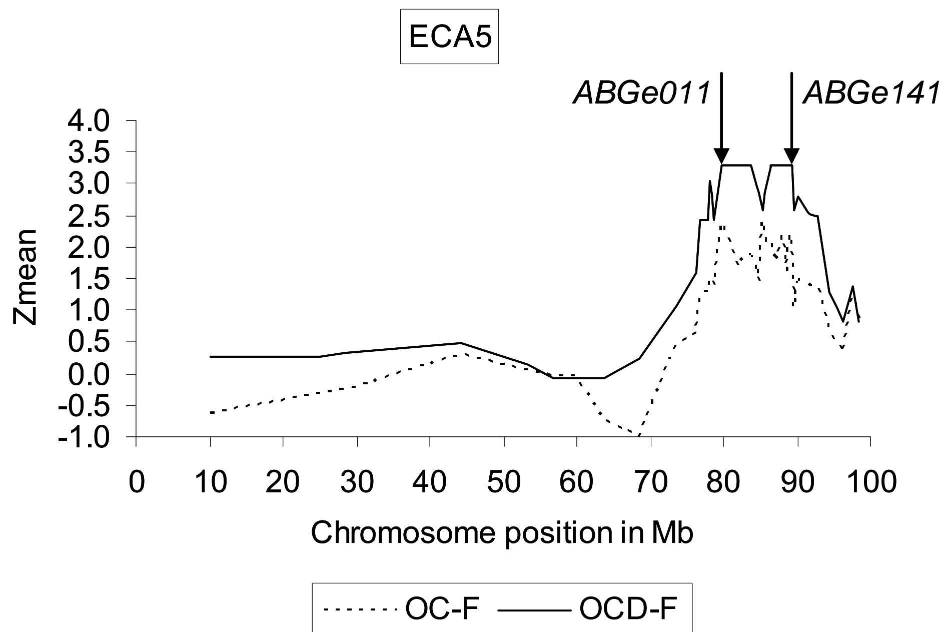

Figure 1 Multipoint chromosome-wide Zmeans of the genomic region on ECA5 harbouring QTL for equine fetlock osteochondrosis (OC-F) and fetlock osteochondrosis dissecans (OCD-F). The proximal and distal maxima of the curves are marked with arrows indicating the genotyped microsatellite makers at the respective positions

Table S1 Number of families analysed, their sizes and prevalences of osteochondrosis (OC), osteochondrosis dissecans (OCD), osteochondrosis in fetlock (OC-F) and hock (OC-H) joints and osteochondrosis dissecans in fetlock (OCD-F) and hock (OCD-H) joints by family and in total

Half-sib family	Number of progeny	Male	Female	Prevalences in % for					
				OC	OCD	OC -F	OCD-F	OC-H	OCD-H
1	4	1	3	100.0	100.0	50.0	50.0	75.5	75.0
2	9	4	5	44.4	33.3	11.1	0.0	33.3	33.3
3	7	5	2	71.4	42.9	71.4	42.9	28.6	14.3
4	15	9	6	53.3	33.3	26.7	6.7	26.7	26.7
5	8	1	7	100.0	25.0	75.0	12.5	37.5	12.5
6	4	2	2	100.0	25.0	50.0	25.0	50.0	0.0
7	5	3	2	100.0	40.0	80.0	40.0	20.0	0.0
8	6	2	4	100.0	66.7	83.3	50.0	66.7	33.3
9	20	12	8	75.0	60.0	50.0	20.0	50.0	45.0
10	8	4	4	62.5	25.0	50.0	12.5	12.5	12.5
11	5	3	2	40.0	20.0	20.0	0.0	20.0	20.0
12	5	2	3	100.0	80.0	100.0	80.0	0.0	0.0
13	5	3	2	40.0	40.0	40.0	40.0	0.0	0.0
14	3	1	2	100.0	66.7	100.0	66.7	0.0	0.0
Total	104	52	52	73.1	45.2	51.9	25.0	32.7	24.0

Table S2 Characteristics of the microsatellites used in this study

Marker	Acc. No	Mb	Ta (°C)	Forward / reverse primer sequence (5'-3')	Repeat motif	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
TKY1175	AB104393	10.10	60	F: TTATCACCAGTTTCCAGAGC R: CTTATTCCACCCACTAATTCAC	TG	6	176 - 204	62.7	60.3
AHT068	AJ507685	25.00	60	F: GGGAGGAAACCCAGTCAATT R: GGTCCCTCATCACTTCCACA	AC	6	292 - 304	64.5	61.5
HMS063		28.65	62	F: GGCACCTCCTAGAATTGTGC R: AGTCTTCTAATCCTCTCCCTG	GT	7	145 - 157	72.5	68.9
AHT050	AJ507667	44.29	56	F: GTTGGCTGGTTTTTGCATTT R: CGTACACACATTTTCACCCA	TG	6	295 - 311	75.9	65.1
COR023	AF101392	53.41	58	F: CGTTTAGCACCTCTCATGAAC R: TCTTTGCAAAATAGGGCTTG	GT	5	269 - 279	31.4	29.4
UCDEQ304	U67402	56.68	55	F: CGCTTTCCTGCTGTCACC R: GAGGGACTGTGGGGGAGGT	CA	6	95 - 105	66.2	58.7
HMS05	X74634	57.36	60	F: TAGTGTATCCGTCAGAGTTCAAAG R: GCAAGGAAGTCAGACTCCTGGA	AC	3	100 - 106	63.8	55.2
HTG015	AF169299	59.83	55	F: TCTTGATGGCAGAGCCAGGATTTG R: AATGTCACCATGCGGCACATGACT	TG	4	130 - 146	45.7	40.3
LEX069		63.74	58	F: TTTCTTTTTCCCACTTAAAGC R: TGGGACTTAGCAGTATGAAAC	CA	6	134 - 162	72.0	71.9
UMNe582	AY735282	68.63	58	F: TCTTGACCTTCTTTACTTAGTACACA R: CCTGGGCATAGACCTACACA	TG	5	165 - 183	59.8	53.4
UMNe534	AY735262	73.63	58	F: ATGTTGTTGCAAATGGTAGGG R: TCCATCAATCCCTCTTCTGG	TG	6	237 - 251	80.3	72.9
LEX034	AF075636	76.17	55	F: GCGGAGGTAAGAAGTGGTAG R: GGCCTAAGATGAGGGTGAA	CA	5	243 - 253	62.9	61.2
ABGe135	AM992887	76.69	58	F: ATTACATCCTGCCCCAACAG R: TGGTTAAAGAACCCCTCGTG	CA	5	169 - 181	52.4	49.8
ABGe136	AM992888	77.35	58	F: TGTATCTGAAAACAGGGCAATAG R: CCTGTCAAACCAGAGATTTTCC	AT	7	196 - 224	84.6	76.8
ABGe137	AM992889	77.74	58	F: CACTAGCCAAGTCCGAGTCC R: TGACTCCAGGACATAATGTGG	TG	8	156 - 172	81.3	79.5
ABGe138	AM992890	78.03	58	F: CGGTGAGTACTGCTGGGTTC R: CCCACTGAACATATAAGGTGGTC	AC	3	128 - 132	51.9	36.1
ASB010	X93524	78.29	58	F: GTTGTCTAGGTGCAGAATCTGG R: GTTATGTCTCCCCTTTCTCTACC	TG	6	142 - 152	72.0	63.2
ABGe010	AM905688	78.76	59	F: CGTGATTGCATAATACTCCTCAG R: CCTTTGCCTTCATGGAATAAAC	TG	9	108 - 130	72.6	67.7
ABGe011	AM905689	79.65	59	F: ATTGCCCACCCTGTTGTAAG R: CAGATTAGCTTTTCCCTTTTGG	GAT	2	181 - 190	25.1	21.7
ABGe012	AM905690	80.98	59	F: TCGAGTGCAACAATGTGTAGG R: AGTCGAAGGCTTCCCACTAC	AC	8	109 - 127	71.6	68.9

Table S2 continued

Marker	Acc. No	Mb	Ta (°C)	Forward / reverse primer sequence (5'-3')	Repeat motif	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
ABGe013	AM905691	81.79	59	F: GGTGCAGATAAGCCTGTTGAC R: GAAGCCCCTAGGTCTCCAAC	AC	7	186 - 198	82.6	80.4
ABGe014	AM905692	83.21	59	F: AAACTAGAATTCTTATGCCCAAGG R: AGATAGATGCTTTGGGGTGTG	TA	6	236 - 246	81.9	73.5
TKY911	AB104129	83.74	58	F: GATCTTTAGAATCAGCTTGTTG R: CTCGCCACGTTAGTTGATG	AC	7	132 - 148	65.3	58.6
TKY525	AB103743	84.44	60	F: TGAGTGGCGTACCTTTCCAT R: GGAACCGTGCTGGGATACTA	AC	7	208 - 232	39.9	37.5
ABGe015	AM905693	84.68	59	F: TTCTCTCCTCCTTCCCCAAC R: TTTGGATGATTCACCTTCTGC	TC	5	162 - 184	58.4	57.0
ABGe016	AM905694	85.21	57	F: CCCTCAGCTTTTGTCACCTC R: GGGACACAGTCACATCATGC	GT	6	234 - 242	56.8	55.1
ABGe017	AM905695	85.63	59	F: AGCCAAAGGAGTGACTGTGG R: TCCCTAGCTTGCCTTCTGTG	GA	4	238 - 244	64.9	55.9
UMNe455	AY731398	86.27	60	F: TGAGGTACTGTGCCTTGCTG R: CTGGGAAGACAGAGCCAGTC	CA	5	124 - 132	70.6	64.3
TKY801	AB104019	86.40	58	F: TCCCTAAGCTAATTCCATCC R: GCGCTTGCTCATCTAAAAGC	TG	4	142 -152	53.8	46.4
ABGe018	AM905696	86.56	59	F: TTTTGGAAAGCATCATTCCTG R: CCTCCAAAAAGATCCCCAAG	TG	4	238 - 244	61.5	52.8
ABGe019	AM905697	87.22	59	F: GCAATGCTTGGGCAAATAC R: CTCCTATCCATTATTTGGCACTG	GT	11	170 - 200	81.8	77.6
ABGe020	AM905698	87.71	59	F: AATAATGTTATTCTGAGATTGCTTCC R: CTTATTCCAAAAATGTAAGCTGTTG	CA	5	110 - 122	66.7	55.8
ABGe021	AM905699	87.96	59	F: TGATCTGTGGGGAGACACTG R: TTCCAGAGAAGAGAGGAAGTGG	CA	9	176 - 196	81.8	75.4
ABGe022	AM905700	88.59	59	F: TGAGAGTATAAAAGGTACATGTGTGTG R: TCCTCTAGTCCCAGCCTAGTTC	GT	5	102 - 110	67.6	63.3
ABGe139	AM992891	88.66	58	F: CTGAAAATCAGAACAACCAAATG R: CAGAGCACTATTTTTGCTTGAAC	CA	7	99 - 115	61.8	68.1
LEX014	AF075616	88.96	55	F: CCTTACTCACTGGGGAATAAA R: AGACTGAACACCTAACTATGA	GT	4	390 - 400	71.3	62.8
ABGe140	AM992892	89.28	58	F: GACCTTAACTTCCTAGATTGCTGAG R: GGAGCAAGGCTCAATTTTTC	AC	5	176 - 190	50.2	43.8
ABGe141	AM992893	89.31	58	F: GAATTAGTTGTTTACTAGATTGGGAAG R: TTGTTGCAAAAATTGATGAGTG	AT	12	181 - 213	85.3	80.3
ABGe023	AM905701	89.59	57	F: TTCTCATGACCATCTTTTCCTG R: GGGGGAGAATGTTCTTAATTTATTC	TA	5	218 - 226	69.1	71.3
ABGe024	AM905702	90.23	56	F: CCACTACCCCTTAGGAGAACAC R: TGCCCCCAAATGTGAAAG	AC	8	216 - 240	68.8	64.4

Table S2 continued

Marker	Acc. No	Mb	Ta (°C)	Forward / reverse primer sequence (5'-3')	Repeat motif	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
ABGe025	AM905703	91.52	59	F: CCTAGCAGCAAGAGCAAAGG R: CATTACAGCATTACCAGGTATCTTTC	GT	3	146 - 162	47.7	45.3
ABGe026	AM905704	91.68	59	F: TAACCCTTTGGTATGGCTCTG R: TGTCAGAGGATTTCAAGAAAGATTG	AC	8	106 - 124	80.0	73.3
TKY344	AB044845	92.50	56	F: GTGTCCATCAATGGATGAAG R: CTTAAGGCTAAATAATATCCC	CA	8	98 - 102	84.5	77.2
ABGe027	AM905705	92.77	62	F: CTCCTCCAGCCCTAAGAAGG R: TGATTTCTTGGATTCTCCAAAC	CA	5	102 - 110	71.9	63.8
ABGe028	AM905706	94.44	59	F: TTTCAGAGATGCCTCCAGATG R: ATGCTCTCATTCCCCTTGTG	GT	7	134 - 164	57.1	49.2
ABGe029	AM905707	95.40	59	F: CGAGAGCTTCCACCTTCTTG R: AAAGATTGCACGGTTCTTGC	TC	6	148 - 162	71.2	60.9
ABGe030	AM905708	96.39	59	F: CAGTCTCTCCCCTCCTGTTC R: TGGAGGTGACAACAGCAGAC	CA	6	230 - 244	75.6	68.6
ABGe031	AM905709	97.55	59	F: TCCTCTTTCATCCCATCAGG R: TGGGGAATTTTAAATGGTTGG	GT	7	138 - 156	74.9	67.6

Mb: Positions on the horse genome assembly EquCab2 in megabases
T_a: Annealing temperature
HET: Observed heterozygosity
PIC: Polymorphism information content
Markers in bold indicate these microsatellites which were used in a previous whole genome scan for osteochondrosis in Hanoverian warmblood horses (Dierks *et al.* 2007)

Permutation sequences were built with all variations of di-, tri- and tetra-repeat motifs with a minimum length of 15 repeats and a maximum length of 30 repeats in the identified QTL region. These sequences were aligned with the horse genome assembly (Broad Institute, version EquCab2, 2007) using the SSAHA2 package (Sequence Search and Alignment by Hashing Algorithm combined with the cross-match sequence alignment program developed by Phil Green at the University of Washington, version 1.0.1, The Wellcome Trust Sanger Institute, UK, 2007). Alignment results that obtained a maximum score per length (100% identity) were used for primer design. For this purpose flanking sequences were taken from EquCab2 and equine PCR primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) after masking repetitive elements with the RepeatMasker (http://www.repeatmasker.org/). The marker

positions on ECA5 on EquCab2 were verified using the Basic Local Alignment Search Tool (BLASTall version 2.2.17) of NCBI.

The average PIC and HET of the 29 newly developed microsatellites was 62.2% and 67.5% with a minimum of 21.7% and 25.1%.

Table S3 Multipoint chromosome-wide test statistics with their genome-wide and chromosome-wide significant error probabilities (P_Z, P_L) and map positions in Mb of microsatellites linked with fetlock OC (OC-F) and fetlock OCD (OCD-F) in Hanoverian warmblood horses

Pos. in Mb	Marker	OC-F				OCD-F			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
76.69	*ABGe135*			0.72	0.03	2.43	0.007	1.70	0.003
77.35	*ABGe136*			0.73	0.03	2.43	0.007	1.70	0.003
77.74	*ABGe137*			0.73	0.03	2.43	0.007	1.70	0.003
78.03	*ABGe138*			1.19	0.01	3.05	0.0012*	2.41	0.0004*
78.29	*ASB010*			1.09	0.012	2.83	0.002*	2.26	0.0006*
78.76	*ABGe010*			1.15	0.011	2.43	0.007	1.70	0.003
79.65	*ABGe011*	2.41	0.008	1.59	0.003	3.30	0.0005*	2.24	0.0007*
80.98	*ABGe012*	1.97	0.02	1.25	0.008	3.30	0.0005*	2.24	0.0007*
81.79	*ABGe013*	1.70	0.04	1.00	0.02	3.30	0.0005*	2.24	0.0007*
83.21	*ABGe014*	1.91	0.03	1.29	0.007	3.30	0.0005*	2.24	0.0007*
83.74	*TKY911*	1.88	0.03	1.07	0.013	3.30	0.0005*	2.24	0.0007*
84.44	*TKY525*			0.60	0.05	2.96	0.002*	1.96	0.0013*
84.68	*ABGe015*					2.85	0.002*	1.80	0.002*
85.21	*ABGe016*	2.44	0.007	0.86	0.02	2.59	0.005	1.07	0.013
85.63	*ABGe017*	2.07	0.02	0.75	0.03	2.86	0.002*	1.82	0.002*
86.27	*UMNe455*	2.05	0.02	0.81	0.03	3.27	0.0005*	2.22	0.0007*
86.40	*TKY801*	1.97	0.02	0.80	0.03	3.29	0.0005*	2.23	0.0007*
86.56	*ABGe018*	1.88	0.03	0.77	0.03	3.30	0.0005*	2.24	0.0007*
87.22	*ABGe019*	1.80	0.04	0.87	0.02	3.30	0.0005*	2.24	0.0007*
87.71	*ABGe020*	2.05	0.02	0.99	0.02	3.30	0.0005*	2.24	0.0007*
87.96	*ABGe021*	2.17	0.015	1.04	0.014	3.30	0.0005*	2.24	0.0007*
88.59	*ABGe022*	1.62	0.05	0.86	0.02	3.30	0.0005*	2.24	0.0007*
88.66	*ABGe139*	1.62	0.05	0.86	0.02	3.30	0.0005*	2.24	0.0007*
88.96	*LEX014*	2.22	0.013	1.08	0.013	3.30	0.0005*	2.24	0.0007*
89.28	*ABGe140*	1.62	0.05	0.86	0.02	3.30	0.0005*	2.24	0.0007*
89.31	*ABGe141*	1.62	0.05	0.86	0.02	3.30	0.0005*	2.24	0.0007*
89.59	*ABGe023*					2.59	0.005	1.07	0.013
90.23	*ABGe024*			0.68	0.04	2.81	0.002*	1.94	0.0014*
91.52	*ABGe025*			0.57	0.05	2.56	0.005	1.61	0.003
91.68	*ABGe026*			0.56	0.05	2.53	0.006	1.55	0.004

Table S3 continued

Pos. in Mb	Marker	OC-F				OCD-F			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
92.50	*TKY344*					2.48	0.006	1.43	0.005
92.77	*ABGe027*					2.48	0.006	1.43	0.005

* genome-wide significant after Bonferroni's correction using $P_{genome\text{-}wide} = 1- (1 - P_{chromosome\text{-}wide})^{1/r}$ with r being the length of equine chromosome 5 (99.7 Mb) divided by the total equine genome length (2680 Mb). *:P<0.05, **:P<0.01, ***:P<0.001

Table S4 Significantly associated microsatellites with osteochondrosis dissecans in fetlock joints in 14 half-sib groups

Marker	Location on ECA5 in Mb	Genotype			Allele			Trend		
		X²	df	P	X²	df	P	X²	df	P
TKY525	84.44	24.42	9	0.004	11.54	6	0.073	14.11	6	0.028
ABGe016	85.21	14.94	9	0.093	10.08	4	0.039	n. e.	-	-
ABGe018	86.56	22.65	8	0.004	12.79	3	0.005	13.48	3	0.004
ABGe025	91.52	11.32	5	0.045	5.62	2	0.060	4.69	2	0.100

n. e.: not estimable

Table S5 Equine articular ESTs in 22 genes, respectively their according hypothetical loci located in the QTL region. Positions correspond to EquCab2 (http://www.ensembl.org/Equus_caballus/index.html)

Gene name	Description	Position start	Position end	EST query
LOC100052327 (LMO4)	*LIM domain only 4*	76,729,078	76,742,003	gi|57723937|gb|CX605455.1|CX605455 gi|57702222|gb|CX594608.1|CX594608 gi|57714660|gb|CX600827.1|CX600827 gi|57702378|gb|CX594686.1|CX594686
LOC100063266 (HS2ST1)	*heparan sulfate 2-O sulfotransferase 1*	76,959,579	77,123,440	gi|57711919|gb|CX599449.1|CX599449
LOC100052383 (SEP15)	*15 kDa selenoprotein*	77,124,128	77,160,069	gi|57707213|gb|CX597105.1|CX597105 gi|57713286|gb|CX600133.1|CX600133 gi|57703190|gb|CX595090.1|CX595090 gi|57711380|gb|CX599180.1|CX599180 gi|57714359|gb|CX600675.1|CX600675
LOC100052441 (SH3GLB1)	*SH3-domain GRB2-like endophilin B1*	77,240,598	77,277,567	gi|57698499|gb|CX592759.1|CX592759
LOC100063932 (ZNHIT6)	*zinc finger, HIT type 6*	78,153,175	78,205,451	gi|57699723|gb|CX593361.1|CX593361 gi|57704253|gb|CX595622.1|CX595622 gi|57724020|gb|CX605496.1|CX605496
LOC100064066 (CYR61)	*cysteine-rich, angiogenic inducer, 61*	78,262,823	78,264,924	gi|57705961|gb|CX596481.1|CX596481 gi|57713975|gb|CX600480.1|CX600480 gi|57713284|gb|CX600132.1|CX600132 gi|57700626|gb|CX593809.1|CX593809
LOC100064154 (DDAH1)	*dimethylarginine dimethylaminohydrolase 1*	78,365,481	78,486,705	gi|57721812|gb|CX604395.1|CX604395
LOC100052828 (PRKACB)	*protein kinase, cAMP-dependent, catalytic, beta*	79,479,582	79,529,127	gi|57724931|gb|CX605951.1|CX605951 gi|57716563|gb|CX601774.1|CX601774 gi|57712694|gb|CX599836.1|CX599836 gi|57709722|gb|CX598343.1|CX598343
LOC100052983 (ELTD1)	*EGF, latrophilin and seven transmembrane domain containing 1*	83,968,157	84,071,915	gi|57699236|gb|CX593125.1|CX593125
LOC100066247 (GIPC2)	*PDZ domain protein GIPC2*	84,690,565	84,763,683	gi|57698831|gb|CX592921.1|CX592921 gi|57720734|gb|CX603858.1|CX603858 gi|57718044|gb|CX602509.1|CX602509 gi|57705329|gb|CX596168.1|CX596168 gi|57713702|gb|CX600342.1|CX600342 gi|57719505|gb|CX603239.1|CX603239
LOC100053123 (DNAJB4)	*DnaJ (Hsp40) homolog, subfamily B, member 4*	84,789,441	84,799,501	gi|57703752|gb|CX595373.1|CX595373 gi|57705469|gb|CX596238.1|CX596238
FUBP1	*far upstream element (FUSE) binding protein 1*	84,833,973	84,863,495	gi|57704139|gb|CX595565.1|CX595565 gi|57704296|gb|CX595644.1|CX595644

Table S5 continued

Gene name	Description	Position start	Position end	EST query
LOC100066569 (FAM73A)	*family with sequence similarity 73, member A*	84,921,341	84,992,403	gi|57710209|gb|CX598589.1|CX598589
LOC100053274 (AK5)	*adenylate kinase 5*	85,191,445	85,413,702	gi|57699852|gb|CX593423.1|CX593423 gi|57709973|gb|CX598471.1|CX598471 gi|57723712|gb|CX605344.1|CX605344
LOC100066781 (PIGK)	*phosphatidylinositol glycan, class K*	85,459,645	85,565,992	gi|57713672|gb|CX600327.1|CX600327 gi|57724662|gb|CX605817.1|CX605817 gi|57717132|gb|CX602053.1|CX602053 gi|57716631|gb|CX601807.1|CX601807
LOC100066966 (ST6GALNAC3)	*ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3*	85,959,558	86,458,375	gi|57703006|gb|CX594999.1|CX594999 gi|57718197|gb|CX602585.1|CX602585
NEGR1	*neuronal growth regulator 1*	89,631,942	90,446,366	gi|57705673|gb|CX596339.1|CX596339
LOC100053515 (ZRANB2)	*zinc finger, RAN-binding domain containing 2*	90,696,003	90,713,392	gi|57708257|gb|CX597616.1|CX597616 gi|57698194|gb|CX592608.1|CX592608 gi|57703097|gb|CX595044.1|CX595044 gi|57708352|gb|CX597663.1|CX597663
LOC100053557 (PTGER3)	*prostaglandin E receptor 3 (subtype EP3)*	90,733,024	90,805,006	gi|57720217|gb|CX603601.1|CX603601
LOC100053614 (CTH)	*cystathionase (cystathionine gamma-lyase)*	91,204,559	91,224,694	gi|57697418|gb|CX592228.1|CX592228
LOC100068423 (SFRS11)	*splicing factor, arginine/serine-rich 11*	91,371,014	91,394,016	gi|57716024|gb|CX601509.1|CX601509
LRRC7	*leucine rich repeat containing 7*	91,483,421	91,775,303	gi|57718928|gb|CX602946.1|CX602946

CHAPTER 4

Refinement of a quantitative gene locus on equine chromosome 16 responsible for osteochondrosis in Hanoverian warmblood horses

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4 Refinement of a quantitative gene locus on equine chromosome 16 responsible for osteochondrosis in Hanoverian warmblood horses

4.1 Abstract

Osteochondrosis is an inherited developmental disease in young horses most frequently observed in thoroughbreds, trotters, warmblood and coldblood horses. Quantitative trait loci (QTL) for equine osteochondrosis have been identified in Hanoverian warmblood horses employing a whole genome scan with microsatellites. A QTL on ECA16 reached the genome-wide significance level for hock OCD. The aim of this study was to refine this QTL on ECA16 using an extended marker set of 34 newly developed microsatellites and 15 single nucleotide polymorphisms (SNPs). We used the same 14 paternal half-sib groups as in the previous whole genome scan. The QTL for OCD in hock joints on ECA16 could be delimited at an interval between 17.60 and 45.18 Mb using multipoint non-parametric linkage analyses. In addition, six microsatellites and one SNP were significantly associated with hock OCD in the QTL region between 24.26 and 42.41 Mb. Furthermore, our analysis revealed a second QTL for fetlock OC between 6.55 and 24.26 Mb on ECA16. This report is a further step towards unravelling the genes underlying QTL for equine osteochondrosis and towards the development of a marker test for osteochondrosis in Hanoverian warmblood horses.

4.2 Introduction

Osteochondrosis (OC) belongs to those developmental disorders of the locomotory system frequently detected radiographically in young horses (Arnan and Hertsch, 2005; Stock *et al.,* 2005a, Wittwer *et al.,* 2006). Articulations mainly affected are fetlock, hock and stifle joints. A disturbance in the process of enchondral ossification of growing cartilage of the growth plates (Van de Lest *et al.,* 1999) leads to the signs of OC including subchondral bone cysts, wear lines, cartilage flaps, osseous fragments and synovitis (Jeffcott and Henson, 1998; Trotter and McIlwraith, 1981).

Osteochondrosis dissecans (OCD) is an advanced stage of OC which is characterized by the presence of osteochondral fragments (joint mice, chips, corpora libera). Available epidemiological data indicate that OC is present in warmblood, coldblood, thoroughbred and trotter horse populations between 10 and 25% across a range of different breeds (Grøndahl and Dolvik, 1993; KWPN, 1994; Philipsson *et al.,* 1993; Stock *et al.,* 2005a; Wittwer *et al.,* 2006). Specific causes are still unknown but the origin of OC is multifactorial, including genetic factors, growth rate, body size, nutrition, mineral imbalance, endocrinological dysfunction and biomechanical trauma (Jeffcott, 1991; Van Weeren, 2005). The heritability estimates in animal threshold models were in the range from 0.10 – 0.34 for trotters, warmblood and coldblood horses for the prevalence of OC and these estimates corroborate the hereditary disposition to osteochondrosis (Grøndahl and Dolvik, 1993; KWPN, 1994; Philipsson *et al.,* 1993, Pieramati *et al*. 2003; Schober, 2003; Stock *et al.,* 2005b; Wittwer *et al.,* 2007a).

Whole genome scans in Hanoverian warmblood and South German coldblood revealed quantitative trait loci (QTL) for OC (Dierks *et al.,* 2007; Wittwer *et al.,* 2007b). The traits analyzed were OC (fetlock and/or hock joints affected), OCD (fetlock and/or hock joints affected), fetlock OC, fetlock OCD, hock OC and hock OCD. In Hanoverian warmblood horses chromosome-wide and genome-wide significant QTL for traits of OC and OCD were on ECA16. These QTL were located at 33 cM (*COR011*) for OC in fetlock and/or hock joints and OCD in fetlock and/or hock joints as well as at 0 – 3.0 cM (*AHT037*, *TKY279*), 33 – 59 cM (*COR011*, *AHT038*, *TKY350*, *TKY871*, *LEX059*, and *LEX048*) for hock OC and hock OCD and at 89 cM (*TKY406*) for hock OC The marker coverage was at approximately 15 - 20 cM which did not allow a clear delineation of the QTL.

Thus, the aim of this study was to refine the QTL on ECA16, especially between 33 and 59 cM as the markers in this region reached genome-wide significant test statistics. For that purpose non–parametric linkage and association analyses were performed using newly developed single nucleotide polymorphisms (SNPs) and microsatellites. The genes selected to design SNPs are for the most part positional candidate genes but also two functional candidate genes which might be involved in

the development of OC. For the development of new microsatellites the horse genome assembly EquCab2 on ECA16 was searched for all variations of di-, tri- and tetra-repeat motifs with a minimum length of 15 repeats and a maximum length of 30 repeats. Those repeats which gave significant BLAST hits to the horse genome were taken into further investigation and employed for refinement of the QTL on ECA16.

4.3 Materials and methods

Pedigree structure and phenotypic traits

From a large sample of Hanoverian warmblood horses including 629 radiographed foals, 168 stallions and more than 600 mares, 14 paternal half-sib families were chosen for genotyping due to their large family size and their high number of affected foals. The average size of the paternal half-sib groups was 7.4 ranging from three to 20. In total, 211 horses were genotyped including 104 foals, 99 of their mares and eight stallions. These horses were identical with the families used in the previous QTL study (Supplemental Table 1). Diagnosis of osteochondrosis was done following the recording and evaluation scheme developed for warmblood horses (Kroll *et al.*, 2001). The sagittal ridge of the 3rd metacarpal/metatarsal bone of fetlock joints, the intermediate ridge of the distal tibia, the lateral trochlea of the talus and the medial malleolus of the tibia were considered as predilection sites for OC. Signs consistent with osteochondrosis were irregular bone trabeculation with variable radiolucency, irregular bone margin, new bone formation or osseous fragments when these changes were located at these predilection sites. Horses showing radiographic changes of osteochondrosis with or without osseous fragments at the predilection sites of the fetlock and/or hock joints were classified as affected by osteochondrosis (OC) and those horses exhibiting radiodense bodies as signs for osteochondral fragments at the above mentioned predilection sites were treated as affected by osteochondrosis dissecans (OCD). Horses with pathological changes in fetlock or hock joints other than osteochondrosis were not employed in our study. Animals without any signs of radiographic changes at all joints examined (fetlock, hock and stifle) were considered as free from OC, and only these horses were included as controls.

Development of microsatellites and single nucleotide polymorphisms (SNPs)

For the refinement of the QTL on ECA16, 34 new microsatellites (ABGe032–ABGe058, ABGe092–ABGe098) were developed. Therefore permutation sequences were built with all variations of di-, tri- and tetra-repeat motifs with a minimum length of 15 repeats and a maximum length of 30 repeats. These sequences were aligned with the horse genome assembly (NCBI, version EquCab2, 2008, http://www.ncbi.nlm.nih.gov/sites/entrez) using the SSAHA2 package (Sequence Search and Alignment by Hashing Algorithm combined with the cross-match sequence alignment program developed by Phil Green at the University of Washington, version 1.0.1, The Wellcome Trust Sanger Institute, UK, 2007). Alignment results that obtained a maximum score per length (100% identity) were selected for primer design. For this purpose flanking sequences of these simple sequence repeats were extracted and investigated for their suitability for primer design. Equine PCR primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) after masking repetitive elements with the RepeatMasker (http://www.repeatmasker.org/).

For SNP development both whole genome shotgun (WGS) sequences or equine ESTs which yielded significant BLAST hits to the syntenic region of ECA16 on HSA3 (http://www.ncbi.nlm.nih.gov/BLAST/) and the UCSC Horse genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=120144566&clade=mammal&org=Horse&db=0) were used to choose functional and positional candidate genes for osteochondrosis in the identified QTL region on ECA16. Equine PCR primers for SNP identification were designed using the Primer3 software after masking repetitive elements with the RepeatMasker.

Screening for SNPs was performed by comparative sequencing of genomic DNA from eight unrelated stallions which sired eight of the 14 paternal progeny groups.

For verifying the marker positions on ECA16 on the horse genome assembly (EquCab2) the Basic Local Alignment Search Tool (BLASTall version 2.2.17) of NCBI was used.

Genotyping

For genotyping of microsatellites, 1.35 µg genomic DNA was isolated from 75 µl EDTA blood using the QIAamp® 96 Spin Blood Kit (Qiagen, Hilden, Germany). PCR reactions for genotyping of microsatellite markers were performed in 12-µl reaction volumes using 10 ng DNA, 1.2µl 10x incubation buffer containing 15 mM $MgCl_2$, 0.5 µl DMSO, 0.15 µl each dNTP (100 µM each) and 0.5 U *Taq* polymerase (Qbiogene, Heidelberg, Germany). The primer amount ranged from 3.0 pmol to 7.0 pmol. All forward primers were fluorescently labelled at the 5' end with IRD700 or IRD800. To increase efficiency all primers were pooled in PCR multiplex groups of two to six markers according to their allele size and the fluorescence labelling. PCR amplification was carried out in PTC 100™ or PTC 200™ thermocyclers (MJ Research, Watertown, MA, USA) with the following standard program with variable annealing temperature (T_a) between 58°C and 62°C: 94 °C for 4 min, followed by 36 cycles at 94 °C for 30 sec, optimum annealing temperature (T_a) for 1 min, 72 °C for 30 sec, and finally storing at 4 °C for 10 min. The PCR products were size-fractionated by gel electrophoresis on 6% polyacrylamide denaturing gels (Rotiphorese Gel40, Carl Roth, Karlsruhe) using an automated capillary sequencer (LI-COR 4200/S-2 and 4300, Lincoln, NE, USA). Prior to loading, PCR products were diluted with formamide loading buffer in ratios of 1:10 according to empirical values. Allele sizes were detected using an IRD700- and IRD800-fluorescence-labelled DNA ladder, and the genotyping data was analyzed by visual examination.

The PCR reactions for SNP genotyping were performed in a total volume of 30 µl containing 10 ng of genomic DNA as template, 10 pmol of each primer and 1 U *Taq* polymerase (Qbiogene, Heidelberg, Germany). Thermocycling was carried out under the following conditions: initial denaturation at 94°C for 4 min was followed by 35 cycles of 94°C for 30 s, optimum annealing temperature (T_a) for 1 min, 72°C for 1 min and a final cooling at 4°C for 10 min.

The amplicons for SNP development were sequenced on a MegaBACE 1000 (GE Healthcare, Freiburg, Germany) automated capillary sequencer. The sequencing reaction was carried out using the DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare, Freiburg, Germany). Amplification started with an initial denaturation

at 94°C for 1.5 min, followed by 34 cycles of 20 sec denaturing at 94°C, 15 sec annealing at 50°C and 2 min elongation at 60°C. Finally, the reaction was cooled down to 4°C for 10 min. The reaction product was cleaned up using a Sephadex G50 filtration (GE Healthcare). Sequence data was analyzed using the Sequencher 4.7 program (GeneCodes, Ann Arbor, MI, USA).

Genotyping of the identified SNPs was performed via restriction fragment length polymorphisms (RFLPs) or, when no RFLP was available, using Custom TaqMan® SNP Genotyping Assays (Applied Biosystems, Darmstadt, Germany). For RFLPs the amplification of the PCR products containing the SNPs was performed as described above for the development of SNPs. RFLPs were done in 20-µl reaction volumes using 2 µl buffer, possible 0.2 µl bovine serum albumin (BSA) dependent on the used endonucleases and 1.5 U endonuclease with 15 µl of the PCR product. The marker genotypes were determined by gel electrophoresis using 2% agarose gels and evaluated by visual examination. The genotyping assays were analyzed on a 7300 Real Time PCR System (Applied Biosystems) in 12 µl volume using 5.3 µl SensiMix DNA kit (Quantance, London, UK), 0.3 µl Custom TaqMan® SNP Genotyping Assays (Applied Biosystems) and a DNA template of 10 ng. After a 10 min initial denaturation at 95°C, 40 cycles of 15 sec at 92°C and 60 sec at 60°C were used.

Mendelian inheritance and correctness of marker transmission in the pedigrees genotyped was confirmed using the Pedstats software (Wigginton and Abecasis 2005).

Data analysis

Multipoint non-parametric linkage analysis (NPL) was performed using the Merlin software (multipoint engine for rapid likelihood inference, version 1.1.2) (Abecasis *et al.,* 2002) and included 34 newly developed microsatellites, one published microsatellite, 15 SNPs and 21 microsatellites already used in the former whole genome scan (Supplemental Table 2). The Zmean and LOD score test statistics were used to test for the proportion of alleles shared by affected individuals identical by descent (IBD) for the considered marker loci (Kong and Cox, 1997; Kruglyak *et al.,* 1996; Whittemore and Halpern, 1994). The maximum (minimum) achievable Zmeans

were 6.65 (-2.16) for OC in hock joints, and 5.78 (-1.63) for OCD in hock joints. The corresponding maximum (minimum) values for LOD scores were 3.87 (-0.37) and 2.56 (-0.25) indicating enough power to detect genome-wide significant linkage. Chromosome-wide error probabilities were obtained as described in Dierks *et al.* (2007). Genome-wide probabilities were calculated by applying a Bonferroni correction: $P_{genome\text{-}wide} = 1 - (1 - P_{chromosome\text{-}wide})^{1/r}$, where r = length of the respective equine chromosome in Mb, which is 87.4 Mb according to the horse genome assembly EquCab2.0, divided by the total equine genome length (2680 Mb).

In this study, six different phenotypes for OC were distinguished: (1) OC present in fetlock and/or hock joints, (2) OCD present in fetlock and/or hock joints, (3) OC present in fetlock joints, (4) OCD present in fetlock joints, (5) OC present in hock joints, (6) OCD present in hock joints. Controls were horses which did not have any signs of OC in any of the joints examined including fetlock, hock and stifle joints.

In addition, the genotypic data was evaluated using the ALLELE and CASECONTROL procedures of the software package SAS/Genetics (Statistical Analysis System, Version 9.2, SAS Institute, Cary, NC, USA 2008) to determine the observed heterozygosity (HET), the polymorphism information content (PIC) and Hardy-Weinberg equilibrium and to evaluate genotypic and allelic associations, haplotype association and the trend of the genotypes with the phenotypic OC traits using χ^2-tests.

4.4 Results

The former whole genome scan was performed with relative marker positions in cM taken from Swinburne *et al.* (2006) and Penedo *et al.* (2005). The release of the horse genome has made it possible to verify the QTL on the horse genome assembly (EquCab2) (Fig. 1). Thus, the QTL of the previous whole genome scan on ECA16 were located at 23.12 Mb (*COR011*) for OC in fetlock and/or hock joints and OCD in fetlock and/or hock joints, at 3.92-6.63 Mb (*AHT037*, *TKY279*) and 23.12-54.20 Mb (*COR011* - *LEX048*) for hock OC and hock OCD and at 75.41 Mb (*TKY406*) for hock OC.

We identified 34 previously unknown microsatellites and 15 SNPs in the region from 0.49 to 52.38 Mb on ECA16. These markers were genotyped in 14 half-sib families

with a total of 211 horses and then used for linkage and association analysis to refine the QTL in Hanoverian warmblood horses. The average polymorphism information content (PIC) of the new microsatellites was 56.1% with a minimum of 17.4% and a maximum of 79.8%, while the mean observed heterozygosity was 62.2% ranging between 19.9% and 80.8%. The non-parametric multipoint linkage analysis showed chromosome-wide significant Zmeans and LOD scores at 14.38 Mb on ECA16 for OC in fetlock and/or hock joints and in the region from 12.10 to 24.26 Mb for OCD in fetlock and/or hock joints (Table 1).

Chromosome-wide significant Zmeans and LOD scores could be detected for hock OCD in the region from 6.55 to 6.63 Mb and 17.60 to 45.18 Mb on ECA16 and in the region from 33.36 to 36.67 Mb and from 38.43 to 43.40 Mb for hock OC (Table 2).

The highest Zmeans and LOD scores were 3.49 and 1.81 for hock OCD, with corresponding genome-wide error probabilities of 0.006 and 0.05 after Bonferroni correction for the regions between 32.90 and 34.61 Mb, between 35.86 and 36.19 Mb and between 38.43 and 42.41 Mb (Fig. 2).

For hock OC, the highest Zmeans and LOD scores were 2.94 and 1.65 at 40.69 to 42.41 Mb with chromosome-wide error probabilities of 0.002 and 0.003 and genome-wide error probabilities of 0.06 and 0.09 (Fig 3).

Furthermore, a chromosome-wide significant linkage with fetlock OC was evident for the region between 6.55 and 24.26 Mb. The highest Zmean was 2.72 at 12.10 Mb with a chromosome-wide error probability of 0.003 and the highest LOD score was 0.99 at 14.38 Mb with a chromosome-wide error probability of 0.02 (Table 3).

All results from the linkage analyses for all six traits of osteochondrosis in fetlock and hock joints are given in Supplemental Tables 4-6.

Association tests using the CASECONTROL procedure of SAS/Genetics revealed significant genotypic and/or allelic association with hock OCD in the QTL region on ECA16 at 24.26 Mb (*ABGe035*), 27.79 Mb (*ABGe042*), 31.82 Mb (*ABGe047*), 33.65 Mb (*TKY871*), 34.61 Mb (*ABGe049*), 36.19 Mb (*DOCK3_SNP*) and at 42.41 Mb (*ABGe057*) (Table 4). The lowest error probabilities had the microsatellite *ABGe049*. The allele 162 of the microsatellite *ABGe049* was associated with hock OCD, whereas the alleles 168 and 172 were significantly more frequently present in the

horses free from signs of osteochondrosis. Haplotype testing did also reveal significant associations for single haplotypes when adjacent pairs of the above mentioned markers (*ABGe035*, *ABGe042*, *ABGe047*, *TKY871*, *ABGe049*, *DOCK3_SNP*, and *ABGe057*) were employed. All markers which were associated with hock OCD were in Hardy-Weinberg equilibrium. This result also confirms the QTL location in the region between 17.60 Mb and 45.18 Mb.
The SNPs in the *CRTAP* and the *PTHR1* genes as potential functional candidate genes did not reveal significant associations.

4.5 Discussion

This study presents an important step towards the identification of genes responsible for equine OCD in hock joints in the QTL region on ECA16. The partial consistence of the QTL for hock OC and hock OCD leads to the assumption that the same genes may play a role in the development of this disease and that OCD is an aggravated form of OC. However, fetlock and hock joints seem to be influenced by different genes located on ECA16, as the hock QTL did not map at the fetlock QTL. The delimitation of the QTL using very dense microsatellite scans allows clarifying how many positional candidate genes have to be tested for linkage disequilibrium with OC and which of these candidate genes may be responsible for OC. In order to reach this goal and to develop a marker test for osteochondrosis in Hanoverian warmblood horses, it is necessary to develop more single nucleotide polymorphisms (SNPs) in the QTL region for hock OCD. Significantly associated markers may indicate locations where SNPs in linkage disequilibrium may be found and where possible positional candidate genes may be located. For that purpose, it is possible to use the SNP table from Broad Institute (http://www.broad.mit.edu/ftp/distribution/horse_snp_release/v2/) although it is not assured that the SNPs reported are detectable in the examined breed. Furthermore, it can be helpful to use the Equine Articular Cartilage cDNA Library to select candidate genes which are at least expressed in cartilage. At the moment a total of 13,964 equine articular ESTs (expressed sequence tag) can be found at the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez), from which ESTs

located in 75 genes are in the QTL region between 17.60 and 45.18 Mb on ECA16. Near to the significantly associated microsatellite *ABGe049* at 36.9 Mb, there are located several hyaluronoglucosaminidase genes, *HYAL1, HYAL2* and *HYAL3*. These genes encode a lysosomal hyaluronidase (HYAL1) or a protein which is similar to hyaluronidases (HYAL2 and HYAL3). Hyaluronidases intracellularly degrade hyaluronan, one of the major glycosaminoglycans of the extracellular matrix. Hyaluronan is an important integral structural component of articular cartilage and other tissues and acts as a lubricant in joints. It contributes to tissue hydrodynamics, movement, cell proliferation, migration and differentiation, and participates in a number of cell surface receptor interactions. Besides association of hyaluronidases with tumor suppression, mutations in the *HYAL1* gene were found to be associated with mucopolysaccharidosis type IX, or hyaluronidase deficiency (Natowicz *et al.,* 1996; Triggs-Raine *et al.,* 1999). Due to their function these *HYAL1, HYAL2* and *HYAL3* genes seem to be suitable functional candidate genes for osteochondrosis.

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Table 1 Multipoint non-parametric chromosome-wide test statistics (Zmean and LOD score) with their chromosome-wide significant error probabilities (P_Z, P_L) and their map positions (POS) on the horse genome assembly 2.0 for OC and OCD in fetlock and/or hock joints in Hanoverian warmblood horses

POS in Mb	Marker	OC				OCD			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
12.10	*AHT081*					1.88	0.03	0.79	0.03
14.38	*ABGe032*	1.76	0.04	0.71	0.04	2.10	0.02	1.03	0.015
17.60	*ABGe033*					1.82	0.03	0.59	0.05
20.68	*ABGe034*	1.86	0.03			1.94	0.03	0.77	0.03
23.12	*COR011*	1.87	0.03			1.74	0.04	0.69	0.04
24.26	*ABGe035*	1.81	0.03			1.64	0.05	0.64	0.04

Table 2 Multipoint non-parametric chromosome-wide test statistics (Zmean and LOD score) with their chromosome-wide significant error probabilities (P_Z, P_L) and their map positions (POS) on the horse genome assembly 2.0 for hock OC (OC-H) and hock OCD (OCD-H) in Hanoverian warmblood horses

POS in Mb	Marker	OC-H				OCD-H			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
3.44	*ABGe095*							0.68	0.04
3.92	*AHT037*							0.80	0.03
5.02	*ABGe096*							0.82	0.03
6.55	*ABGe097*					1.94	0.03	0.74	0.03
6.63	*TKY279*					1.96	0.03	0.75	0.03
17.60	*ABGe033*	1.68	0.05	0.64	0.04	2.57	0.005	0.69	0.04
20.68	*ABGe034*					3.05	0.0011*	1.70	0.003
23.12	*COR011*					2.73	0.003	1.60	0.003
24.26	*ABGe035*					2.59	0.005	1.54	0.004
24.88	*ABGe036*					2.59	0.005	1.54	0.004
25.31	*ABGe037*					2.59	0.005	1.54	0.004
25.59	*ABGe038*					2.45	0.007	1.48	0.005
25.93	*ABGe039*					2.29	0.011	1.39	0.006
26.53	*ABGe040*					1.70	0.04	0.70	0.04
26.67	*CADPS_SNP*					1.77	0.04	0.68	0.02
27.33	*ABGe041*					2.20	0.014	1.33	0.007
27.79	*ABGe042*					2.59	0.005	1.54	0.004
27.90	*ABGe043*					2.59	0.005	1.54	0.004
27.97	*HMS020*					2.59	0.005	1.54	0.004
28.44	*ABGe044*					2.59	0.005	1.54	0.004
29.46	*ABGe045*					2.59	0.005	1.54	0.004
30.27	*AHT038*			0.68	0.04	2.59	0.005	1.54	0.004
30.49	*ABGe046*					2.59	0.005	1.54	0.004
31.48	*ARHGEF3_SNP*					2.59	0.005	1.54	0.004
31.82	*ABGe047*					2.81	0.003	1.62	0.003
32.65	*WNT5A_SNP*					3.33	0.0004*	1.77	0.002
32.90	*ABGe048*					3.49	0.0002**	1.81	0.002

Table 2 continued

POS in Mb	Marker	OC-H				OCD-H			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
33.36	*BIEC2-355700*	1.89	0.03	0.79	0.03	3.49	0.0002**	1.81	0.002
33.48	*TKY350*	2.01	0.02	0.88	0.02	3.49	0.0002**	1.81	0.002
33.65	*TKY871*	2.12	0.02	0.94	0.02	3.49	0.0002**	1.81	0.002
33.75	*LEX059*	2.12	0.02	0.94	0.02	3.49	0.0002**	1.81	0.002
34.06	*CHDH_SNP*	2.12	0.02	0.94	0.02	3.49	0.0002**	1.81	0.002
34.61	*ABGe049*	2.12	0.02	0.94	0.02	3.49	0.0002**	1.81	0.002
34.93	*ABGe050*	1.60	0.06	0.68	0.04	2.59	0.005	1.54	0.004
35.29	*TKY1069*	1.80	0.04	0.80	0.03	2.94	0.002	1.67	0.003
35.86	*ABGe051*	2.12	0.02	0.94	0.02	3.49	0.0002**	1.81	0.002
36.19	*DOCK3_SNP*	2.12	0.02	0.94	0.02	3.49	0.0002**	1.81	0.002
36.67	*ABGe052*	1.69	0.05	0.72	0.03	2.94	0.002	1.55	0.004
37.10	*ABGe053*					2.45	0.007	1.00	0.02
38.43	*ABGe054*	2.43	0.008	1.33	0.007	3.49	0.0002**	1.81	0.002
39.46	*SCAP_SNP*	2.66	0.004	1.51	0.004	3.49	0.0002**	1.81	0.002
39.93	*PTHR1_SNP*	2.77	0.003	1.57	0.004	3.49	0.0002**	1.81	0.002
39.97	*MYL3_SNP*	2.78	0.003	1.57	0.004	3.49	0.0002**	1.81	0.002
40.69	*ABGe055*	2.94	0.002	1.65	0.003	3.49	0.0002**	1.81	0.002
41.30	*ABGe056*	2.94	0.002	1.65	0.003	3.49	0.0002**	1.81	0.002
42.41	*ABGe057*	2.94	0.002	1.65	0.003	3.49	0.0002**	1.81	0.002
43.40	*ABGe058*	1.83	0.03	0.80	0.03	3.12	0.0009*	1.65	0.003
45.18	*ENTPD3_SNP*					1.94	0.03	0.74	0.03

genome-wide significant after Bonferroni's correction using $P_{genome\text{-}wide} = 1- (1 - P_{chromosome\text{-}wide})^{1/r}$ with r being the length of equine chromosome 16 (87.4 Mb) divided by the total equine genome length (2680 Mb). *: P<0.05, **: P<0.01.

Table 3 Multipoint non-parametric chromosome-wide test statistics (Zmean and LOD score) with their chromosome-wide significant error probabilities (P_Z, P_L) and their map positions (POS) on the horse genome assembly 2.0 for fetlock OC (OC-F) in Hanoverian warmblood horses

POS in Mb	Marker	OC-F			
		Zmean	P_Z	LOD	P_L
6.55	*ABGe097*	2.53	0.006	0.74	0.03
6.63	*TKY279*	2.54	0.006	0.74	0.03
7.95	*ABGe098*	2.33	0.010	0.57	0.05
8.15	*HTG03*	2.34	0.010	0.57	0.05
12.10	*AHT081*	2.72	0.003	0.87	0.02
14.38	*ABGe032*	2.69	0.004	0.99	0.02
17.60	*ABGe033*	2.41	0.008	0.90	0.02
20.68	*ABGe034*	2.30	0.011	0.90	0.02
23.12	*COR011*	2.12	0.02	0.79	0.03
24.26	*ABGe035*	2.03	0.02	0.72	0.03

Table 4 Results of association analysis of microsatellites located in the QTL for OCD in hock joints using χ^2-tests for genotypes, alleles and trend of alleles

Marker	Location on ECA16 in Mb	Genotype			Allele			Trend		
		X²	d.f.	P	X²	d.f.	P	X²	d.f.	P
ABGe033	17.60	6.02	11	0.872	1.55	4	0.818	1.89	4	0.756
ABGe034	20.68	37.14	25	0.056	15.26	10	0.123	16.03	10	0.099
COR011	23.12	11.73	9	0.229	7.12	3	0.068	7.07	3	0.070
ABGe035	24.26	30.60	25	0.200	15.43	7	**0.031**	n. e.	-	-
ABGe036	24.88	5.38	3	0.146	2.01	2	0.365	2.09	2	0.352
ABGe037	25.31	10.63	13	0.641	2.70	6	0.845	2.88	6	0.823
ABGe038	25.59	13.43	11	0.266	7.56	5	0.182	6.45	5	0.265
ABGe039	25.93	2.29	5	0.808	1.44	2	0.487	1.55	2	0.461
ABGe040	26.53	1.24	2	0.539	1.11	2	0.574	1.24	2	0.539
CADPS_SNP	26.67	1.81	1	0.178	1.57	1	0.211	1.81	1	0.178
ABGe041	27.33	0.91	2	0.633	0.68	1	0.408	0.76	1	0.383
ABGe042	27.79	13.56	8	0.094	9.62	4	**0.047**	11.14	4	**0.025**
ABGe043	27.90	3.29	5	0.656	0.66	2	0.719	n. e.	-	-
HMS020	27.97	8.92	10	0.540	2.71	4	0.608	2.94	4	0.569
ABGe044	28.44	20.51	19	0.364	11.87	7	0.105	10.70	7	0.152
ABGe045	29.46	5.78	6	0.449	5.03	4	0.284	5.76	4	0.218
AHT038	30.27	13.31	17	0.715	7.50	5	0.186	n. e.	-	-
ABGe046	30.49	5.18	7	0.638	2.76	3	0.431	2.80	3	0.423
ARHGEF3_SNP	31.48	3.25	2	0.197	0.83	1	0.362	0.87	1	0.350
ABGe047	31.82	32.63	21	**0.050**	14.31	7	**0.046**	n. e.	-	-
WNT5A_SNP	32.65	0.71	2	0.700	0.53	1	0.468	0.55	1	0.459
ABGe048	32.90	12.25	12	0.426	4.51	5	0.479	n. e.	-	-
BIEC2-355700	33.36	0.48	2	0.788	0.04	1	0.847	0.03	1	0.861
TKY350	33.48	23.02	16	0.113	9.60	5	0.087	n. e.	-	-
TKY871	33.65	16.37	9	0.060	10.35	4	**0.035**	9.80	4	**0.044**
LEX059	33.75	4.98	5	0.418	4.90	3	0.180	4.42	3	0.220
CHDH_SNP	34.06	5.43	2	0.066	3.03	1	0.082	2.98	1	0.084
ABGe049	34.61	17.42	6	**0.008**	12.96	3	**0.005**	13.06	3	**0.005**
ABGe050	34.93	14.69	12	0.259	4.34	5	0.501	4.85	5	0.434
TKY1069	35.29	10.05	9	0.346	5.11	3	0.164	4.63	3	0.201

Table 4 continued

Marker	Location on ECA16 in Mb	Genotype			Allele			Trend		
		X²	d.f.	P	X²	d.f.	P	X²	d.f.	P
ABGe051	35.86	5.41	4	0.248	3.86	2	0.145	4.23	2	0.121
DOCK3_SNP	36.19	4.85	2	0.088	5.00	1	**0.025**	4.63	1	**0.032**
ABGe052	36.67	9.66	6	0.140	4.43	3	0.219	5.37	3	0.146
ABGe053	37.10	10.94	7	0.141	5.47	3	0.141	n. e.	-	-
ABGe054	38.43	3.84	6	0.698	3.22	3	0.359	3.27	3	0.352
SCAP_SNP	39.46	1.35	2	0.510	0.25	1	0.620	0.27	1	0.606
PTHR1_SNP	39.93	0.74	2	0.691	0.77	1	0.379	0.71	1	0.401
MYL3_SNP	39.97	1.26	2	0.532	1.20	1	0.274	0.96	1	0.326
ABGe055	40.69	18.05	15	0.260	7.23	5	0.204	n. e.	-	-
ABGe056	41.30	11.37	14	0.656	2.77	5	0.736	n. e.	-	-
ABGe057	42.41	30.87	27	0.277	14.31	7	**0.046**	n. e.	-	-
ABGe058	43.40	27.44	25	0.334	13.17	8	0.106	n. e.	-	-
ENTPD3_SNP	45.18	0.12	1	0.734	0.08	1	0.774	0.12	1	0.734

n. e.: not estimable

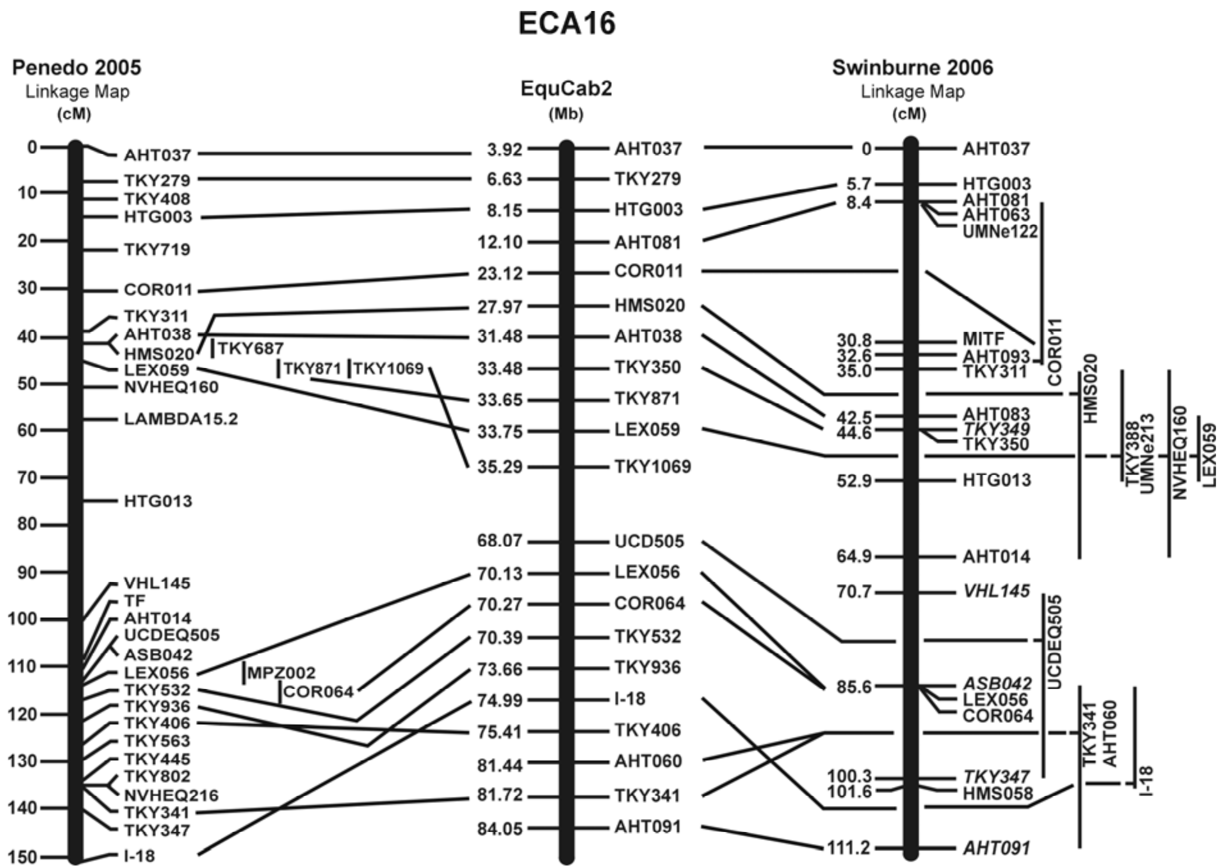

Figure 1 Comparison of the marker order on the horse genome assembly (EquCab2) in Mb with the linkage map reported by Penedo et al. (2005) and Swinburne et al. (2006) in cM. For positions of the 34 newly developed microsatellites see Supplemental Table 2.

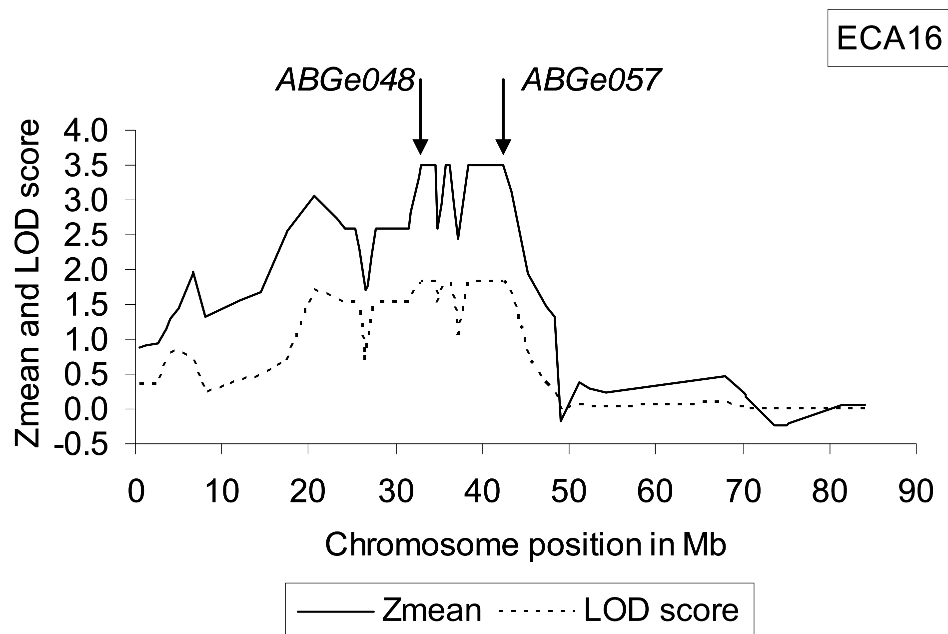

Figure 2 Zmeans and LOD scores for 56 microsatellite markers and 15 SNPs on ECA16 harbouring a quantitative trait locus (QTL) for equine osteochondrosis dissecans in hock joints (OCD-H)

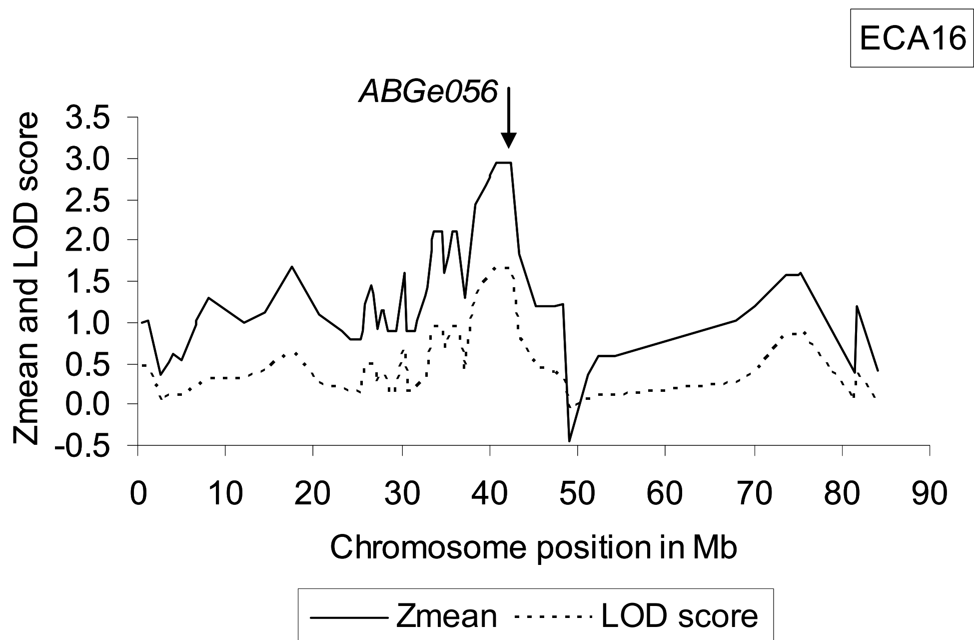

Figure 3 Zmeans and LOD scores for 56 microsatellite markers and 15 SNPs on ECA16 harbouring a quantitative trait locus (QTL) for equine osteochondrosis in hock joints (OC-H)

Supplemental Table 1 Number of families analysed, their sizes and prevalences of osteochondrosis (OC), osteochondrosis dissecans (OCD), osteochondrosis in fetlock (OC-F) and hock (OC-H) joints and osteochondrosis dissecans in fetlock (OCD-F) and hock (OCD-H) joints by family and in total

Half-sib family	Number of progeny	Male	Female	Prevalences in % for					
				OC	OCD	OC -F	OCD-F	OC-H	OCD-H
1	4	1	3	100.0	100.0	50.0	50.0	75.5	75.0
2	9	4	5	44.4	33.3	11.1	0.0	33.3	33.3
3	7	5	2	71.4	42.9	71.4	42.9	28.6	14.3
4	15	9	6	53.3	33.3	26.7	6.7	26.7	26.7
5	8	1	7	100.0	25.0	75.0	12.5	37.5	12.5
6	4	2	2	100.0	25.0	50.0	25.0	50.0	0.0
7	5	3	2	100.0	40.0	80.0	40.0	20.0	0.0
8	6	2	4	100.0	66.7	83.3	50.0	66.7	33.3
9	20	12	8	75.0	60.0	50.0	20.0	50.0	45.0
10	8	4	4	62.5	25.0	50.0	12.5	12.5	12.5
11	5	3	2	40.0	20.0	20.0	0.0	20.0	20.0
12	5	2	3	100.0	80.0	100.0	80.0	0.0	0.0
13	5	3	2	40.0	40.0	40.0	40.0	0.0	0.0
14	3	1	2	100.0	66.7	100.0	66.7	0.0	0.0
Total	104	52	52	73.1	45.2	51.9	25.0	32.7	24.0

Supplemental Table 2 Characteristics of the microsatellites used in this study

Marker	Acc. No	Mb	Ta (°C)	Forward / reverse primer sequence (5'-3')	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
ABGe092	AM942733	0.49	58	F: TGCAGGCTCTAATACTCTGCTG R: GCCTGGTGTGTTTGGTCTTAG	3	88 - 114	41.5	31.7
ABGe093	AM942734	1.10	58	F: TCAAGAGACCAGCCTTGAGC R: GCAGCAACAGTCAAAGGTAGG	6	156 - 172	72.8	64.3
ABGe094	AM942735	2.65	60	F: AACTGCTGGCTGGATCTCTG R: AAGACTGCCCCATTCAATACTC	7	94 - 108	80.3	76.7
ABGe095	AM942736	3.44	58	F: GCTTATCATTCTTTCCCTGAAAC R: CTCATGTTCCTCCCCAAATG	4	153 - 163	35.7	30.4
AHT037	AJ271522	3.92	58	F: ATTTCACCCACTCCCAACC R: TTTCGTTTGCGACAATATGG	4	204 - 218	42.4	42.4
ABGe096	AM942737	5.02	58	F: GAGGAGGATTTTGGCCTACC R: ACCACCCAAACCTCTCCAC	5	200 - 214	56.3	55.7
ABGe097	AM942738	6.55	58	F: CCACCCAGTATTGGATTTGC R: GTCAACGTGTGGGTTTGTTG	3	188 - 196	50.2	37.8
TKY279	AB033930	6.63	60	F: AATGAATGAGACTTGAACCC R: TCTGCTGTTTTAGGCTCGG	6	118 - 132	77.3	71.4
ABGe098	AM942739	7.95	58	F: TTGATCCCAATTGTTTGAAGC R: CACCCATAATCTCTCACTGTCTTG	6	124 - 140	76.5	70.3
HTG003	AF169164	8.15	55	F: TAACCTGGGTGCAAAGCCACCCAT R: TCAGGGCCAATCTTCCTCAC	5	114 - 124	77.7	72.8
AHT081	AJ507698	12.10	58	F: AACTCAGGGGCAGACAAATG R: TTGGAACTTTCAGTCCAGGG	3	201 - 205	49.8	40.4
ABGe032	AM919471	14.38	59	F: CCGAACCTAGGCTGAGGAAG R: CTTCACCTGAAGCGACCAAC	4	200 - 216	48.6	42.6
ABGe033	AM919472	17.60	59	F: GGGTTTGCTTGTGAACTTCTG R: GTGAAGCCCTGACTTTGAGC	5	234 - 248	75.1	69.2
ABGe034	AM919473	20.68	59	CCACATAAGCAGATTTGTTTGG TGTGTCTGGGGAACACATTATC	11	196 - 226	71.4	70.1
COR011	AF083454	23.12	62	F: CCTTCCGGTCTTTATTCACA R: GGTGGCTGGAGACACAATAG	4	267 - 277	60.4	55.1
ABGe035	AM919474	24.26	59	F: GATATCCACCCCAGCAATTAG R: CATGGAATTAGGTGCTCCTTC	9	184 - 208	79.5	77.0
ABGe036	AM919475	24.88	59	F: AAAAAGGGAAAACGAGATGG R: TGAGCTAACCAGCAACCTTC	3	120 - 125	46.5	37.0
ABGe037	AM919476	25.31	59	F: CTCTTACCATGCCAATCCAAG R: TCTAATTTGAGTTTTACCAGGTTCC	7	94 - 110	62.2	59.3
ABGe038	AM919477	25.59	59	F: GCCTAATGGCCCAATGTAAG R: ACAATTGTCCATGACAGCATC	6	190 - 202	62.7	55.9
ABGe039	AM919478	25.93	59	F: CCAGAAGAGGGTGCTAGAGTTC R: TTGACTTTTGAACTGCCTGTG	3	190 - 216	67.8	54.8

Supplemental Table 2 continued

Marker	Acc. No	Mb	Ta (°C)	Forward / reverse primer sequence (5'-3')	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
ABGe040	AM919479	26.53	59	F: ACCTTGGACTGACAGGATGG R: GCCAGGAAGATGGTGTTAGG	3	210 - 222	19.9	17.4
ABGe041	AM919480	27.33	59	F: GAAACAGCAGGGAAAACACC R: CTCTGGGTTGTGAGGCAATC	2	96 - 98	32.4	26.5
ABGe042	AM919481	27.79	59	F: CCCTGTAATGAAGAAATGGACAG R: TGTGTGTACCAATAACCCATGC	5	144 - 152	68.4	54.6
ABGe043	AM919482	27.90	59	F: AAGGGATTTGCAGCTCATTG R: GGTTTTCTTTCCTCCACAGG	5	126 - 134	63.3	60.8
HMS020	U35402	27.97	60	F: TGGGAGAGGTACCTGAAATGTAC R: GTTGCTATAAAAAATTGTCTCCCTAC	6	122 - 136	63.8	68.4
ABGe044	AM919483	28.44	59	F: GATTCGTAGAGAATGACACAATCG R: GACCCTTACCACTGCTTTTCC	9	130 - 158	74.8	71.4
ABGe045	AM919484	29.46	62	F: TGAAGGGGATCACTTACATGG R: AATCTTATCACCTTTGGGTAGTCAC	5	130 - 154	46.1	41.8
AHT038	AJ271523	30.27	58	F: TTCATGGCCTTCAAAACTCC R: CCAGCTGGGGATACTTACCA	7	128 - 140	70.5	67.4
ABGe046	AM919485	30.49	59	F: TTTTTAAAGACTATCTGCCTTTTTAGC R: GGAAATATTTGGTGAGTTTCTGC	4	140 - 152	61.7	55.7
ABGe047	AM919486	31.82	59	F: ATGCAAATGCATGTGTCCAG R: GATGTTTCCTAAGGCGATGG	9	120 - 148	74.2	67.5
ABGe048	AM919487	32.90	59	F: CCTCTGCACTTTACGCTTCC R: TAGGCTCCTTGCTCCTCTTG	7	180 – 206	74.8	63.0
TKY350	AB044850	33.48	56	F: TCCTAGGGAATTCACAGTTG R: TAACAGAACTACAAGGCCC	7	165 - 195	68.5	65.1
TKY871	AB104089	33.65	58	F: CACACTACTCATGAAGCCAT R: ACTTGTCCCAGCACTGTTTG	5	246 - 254	58.2	55.8
LEX059	AF075660	33.75	55	F: TGAAATGTCACCTTCTCAGAG R: GTGGACACTTGCCNTCAT	4	225 - 231	37.2	35.1
ABGe049	AM919488	34.61	59	F: AGCCAGGTCAGTACCATTCG R: TGGGGGACTAGTGATGTGTTG	4	160 - 172	51.2	45.1
ABGe050	AM919489	34.93	59	F: CTCACATTTTTGGCTTTACCG R: CTCCCAGGGAACAGAAACTG	6	82 - 140	67.6	60.4
TKY1069	AB104287	35.29	58	F: TTTGAACCACCAACCAAAGG R: CCCAAAGGGAAAGGATGATG	4	132 - 146	68.1	59.5
ABGe051	AM919490	35.86	59	F: TTGGTAAGGCTTTCCCTCAC R: TGCTGTGTTATTGCCACCTG	3	176 - 180	58.4	45.9
ABGe052	AM919491	36.67	59	F: CCCCACTTCTGAGCAACATC R: TGGCCATCTACCAGAAGGAG	4	116 - 130	61.4	53.6
ABGe053	AM919492	37.10	59	F: ACATTTTCCCTGGGACACAC R: AGCTATGTGGGGTGGAAATG	5	212 - 222	57.9	53.4

Supplemental Table 2 continued

Marker	Acc. No	Mb	Ta (°C)	Forward / reverse primer sequence (5'-3')	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
ABGe054	AM919493	38.43	59	F: TGGGGACCCAGGACTATCTC R: TGTTTGGTGACCCTCCCTAC	4	216 - 228	70.8	61.6
ABGe055	AM919494	40.69	58	F: CCAGGATTGGCTTTTATTTTATTC R: AAAGTTGTCAGGAGGTTGTTCAC	7	96 - 118	72.6	71.4
ABGe056	AM919495	41.30	58	F: TGAACAACCTGAGACCTTCTAGC R: TACCTCAAGCCATGAAGTGC	7	202 - 224	74.2	65.4
ABGe057	AM919496	42.41	58	F: AGGAAACTGCCAAGATGCAG R: GTCTTCTGGCCCTGCCTAC	9	97 - 117	80.8	79.8
ABGe058	AM919497	43.40	58	F: CCACACAGTATTCCCCCAAG R: GGAGAGAGGGTTCAGTGCAG	10	186 - 206	79.3	78.1
LEX048	AF075650	54.20	55	F: CATAGTGCCCTCAAGTTC R: TGAATTGGATAAAGAAGATGTA	2	164 - 166	27.3	25.5
UCDEQ505	U67421	68.07	55	F: ATCACTCTCTTGTTGAGATAAC R: GGGATTTCCTTCTTTCTC	6	183 - 197	73.5	71.1
LEX056	AF075658	70.13	55	F: GACCTACAGGCCACTCATCAA R: GGCAGTTTCCTCCATCCTTA	8	211 - 227	85.2	82.6
COR064	AF142601	70.27	58	F: TCCATACATGTGTGAGGGC R: AAGATGGCTTACAAGGATTATG	4	192 - 202	51.7	49.1
TKY532	AB103750	70.39	58	F: ACAAACACTCGTGGTTGCTG R: TATTTTCAGCAAGGGGCAAC	9	160 - 178	67.1	62.4
TKY936	AB104154	73.66	58	F: ACCACTGTACTGAATACTGG R: ACAAAGCATCTCCTCGAATAG	6	106 - 126	81.7	73.3
I-18	Y10244	74.99	58	F: CAACAAAGATGTTGCAAGGG R: TGTGCCTCTTGTCTCTTAGG	7	97 - 111	72.3	65.5
TKY406	AB103624	75.41	60	F: CCACTAGGGGCCAGTGATT R: GAACTCCACCCTTTGGGATT	10	99 - 123	88.1	82.8
AHT060	AJ507677	81.44	62	F: GGTCAAGCTTTTGGTTTTTCC R: CCTAAGGAAGAGCTGTTCTTGC	10	282 - 308	84.8	79.3
TKY341	AB044842	81.72	56	F: TATCCAGTCACCCATTTTAC R: TTGTGTCAGTACACTCTATG	8	138 - 154	71.8	69.8
AHT091	AJ507708	84.05	62	F: TAGCTGTCTGCAAAGGCTCA R: CCAGTGTTCCACATGCCTC	4	108 - 126	19.0	20.2

Mb: Location on the horse genome assembly 2.0 in megabases
T_a: Annealing temperature in °C
HET: Observed heterozygosity
PIC: Polymorphism information content
Markers in bold indicate these microsatellites which were used in a previous whole genome scan for osteochondrosis in Hanoverian warmblood horses (Dierks *et al.*, 2007)

Supplemental Table 3 Primer sequences, product size, annealing temperature (Ta) and the corresponding location in the human gene, polymorphism information content (PIC), observed heterozygosity (HET) and SNP ID for 15 intragenic polymorphisms of Hanoverian Warmblood horses and RFLP information about 12 intragenic single nucleotide polymorphisms

Gene symbol	Primer forward (5´ -> 3´), primer reverse (5´ -> 3´)	bp	Ta	Corresponding location in the human gene	PIC	HET	SNP ID	RFLP (enzyme, recognition sequence, number of cutting sites in PCR product)
CADPS	CTTGCCGTGGCAATTAAGAG ATGCCCATGAGATGTTTACC	533	58	Exon 27, UTR	19.4	23.0	AAWR02008313:g.35989 T>C	HphI GGTGA$(N)_8$, 1
ARHGEF3	CTTTGGCCAATGTCAGGTTC CATGTGTCTGGAACCAGGTG	529	60	Intron 3	23.8	28.4	AAWR02008391:g.7772 G>A	
WNT5A	TATTCGCTTCCCCTCAGTTG AGCCAGCTTTAAGCCATCTG	683	58	Exon 5, UTR	18.5	22.3	AAWR02008412:g.6816 A>G	BbsI GAAGAC$(N)_2$, 1
BIEC2-355700	CTAACTGCAGAAGCCCGTTC GTACTTTCCCCGCTGGAATC	355	60	Intergenic	37.1	45.9	AAWR02008419:g.31720 G>C	AvaI CYCGRG, 1
CHDH	CCACCCCATCATTCTAGCTG CTGGAAGATTCTCGGAATGG	557	60	Intron 8	11.6	12.3	AAWR02008427:g.59870 G>A	HhaI GCGC, 2
DOCK3	ACAGCTGAGAGCTTCAATGG GTCCCTGTTGCTTTGGAAAC	493	60	Intron 49	35.7	47.3	AAWR02008465:g.19744 C>T	NspI RCATGY, 2
SCAP	ACACAGTGGGTGCCTCATC GCTGGAAGACCAGGGTAATG	531	60	Intron 4	26.1	34.4	AAWR02008534:g.51496 C>T	BceAI ACGGC$(N)_{12}$, 2
MYL3	GCCTGAGACCTTGCCATTAG TCCTCCCCAGTGTCATTTTC	530	59	Intron 3	35.8	44.0	AAWR02008554:g.8045 C>T	Tsp509I AATT, 2

Supplemental Table 3 continued

Gene symbol	Primer forward (5´ -> 3´), primer reverse (5´ -> 3´)	bp	Ta	Corresponding location in the human gene	PIC	HET	SNP ID	RFLP (enzyme, recognition sequence, number of cutting sites in PCR product)
PTHR1	TCAAATCCACACTGGTGCTC						AAWR02008550:g.1692	
	CCAGGATTTCTTGATCTCAGC	807	60	Intron 14-16	10.4	10.2	G>A	
ENTPD3	GCAGTTCCATAGCCTTCTGG						AAWR02008660:g.22358	TspRI
	CTCCACTCTGGACCCAAGAC	584	58	Intron 6	29.9	46.5	T>C	NNCASTGNN, 1
ITGA9	GCAGATGAACAGGCTGAGAAC						AAWR02008700:g.83581	AciI
	GCCAATAAAACAACTAACAACAACC	569	59	Intron 18	26.3	32.7	C>A	CCGC, 2
DCAMKL3	ATTGGAAAATCAGGGGAAGG						AAWR02008714:g.48628	AccI
	CAGGCTCAGGGATAATCAGC	594	58	Exon 4, UTR	37.4	54.3	C>T	GTMKAC, 1
ARPP-21	TATCCTCAGCCCTCCTACCC						AAWR02008722:g.21082	HpyCH4III
	AGAGAGAACCCTGCATGACG	542	58	Intron 19	28.2	35.4	T>C	ACNGT, 2
CRTAP	CTCTTACAGGGAGAGGTTGTGG						AAWR02008746:g.65672	AluI
	TGTCTTTCAAACCAAAATGGTG	503	60	Exon 7, UTR	8.6	8.5	G>C	AGCT, 2
OSBPL10	TTTGTAAAGGCTGGGGACTG						AAWR02008775:g.30806	
	GCTCATTCCTGGGAGAAGTG	575	60	Exon 13, UTR	19.9	23.8	A>G	

Supplemental Table 4 Results of multipoint non-parametric linkage analysis for the traits OC in fetlock and/or hock joints and OCD in fetlock and/or hock joints on ECA16

POS in Mb	Marker	OC				OCD			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
0.49	*ABGe092*	-0.46	0.7	-0.06	0.7	0.64	0.3	0.11	0.2
1.10	*ABGe093*	-0.47	0.7	-0.06	0.7	0.65	0.3	0.11	0.2
2.65	*ABGe094*	-0.29	0.6	-0.03	0.6	0.59	0.3	0.09	0.3
3.44	*ABGe095*	-0.19	0.6	-0.01	0.6	0.77	0.2	0.16	0.2
3.92	*AHT037*	-0.14	0.6	-0.01	0.6	0.79	0.2	0.18	0.2
5.02	*ABGe096*	0.23	0.4	0.02	0.4	0.92	0.2	0.26	0.14
6.55	*ABGe097*	1.21	0.11	0.42	0.08	1.44	0.07	0.41	0.08
6.63	*TKY279*	1.23	0.11	0.43	0.08	1.46	0.07	0.42	0.08
7.95	*ABGe098*	0.93	0.2	0.18	0.2	1.14	0.13	0.23	0.2
8.15	*HTG003*	0.95	0.2	0.19	0.2	1.14	0.13	0.23	0.2
12.10	*AHT081*	1.40	0.08	0.51	0.06	1.88	**0.03**	0.79	**0.03**
14.38	*ABGe032*	1.76	**0.04**	0.71	**0.04**	2.10	**0.02**	1.03	**0.015**
17.60	*ABGe033*	1.58	0.06	0.49	0.07	1.82	**0.03**	0.59	**0.05**
20.68	*ABGe034*	1.86	**0.03**	0.53	0.06	1.94	**0.03**	0.77	**0.03**
23.12	*COR011*	1.87	**0.03**	0.51	0.06	1.74	**0.04**	0.69	**0.04**
24.26	*ABGe035*	1.81	**0.03**	0.48	0.07	1.64	**0.05**	0.64	**0.04**
24.88	*ABGe036*	0.89	0.2	0.12	0.2	1.09	0.14	0.31	0.11
25.31	*ABGe037*	0.45	0.3	0.03	0.4	0.10	0.5	0.00	0.5
25.59	*ABGe038*	0.30	0.4	0.01	0.4	0.46	0.3	0.05	0.3
25.93	*ABGe039*	0.51	0.3	0.04	0.3	0.33	0.4	0.03	0.4
26.53	*ABGe040*	1.11	0.13	0.17	0.2	-0.17	0.6	-0.01	0.6
26.67	CADPS_SNP	0.89	0.2	0.12	0.2	-0.15	0.6	-0.01	0.6
27.33	*ABGe041*	0.38	0.4	0.02	0.4	0.03	0.5	0.00	0.5
27.79	*ABGe042*	0.48	0.3	0.04	0.3	0.22	0.4	0.01	0.4
27.90	*ABGe043*	0.45	0.3	0.03	0.4	0.20	0.4	0.01	0.4
27.97	*HMS020*	0.42	0.3	0.03	0.4	0.18	0.4	0.01	0.4
28.44	*ABGe044*	0.10	0.5	0.00	0.5	0.01	0.5	0.00	0.5
29.46	*ABGe045*	0.11	0.5	0.00	0.5	0.01	0.5	0.00	0.5
30.27	*AHT038*	0.37	0.4	0.02	0.4	0.67	0.3	0.09	0.3
30.49	*ABGe046*	0.32	0.4	0.02	0.4	0.01	0.5	0.00	0.5
31.48	ARHGEF3_SNP	0.30	0.4	0.01	0.4	0.01	0.5	0.00	0.5
31.82	*ABGe047*	0.36	0.4	0.02	0.4	0.13	0.4	0.00	0.5
32.65	WNT5A_SNP	0.39	0.3	0.02	0.4	0.42	0.3	0.03	0.4
32.90	*ABGe048*	0.39	0.3	0.02	0.4	0.51	0.3	0.04	0.3

Supplemental Table 4 continued

POS in Mb	Marker	OC				OCD			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
33.36	BIEC2-355700	0.30	0.4	0.02	0.4	0.51	0.3	0.04	0.3
33.48	*TKY350*	0.27	0.4	0.01	0.4	0.51	0.3	0.04	0.3
33.65	*TKY871*	0.33	0.4	0.02	0.4	0.51	0.3	0.04	0.3
33.75	*LEX059*	0.33	0.4	0.02	0.4	0.51	0.3	0.04	0.3
34.06	*CHDH_SNP*	0.37	0.4	0.03	0.4	0.51	0.3	0.04	0.3
34.61	*ABGe049*	0.45	0.3	0.04	0.3	0.51	0.3	0.04	0.3
34.93	*ABGe050*	0.18	0.4	0.01	0.4	0.01	0.5	0.00	0.5
35.29	*TKY1069*	0.36	0.4	0.03	0.4	0.20	0.4	0.01	0.4
35.86	*ABGe051*	0.56	0.3	0.06	0.3	0.51	0.3	0.04	0.3
36.19	*DOCK3_SNP*	0.56	0.3	0.06	0.3	0.51	0.3	0.04	0.3
36.67	*ABGe052*	0.44	0.3	0.04	0.3	0.15	0.4	0.00	0.4
37.10	*ABGe053*	0.34	0.4	0.02	0.4	-0.16	0.6	-0.00	0.6
38.43	*ABGe054*	0.59	0.3	0.07	0.3	0.51	0.3	0.04	0.3
39.46	*SCAP_SNP*	0.61	0.3	0.08	0.3	0.50	0.3	0.04	0.3
39.93	*PTHR1_SNP*	1.23	0.11	0.27	0.13	1.12	0.13	0.19	0.2
39.97	*MYL3_SNP*	1.28	0.10	0.29	0.12	1.17	0.12	0.20	0.2
40.69	*ABGe055*	0.94	0.2	0.19	0.2	0.81	0.2	0.11	0.2
41.30	*ABGe056*	0.64	0.3	0.09	0.3	0.5	0.3	0.04	0.3
42.41	*ABGe057*	0.63	0.3	0.09	0.3	0.5	0.3	0.04	0.3
43.40	*ABGe058*	0.51	0.3	0.06	0.3	0.24	0.4	0.01	0.4
45.18	*ENTPD3_SNP*	0.09	0.5	0.00	0.5	-0.02	0.5	-0.00	0.5
47.46	*ITGA9_SNP*	-0.76	0.8	-0.11	0.8	-0.18	0.6	-0.01	0.6
48.33	*DCAMKL3_SNP*	-0.69	0.8	-0.10	0.7	-0.01	0.5	-0.00	0.5
49.04	*ARPP-21_SNP*	-0.98	0.8	-0.14	0.8	-0.13	0.6	-0.01	0.6
51.13	*CRTAP_SNP*	-0.82	0.8	-0.12	0.8	-0.02	0.5	-0.00	0.5
52.38	*OSBPL10_SNP*	-0.86	0.8	-0.13	0.8	-0.25	0.6	-0.02	0.6
54.20	*LEX048*	-0.69	0.8	-0.10	0.7	0.01	0.5	0.00	0.5
68.07	*UCDEQ505*	-0.45	0.7	-0.03	0.7	0.87	0.2	0.25	0.14
70.13	*LEX056*	-1.03	0.8	-0.12	0.8	0.24	0.4	0.02	0.4
70.27	*COR064*	-0.50	0.7	-0.04	0.7	0.80	0.2	0.20	0.2
70.39	*TKY532*	-0.50	0.7	-0.04	0.7	0.79	0.2	0.19	0.2
73.66	*TKY936*	-0.60	0.7	-0.06	0.7	0.32	0.4	0.03	0.4
74.99	*I-18*	-0.40	0.7	-0.03	0.6	-0.04	0.5	-0.00	0.5
75.41	*TKY406*	-0.40	0.7	-0.03	0.6	-0.04	0.5	-0.00	0.5
81.44	*AHT060*	-1.68	1.0	-0.25	0.9	-0.13	0.6	-0.01	0.6
81.72	*TKY341*	-1.62	0.9	-0.24	0.9	-0.13	0.6	-0.01	0.6
84.05	*AHT091*	-1.30	0.9	-0.20	0.8	-0.12	0.5	-0.01	0.6

Supplemental Table 5 Results of multipoint non-parametric linkage analysis for the traits OC in fetlock joints (OC-F) and OCD in fetlock joints (OCD-F) on ECA16

POS in Mb	Marker	OC-F				OCD-F			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
0.49	*ABGe092*	1.56	0.06	0.27	0.13	1.00	0.2	0.21	0.2
1.10	*ABGe093*	1.58	0.06	0.27	0.13	1.01	0.2	0.21	0.2
2.65	*ABGe094*	1.59	0.06	0.26	0.13	0.96	0.2	0.18	0.2
3.44	*ABGe095*	1.68	**0.05**	0.30	0.12	0.96	0.2	0.18	0.2
3.92	*AHT037*	1.74	**0.04**	0.33	0.11	0.96	0.2	0.18	0.2
5.02	*ABGe096*	2.07	**0.02**	0.50	0.06	1.05	0.15	0.23	0.10
6.55	*ABGe097*	2.53	**0.006**	0.74	**0.03**	1.29	0.10	0.35	0.10
6.63	*TKY279*	2.54	**0.006**	0.74	**0.03**	1.31	0.09	0.36	0.3
7.95	*ABGe098*	2.33	**0.010**	0.57	**0.05**	0.74	0.2	0.10	0.3
8.15	*HTG003*	2.34	**0.010**	0.57	**0.05**	0.75	0.2	0.10	0.2
12.10	*AHT081*	2.72	**0.003**	0.87	**0.02**	0.91	0.2	0.15	0.2
14.38	*ABGe032*	2.69	**0.004**	0.99	**0.02**	0.65	0.3	0.10	0.4
17.60	*ABGe033*	2.41	**0.008**	0.90	**0.02**	0.24	0.4	0.02	0.4
20.68	*ABGe034*	2.30	**0.011**	0.90	**0.02**	0.25	0.4	0.02	0.4
23.12	*COR011*	2.12	**0.02**	0.79	**0.03**	0.23	0.4	0.02	0.4
24.26	*ABGe035*	2.03	**0.02**	0.72	**0.03**	0.21	0.4	0.01	0.6
24.88	*ABGe036*	1.09	0.14	0.21	0.2	-0.25	0.6	-0.02	0.7
25.31	*ABGe037*	0.81	0.2	0.12	0.2	-0.60	0.7	-0.10	0.8
25.59	*ABGe038*	0.32	0.4	0.02	0.4	-0.66	0.7	-0.12	0.8
25.93	*ABGe039*	0.26	0.4	0.01	0.4	-0.70	0.8	-0.14	0.9
26.53	*ABGe040*	0.42	0.3	0.03	0.4	-0.84	0.8	-0.24	0.9
26.67	*CADPS_SNP*	0.21	0.4	0.01	0.4	-0.87	0.8	-0.26	0.9
27.33	*ABGe041*	-0.05	0.5	-0.00	0.5	-0.99	0.8	-0.34	0.9
27.79	*ABGe042*	-0.08	0.5	-0.00	0.5	-1.06	0.9	-0.37	0.9
27.90	*ABGe043*	-0.14	0.6	-0.00	0.6	-1.08	0.9	-0.38	0.9
27.97	*HMS020*	-0.17	0.6	-0.01	0.6	-1.11	0.9	-0.40	0.9
28.44	*ABGe044*	-0.53	0.7	-0.06	0.7	-1.32	0.9	-0.49	0.9
29.46	*ABGe045*	-0.53	0.7	-0.06	0.7	-1.32	0.9	-0.49	0.9
30.27	*AHT038*	-0.49	0.7	-0.05	0.7	-0.50	0.7	-0.05	0.7
30.49	*ABGe046*	-0.54	0.7	-0.06	0.7	-1.32	0.9	-0.49	0.9
31.48	*ARHGEF3_SNP*	-0.56	0.7	-0.06	0.7	-1.32	0.9	-0.49	0.9
31.82	*ABGe047*	-0.57	0.7	-0.06	0.7	-1.32	0.9	-0.49	0.9
32.65	*WNT5A_SNP*	-0.71	0.8	-0.10	0.7	-1.32	0.9	-0.49	0.9
32.90	*ABGe048*	-0.76	0.8	-0.11	0.8	-1.32	0.9	-0.49	0.9
33.36	*BIEC2-355700*	-0.79	0.8	-0.12	0.8	-1.32	0.9	-0.49	0.9

Supplemental Table 5 continued

POS in Mb	Marker	OC-F				OCD-F			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
33.48	*TKY350*	-0.80	0.8	-0.12	0.8	-1.32	0.9	-0.49	0.9
33.65	*TKY871*	-0.74	0.8	-0.10	0.8	-1.32	0.9	-0.49	0.9
33.75	*LEX059*	-0.74	0.8	-0.10	0.8	-1.32	0.9	-0.49	0.9
34.06	*CHDH_SNP*	-0.69	0.8	-0.09	0.7	-1.32	0.9	-0.49	0.9
34.61	*ABGe049*	-0.61	0.7	-0.07	0.7	-1.32	0.9	-0.49	0.9
34.93	*ABGe050*	-0.56	0.7	-0.06	0.7	-1.32	0.9	-0.49	0.9
35.29	*TKY1069*	-0.50	0.7	-0.05	0.7	-1.32	0.9	-0.49	0.9
35.86	*ABGe051*	-0.50	0.7	-0.05	0.7	-1.32	0.9	-0.49	0.9
36.19	*DOCK3_SNP*	-0.50	0.7	-0.05	0.7	-1.32	0.9	-0.49	0.9
36.67	*ABGe052*	-0.50	0.7	-0.05	0.7	-1.32	0.9	-0.49	0.9
37.10	*ABGe053*	-0.50	0.7	-0.05	0.7	-1.32	0.9	-0.49	0.9
38.43	*ABGe054*	-0.56	0.7	-0.06	0.7	-1.32	0.9	-0.49	0.9
39.46	*SCAP_SNP*	-0.61	0.7	-0.07	0.7	-1.32	0.9	-0.50	0.9
39.93	*PTHR1_SNP*	0.03	0.5	0.00	0.5	-0.57	0.7	-0.07	0.7
39.97	*MYL3_SNP*	0.08	0.5	0.00	0.5	-0.50	0.7	-0.05	0.7
40.69	*ABGe055*	-0.33	0.6	-0.02	0.6	-0.95	0.8	-0.31	0.9
41.30	*ABGe056*	-0.66	0.7	-0.09	0.7	-1.32	0.9	-0.50	0.9
42.41	*ABGe057*	-0.67	0.7	-0.09	0.7	-1.33	0.9	-0.50	0.9
43.40	*ABGe058*	-0.10	0.5	-0.00	0.5	-1.35	0.9	-0.52	0.9
45.18	*ENTPD3_SNP*	-0.09	0.5	-0.00	0.6	-1.12	0.9	-0.47	0.9
47.46	*ITGA9_SNP*	-0.84	0.8	-0.17	0.8	-1.55	0.9	-0.68	1.0
48.33	*DCAMKL3_SNP*	-0.85	0.8	-0.17	0.8	-1.44	0.9	-0.63	1.0
49.04	*ARPP-21_SNP*	-0.86	0.8	-0.17	0.8	-1.20	0.9	-0.47	0.9
51.13	*CRTAP_SNP*	-0.44	0.7	-0.08	0.7	-0.92	0.8	-0.37	0.9
52.38	*OSBPL10_SNP*	-0.37	0.6	-0.06	0.7	-0.83	0.8	-0.28	0.9
54.20	*LEX048*	-0.14	0.6	-0.01	0.6	-0.48	0.7	-0.07	0.7
68.07	*UCDEQ505*	0.43	0.3	0.05	0.3	0.26	0.4	0.02	0.4
70.13	*LEX056*	-0.30	0.6	-0.02	0.6	-0.47	0.7	-0.05	0.7
70.27	*COR064*	0.26	0.4	0.02	0.4	0.23	0.4	0.01	0.4
70.39	*TKY532*	0.26	0.4	0.02	0.4	0.22	0.4	0.01	0.4
73.66	*TKY936*	-0.04	0.5	-0.00	0.5	-0.06	0.5	-0.00	0.5
74.99	*I-18*	0.73	0.2	0.10	0.3	-0.04	0.5	-0.00	0.5
75.41	*TKY406*	0.74	0.2	0.10	0.2	-0.05	0.5	-0.00	0.5
81.44	*AHT060*	0.47	0.3	0.05	0.3	1.11	0.13	0.25	0.14
81.72	*TKY341*	0.43	0.3	0.04	0.3	1.11	0.13	0.25	0.14
84.05	*AHT091*	0.42	0.3	0.05	0.3	1.01	0.2	0.25	0.14

Supplemental Table 6 Results of multipoint non-parametric linkage analysis for the traits OC in hock joints (OC-H) and OCD in hock joints (OCD-H) on ECA16

POS in Mb	Marker	OC-H				OCD-H			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
0.49	*ABGe092*	0.99	0.2	0.46	0.07	0.89	0.2	0.36	0.10
1.10	*ABGe093*	1.02	0.2	0.47	0.07	0.91	0.2	0.36	0.10
2.65	*ABGe094*	0.36	0.4	0.04	0.3	0.95	0.2	0.36	0.10
3.44	*ABGe095*	0.52	0.3	0.10	0.3	1.16	0.12	0.68	**0.04**
3.92	*AHT037*	0.62	0.3	0.14	0.2	1.29	0.10	0.80	**0.03**
5.02	*ABGe096*	0.54	0.3	0.10	0.2	1.44	0.08	0.82	**0.03**
6.55	*ABGe097*	0.98	0.2	0.20	0.2	1.94	**0.03**	0.74	**0.03**
6.63	*TKY279*	1.01	0.2	0.21	0.2	1.96	**0.03**	0.75	**0.03**
7.95	*ABGe098*	1.29	0.10	0.30	0.12	1.33	0.09	0.25	0.14
8.15	*HTG003*	1.31	0.10	0.31	0.12	1.33	0.09	0.25	0.14
12.10	*AHT081*	1.00	0.2	0.30	0.12	1.56	0.06	0.40	0.09
14.38	*ABGe032*	1.13	0.13	0.40	0.09	1.69	**0.05**	0.48	0.07
17.60	*ABGe033*	1.68	**0.05**	0.64	**0.04**	2.57	**0.005**	0.69	**0.04**
20.68	*ABGe034*	1.10	0.14	0.25	0.14	3.05	**0.0011**	1.70	**0.003**
23.12	*COR011*	0.90	0.2	0.18	0.2	2.73	**0.003**	1.60	**0.003**
24.26	*ABGe035*	0.80	0.2	0.15	0.2	2.59	**0.005**	1.54	**0.004**
24.88	*ABGe036*	0.79	0.2	0.15	0.2	2.59	**0.005**	1.54	**0.004**
25.31	*ABGe037*	0.78	0.2	0.14	0.2	2.59	**0.005**	1.54	**0.004**
25.59	*ABGe038*	0.89	0.2	0.20	0.2	2.45	**0.007**	1.48	**0.005**
25.93	*ABGe039*	1.23	0.11	0.50	0.06	2.29	**0.011**	1.39	**0.006**
26.53	*ABGe040*	1.46	0.07	0.50	0.06	1.70	**0.04**	0.70	**0.04**
26.67	*CADPS_SNP*	1.36	0.09	0.49	0.07	1.77	**0.04**	0.86	**0.02**
27.33	*ABGe041*	0.93	0.2	0.27	0.13	2.20	**0.014**	1.33	**0.007**
27.79	*ABGe042*	1.15	0.13	0.39	0.09	2.59	**0.005**	1.54	**0.004**
27.90	*ABGe043*	1.14	0.13	0.38	0.09	2.59	**0.005**	1.54	**0.004**
27.97	*HMS020*	1.12	0.13	0.36	0.10	2.59	**0.005**	1.54	**0.004**
28.44	*ABGe044*	0.88	0.2	0.17	0.2	2.59	**0.005**	1.54	**0.004**
29.46	*ABGe045*	0.89	0.2	0.17	0.2	2.59	**0.005**	1.54	**0.004**
30.27	*AHT038*	1.60	0.06	0.68	**0.04**	2.59	**0.005**	1.54	**0.004**
30.49	*ABGe046*	0.89	0.2	0.17	0.2	2.59	**0.005**	1.54	**0.004**
31.48	*ARHGEF3_SNP*	0.89	0.2	0.17	0.2	2.59	**0.005**	1.54	**0.004**
31.82	*ABGe047*	1.02	0.2	0.22	0.2	2.81	**0.003**	1.62	**0.003**
32.65	*WNT5A_SNP*	1.32	0.09	0.32	0.11	3.33	**0.0004**	1.77	**0.002**
32.90	*ABGe048*	1.42	0.08	0.35	0.10	3.49	**0.0002**	1.81	**0.002**
33.36	*BIEC2-355700*	1.89	**0.03**	0.79	**0.03**	3.49	**0.0002**	1.81	**0.002**

Supplemental Table 6 continued

POS in Mb	Marker	OC-H				OCD-H			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
33.48	*TKY350*	2.01	**0.02**	0.88	**0.02**	3.49	**0.0002**	1.81	**0.002**
33.65	*TKY871*	2.12	**0.02**	0.94	**0.02**	3.49	**0.0002**	1.81	**0.002**
33.75	*LEX059*	2.12	**0.02**	0.94	**0.02**	3.49	**0.0002**	1.81	**0.002**
34.06	*CHDH_SNP*	2.12	**0.02**	0.94	**0.02**	3.49	**0.0002**	1.81	**0.002**
34.61	*ABGe049*	2.12	**0.02**	0.94	**0.02**	3.49	**0.0002**	1.81	**0.002**
34.93	*ABGe050*	1.60	0.06	0.68	**0.04**	2.59	**0.005**	1.54	**0.004**
35.29	*TKY1069*	1.80	**0.04**	0.80	**0.03**	2.94	**0.002**	1.67	**0.003**
35.86	*ABGe051*	2.12	**0.02**	0.94	**0.02**	3.49	**0.0002**	1.81	**0.002**
36.19	*DOCK3_SNP*	2.12	**0.02**	0.94	**0.02**	3.49	**0.0002**	1.81	**0.002**
36.67	*ABGe052*	1.69	**0.05**	0.72	**0.03**	2.94	**0.002**	1.55	**0.004**
37.10	*ABGe053*	1.31	**0.10**	0.40	0.09	2.45	**0.007**	1.00	**0.02**
38.43	*ABGe054*	2.43	**0.008**	1.33	**0.007**	3.49	**0.0002**	1.81	**0.002**
39.46	*SCAP_SNP*	2.66	**0.004**	1.51	**0.004**	3.49	**0.0002**	1.81	**0.002**
39.93	*PTHR1_SNP*	2.77	**0.003**	1.57	**0.004**	3.49	**0.0002**	1.81	**0.002**
39.97	*MYL3_SNP*	2.78	**0.003**	1.57	**0.004**	3.49	**0.0002**	1.81	**0.002**
40.69	*ABGe055*	2.94	**0.002**	1.65	**0.003**	3.49	**0.0002**	1.81	**0.002**
41.30	*ABGe056*	2.94	**0.002**	1.65	**0.003**	3.49	**0.0002**	1.81	**0.002**
42.41	*ABGe057*	2.94	**0.002**	1.65	**0.003**	3.49	**0.0002**	1.81	**0.002**
43.40	*ABGe058*	1.83	**0.03**	0.80	**0.03**	3.12	**0.0009**	1.65	**0.003**
45.18	*ENTPD3_SNP*	1.19	0.12	0.45	0.07	1.94	**0.03**	0.74	**0.03**
47.46	*ITGA9_SNP*	1.19	0.12	0.38	0.09	1.46	0.07	0.36	0.10
48.33	*DCAMKL3_SNP*	1.21	0.11	0.34	0.11	1.31	0.09	0.25	0.14
49.04	*ARPP-21_SNP*	-0.46	0.7	-0.06	0.7	-0.19	0.6	-0.02	0.6
51.13	*CRTAP_SNP*	0.35	0.4	0.05	0.3	0.38	0.4	0.05	0.3
52.38	*OSBPL10_SNP*	0.60	0.3	0.11	0.2	0.29	0.4	0.03	0.3
54.20	*LEX048*	0.59	0.3	0.11	0.2	0.24	0.4	0.03	0.4
68.07	*UCDEQ505*	1.01	0.2	0.26	0.14	0.47	0.3	0.08	0.3
70.13	*LEX056*	1.19	0.12	0.39	0.09	0.23	0.4	0.02	0.4
70.27	*COR064*	1.21	0.11	0.40	0.09	0.21	0.4	0.02	0.4
70.39	*TKY532*	1.21	0.11	0.41	0.09	0.19	0.4	0.01	0.4
73.66	*TKY936*	1.57	0.06	0.84	**0.02**	-0.23	0.6	-0.01	0.6
74.99	*I-18*	1.57	0.06	0.84	**0.02**	-0.23	0.6	-0.01	0.6
75.41	*TKY406*	1.59	0.06	0.90	**0.02**	-0.20	0.6	-0.01	0.6
81.44	*AHT060*	0.39	0.4	0.05	0.3	0.07	0.5	0.00	0.5
81.72	*TKY341*	1.19	0.12	0.38	0.09	0.06	0.5	0.00	0.5
84.05	*AHT091*	0.41	0.3	0.04	0.3	0.06	0.5	0.00	0.5

CHAPTER 5

Identification of a new quantitative trait locus on equine chromosome 18 responsible for osteochondrosis in Hanoverian warmblood horses

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5 Identification of a new quantitative trait locus on equine chromosome 18 responsible for osteochondrosis in Hanoverian warmblood horses

5.1 Abstract

In this study we present a newly detected QTL associated with osteochondrosis in Hanoverian warmblood horses on equine chromosome 18 (ECA18). We developed a highly polymorphic and on ECA18 evenly distributed marker set employing the horse genome assembly EquCab2. The marker set included 11 newly developed microsatellites. Average polymorphism information content was 62.1 % at an average spacing of 3 Mb. For genotyping of this marker set comprising a total of 27 highly polymorphic microsatellites, we used the same 14 paternal half-sib families as in the previous whole genome scan. The chromosome-wide linkage analysis revealed a QTL for osteochondrosis in fetlock and/or hock joints as well as for osteochondrosis dissecans in hock joints between 74.94 and 82.25 Mb. Within this QTL for equine osteochondrosis, the parathyroid hormone 2 receptor gene could be identified as positional candidate gene. This report is a further step towards the identification of genes responsible for osteochondrosis in horses.

Key Words: ECA18, *Equus caballus,* horse, osteochondrosis, QTL

5.2 Introduction

Osteochondrosis (OC) is an inherited developmental orthopaedic disorder in young horses (Grøndahl and Dolvik, 1993; Philipsson et al., 1993; KWPN, 1994; Stock et al., 2005; Wittwer et al., 2006; Ytrehus et al., 2007). Abnormal chondrocyte differentiation and maturation causes altered enchondral ossification of the joints (Jeffcott and Henson, 1998). Articulations most commonly affected in horses are fetlock, hock and stifle joints. Osteochondrotic lesions can be identified as subchondral bone cysts, fissures, cartilage flaps and osteochondrosis dissecans (OCD) (Van Weeren, 2005). Whole genome scans in Hanoverian warmblood and

South German coldblood revealed QTL for osteochondrosis (Dierks et al., 2007; Wittwer et al., 2007). The chromosome-wide significant QTL in Hanoverian warmblood horses were located on ECA2, 3, 4, 5, 15, 16, 19 and 21. For South German coldblood horses, a genome-wide significant QTL was located at 45.9 cM on ECA18 for fetlock OCD. In addition, at 78.2 cM on ECA18 the microsatellite *TKY016* was chromosome-wide significantly linked with hock OC and palmar/plantar osteochondral fragments in the fetlock joints (POFs) of South German Coldblood horses (Wittwer et al., 2007). Reasons to a further investigation of ECA18 in Hanoverian warmblood horses were that this genomic region on ECA18 showed one of the highest test statistics among the OC-QTL detected in South German coldblood horses and markers in this region were chromosome-wide significant for several signs of OC. Furthermore, SNPs in the *XIRP2* gene at 46.8 Mb were significantly associated with fetlock OC, fetlock OCD and hock OC, suggesting that *XIRP2* may be involved in pathogenesis of osteochondrosis in South German Coldblood horses (Wittwer et al., 2009).

The objective of this study was to increase the marker density on ECA18 in order to verify whether QTL for osteochondrosis in Hanoverian warmblood horses could be detected employing a very dense and highly polymorphic marker set.

5.3 Material and Methods

Pedigree structure and phenotypic traits

From a large sample of Hanoverian warmblood horses including 629 radiographed foals, 168 stallions and more than 600 mares, 14 paternal half-sib families were chosen for genotyping due to their large family size and their high number of affected foals. The average size of paternal half-sib groups was 7.4, ranging from three to 20. In total, 211 horses were genotyped including 104 foals, 99 mares and eight stallions (Supplementary Table I). Diagnosis of osteochondrosis was done following the recording and evaluation scheme developed for warmblood horses (Kroll et al., 2001). The sagittal ridge of the 3rd metacarpal/metatarsal bone of fetlock joints, the intermediate ridge of the distal tibia, the lateral trochlea of the talus and the medial malleolus of the tibia were considered as predilection sites for OC. Signs consistent

with osteochondrosis were irregular bone trabeculation with variable radiolucency, irregular bone margin, new bone formation or osteochondral fragments when these changes were located at the predilection sites of fetlock and hock joints. Horses showing radiographic changes of osteochondrosis with or without osseous fragments at the predilection sites of the fetlock and/or hock joints were classified as affected by osteochondrosis (OC) and those horses exhibiting radiodense bodies as signs for osteochondral fragments at the above mentioned predilection sites were treated as affected by osteochondrosis dissecans (OCD). Horses with pathological changes in fetlock or hock joints other than osteochondrosis were not employed in our study. Animals without any signs of radiographic changes at all joints examined (fetlock, hock and stifle) were considered as free from OC, and only these horses were included as controls.

Identification of microsatellites

We supplemented the previously employed marker set for ECA18 with a total of 20 informative and evenly spaced microsatellites. From previously published equine linkage maps 9 microsatellites could be selected for improvement of the marker set for ECA18. In order to achieve a uniform coverage of ECA18, 11 microsatellites had to be newly developed (Supplementary Table II). For that purpose permutation sequences were built with variations of di-, tri- and tetra-repeat motifs with a minimum length of 15 repeats and a maximum length of 30 repeats. These sequences were aligned with the horse genome assembly EquCab2 (http://www.broad.mit.edu/ftp/pub/assemblies/mammals/horse/Equus2/) using the SSAHA2 package (Sequence Search and Alignment by Hashing Algorithm combined with the cross-match sequence alignment program developed by Phil Green at the University of Washington, version 1.0.1, The Wellcome Trust Sanger Institute, UK, 2007). Alignment results that obtained a maximum score per length (100% identity) were selected for primer design. For this purpose flanking sequences of these simple sequence repeats were extracted and investigated for their suitability for primer design. Equine PCR primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) after masking repetitive

elements using RepeatMasker (http://www.repeatmasker.org/). For verifying the marker positions on ECA16 on the horse genome assembly (EquCab2) the Basic Local Alignment Search Tool (BLASTall version 2.2.17) of NCBI was used.

Genotyping of microsatellites

For genotyping of microsatellites, 1.35 µg genomic DNA was isolated from 75 µl EDTA blood using the QIAamp® 96 Spin Blood Kit (Qiagen, Hilden, Germany). PCR reactions for genotyping of microsatellite markers were performed in 12-µl reaction volumes using 10 ng DNA, 1.2µl 10x incubation buffer containing 15 mM $MgCl_2$, 0.5 µl DMSO, 0.15 µl each dNTP (100 µM each) and 0.5 U Taq polymerase (Qbiogene, Heidelberg, Germany). The primer amount ranged from 3.0 pmol to 10.0 pmol. All forward primers were fluorescently labelled at the 5' end with IRD700 or IRD800. To increase efficiency all primers were pooled in PCR multiplex groups of two to six markers according to their allele size and the fluorescence labelling. PCR amplification was carried out in PTC 100™ or PTC 200™ thermocyclers (MJ Research, Watertown, MA, USA) using the following standard program with variable annealing temperature (Ta) between 55°C and 60°C: 94 °C for 4 min, followed by 36 cycles at 94 °C for 30 sec, optimum annealing temperature (Ta) for 1 min, 72 °C for 30 sec, and finally storing at 4 °C for 10 min. The PCR products were size-fractionated by gel electrophoresis on 6% polyacrylamide denaturing gels (Rotiphorese Gel40, Carl Roth, Karlsruhe) using an automated sequencer (LI-COR 4200/S-2 and 4300, Lincoln, NE, USA). Prior to loading, PCR products were diluted with formamide loading buffer in ratios of 1:10 according to empirical values. Allele sizes were detected using an IRD700- and IRD800-fluorescence-labelled DNA ladder, and the genotyping data was analyzed by visual examination. Mendelian inheritance and correctness of marker transmission in the pedigrees genotyped was confirmed using the Pedstats software (Wigginton and Abecasis, 2005).

Data analysis

Multipoint non-parametric linkage analysis (NPL) was performed using the Merlin software (multipoint engine for rapid likelihood inference, version 1.1.2) (Abecasis et

al., 2002). The Zmean and LOD score test statistics were used to test for the proportion of alleles shared by affected individuals identical-by-descent (IBD) for the considered marker loci (Whittemore and Halpern, 1994; Kruglyak et al., 1996; Kong and Cox, 1997). Error probabilities for chromosome-wide significant linkage were determined using a chromosome-wide permutation approach as described by Dierks et al. (2007). Linkage analyses were performed for the different phenotypic traits: (1) OC (fetlock and/or hock joints affected), (2) OCD (fetlock and/or hock joints affected, (3) fetlock OC, (4) fetlock OCD, (5) hock OC, (6) hock OCD.

In addition, the genotypic data was evaluated using the ALLELE and CASECONTROL procedures of the software package SAS/Genetics (Statistical Analysis System, Version 9.2, SAS Institute, Cary, NC, USA 2009) to determine the observed heterozygosity (HET), the polymorphism information content (PIC) and Hardy-Weinberg equilibrium and to evaluate genotypic and allelic associations, and the trend of the alleles with the phenotypic OC traits using χ^2-tests.

5.4 Results and discussion

The increase of the marker density from 7 microsatellites in the previous whole genome scan to a total of 27 equally and highly informative markers in Hanoverian warmblood horses made it possible to discover a QTL for osteochondrosis in fetlock and/or hock joints on ECA18. However, the position of this QTL did not coincide with the locations of the QTL in South German Coldblood horses for fetlock OCD at 45.9 – 54.0 cM (corresponds to 37.31 – 51.61 Mb on EquCab2), but mapped nearby the QTL for hock OC and POFs at 78.2 cM (corresponds to 66.84 Mb on EquCab2). The average polymorphism information content (PIC) of the microsatellites used in this study was 62.1% with a minimum of 39.6% and a maximum of 79.9%, while the mean observed heterozygosity (HET) was 68.3% ranging between 44.6% and 88.3%. The non-parametric multipoint linkage analysis showed chromosome-wide significant Zmeans and LOD scores in the region between 74.94 and 82.25 Mb for fetlock and/or hock OC (Figure 1). The highest Zmeans and LOD scores for OC in fetlock and/or hock joints were 2.43 and 0.83 at 75.25 Mb with chromosome-wide significant error probabilities of 0.008 and 0.03 (Table 1). The maximum (minimum)

achievable Zmeans and LOD scores were 42.43 (-2.28) and 10.17 (-0.03) for this trait and thus, high enough to achieve genome-wide significant test statistics for linkage. A further chromosome-wide significant linkage was found for OCD in hock joints with the highest Zmean and LOD score at 1.66 (P = 0.05) and 0.76 (P = 0.03) for the marker *UCD387* (Table 1). Zmeans and LOD scores for OC in hock joints were at the significance threshold for the markers in the region at 74.94 – 82.25 Mb. Zmean and LOD score were highest for *HLM003* with values of 1.68 (Zmean, P = 0.05) and 0.51 (LOD score, P = 0.06). In the region between 74.94 and 82.25 Mb, Zmeans and LOD scores for OCD in fetlock and/or hock joints were at 0.88 – 1.06 and 0.19 – 0.29 with chromosome-wide error probabilities of 0.12 – 0.2. Corresponding test statistics for OC in fetlock joints were at 0.68 – 1.04 (Zmeans) and 0.07 – 0.17 (LOD scores) with chromosome-wide error probabilities of 0.15 – 0.3. For OCD in fetlock joints, Zmeans and LOD scores reached values of 0.55 and 0.04 with chromosome-wide error probabilities of 0.3. Association tests using the CASECONTROL procedure of SAS/Genetics did not reveal any significant genotypic and/or allelic association with OC in fetlock and/or hock joints in the QTL region on ECA18.

This study presents an important step towards the identification of genes responsible for equine osteochondrosis and shows that a chromosome-wide linkage signal for the microsatellites *HLM003*, *UCD387*, *ABGe158* and *ABGe159* could be detected through a much more evenly and densely distributed marker set. In the previous whole genome scan the genomic region between 38.5 Mb and the distal end of ECA18 was covered only by two microsatellites and this fact was the reason why we did not detect this QTL in our previous study in Hanoverian horses (Dierks et al., 2007). The QTL at 45.9 – 54 cM in South German Coldblood horses was not seen as there was not an osteochondrosis associated QTL segregating in many Hanoverian warmblood families. Only one family of the present study segregated for the QTL at that position like in South German Coldblood horses, but this linkage disequilibrium in one family was not large enough for an overall chromosome-wide significant linkage in the whole data set. Thus, we can not completely preclude that in addition to the QTL at 74.94 – 82.25 Mb, a further QTL at 37.31 – 51.61 Mb is segregating in Hanoverian warmblood horses.

In order to develop a marker test for osteochondrosis in Hanoverian warmblood horses it is necessary to detect associated and single nucleotide polymorphisms (SNPs) in the QTL region. Thus, screening of this QTL region for potential candidate genes is a first step to prioritize a set of SNPs for association tests. The Equine Articular Cartilage cDNA Library is a useful tool to select candidate genes which are at least expressed in cartilage. At the moment a total of 13,964 equine articular ESTs (expressed sequence tag) can be found at the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez), from which ESTs located in 17 genes are in the QTL region between 74.94 and 82.25 Mb on ECA18. At 82.28 Mb, there is a gene which encodes the parathyroid hormone 2 receptor (*PTH2R*). Meulenbelt et al. (2006) performed linkage analysis in man using families with early-onset osteoarthritis and found significant linkage to a region which includes the *PTH2R* gene. This receptor is more selective in ligand recognition and has a more specific tissue distribution compared to parathyroid hormone receptor 1 (*PTHR1*). It is activated only by parathyroid hormone (PTH) and not by parathyroid hormone-like hormone (PTHLH). The function of PTH2R is not yet well characterized but as parathyroid hormone is a key regulator of calcium metabolism, this gene seems to be a functional candidate gene for osteochondrosis.

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Table 1 Multipoint non-parametric chromosome-wide test statistics (Zmean and LOD score) with their chromosome-wide error probabilities (P_Z, P_L) and their map positions (POS) in megabases (Mb) on the horse genome assembly EquCab2 for osteochondrosis (OC) in fetlock and/or hock joints and osteochondrosis dissecans (OCD) in hock joints in Hanoverian warmblood horses.

POS in Mb	Marker	OC			
		Zmean	P_Z	LOD	P_L
74.94	*HLM003*	2.17	0.015	0.61	0.05
75.25	*UCD387*	2.43	0.008	0.83	0.03
78.86	*ABGe158*	2.24	0.012	0.65	0.04
82.25	*ABGe159*	2.09	0.02	0.52	0.06
		OCD-hock			
74.94	*HLM003*	1.60	0.05	0.75	0.03
75.25	*UCD387*	1.66	0.05	0.76	0.03
78.86	*ABGe158*	1.45	0.07	0.51	0.06
82.25	*ABGe159*	1.38	0.08	0.48	0.07

Supplementary Table 1 Number of families analysed, their sizes and prevalences of osteochondrosis (OC), osteochondrosis dissecans (OCD), osteochondrosis in fetlock (OC-F) and hock (OC-H) joints and osteochondrosis dissecans in fetlock (OCD-F) and hock (OCD-H) joints by family and in total

Half-sib family	Number of progeny	Male	Female	Prevalences (%)					
				OC	OCD	OC -F	OCD-F	OC-H	OCD-H
1	4	1	3	100.0	100.0	50.0	50.0	75.5	75.0
2	9	4	5	44.4	33.3	11.1	0.0	33.3	33.3
3	7	5	2	71.4	42.9	71.4	42.9	28.6	14.3
4	15	9	6	53.3	33.3	26.7	6.7	26.7	26.7
5	8	1	7	100.0	25.0	75.0	12.5	37.5	12.5
6	4	2	2	100.0	25.0	50.0	25.0	50.0	0.0
7	5	3	2	100.0	40.0	80.0	40.0	20.0	0.0
8	6	2	4	100.0	66.7	83.3	50.0	66.7	33.3
9	20	12	8	75.0	60.0	50.0	20.0	50.0	45.0
10	8	4	4	62.5	25.0	50.0	12.5	12.5	12.5
11	5	3	2	40.0	20.0	20.0	0.0	20.0	20.0
12	5	2	3	100.0	80.0	100.0	80.0	0.0	0.0
13	5	3	2	40.0	40.0	40.0	40.0	0.0	0.0
14	3	1	2	100.0	66.7	100.0	66.7	0.0	0.0
Total	104	52	52	73.1	45.2	51.9	25.0	32.7	24.0

Supplementary Table 2 Characteristics of the microsatellites used in this study

Marker	Acc. No.	Mb	Ta (°C)	Forward / Reversed Primer Sequence (5'-3')	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
UCD136	U67401	0.54	58	F: CTTTGGGCCTTTCCTCCAT R: CGAGCCTGGGAGTGATAC	5	111-119	68.1	65.7
ABGe149	FM177587	1.48	58	F: GACACTTAAGCCAGGATTGG R: GTGCTGTGTTCTTTCCTATGC	7	114-126	44.6	41.4
ABGe150	FM177588	3.0	58	F: CAAGTTTGACTTTTTGTTTGTGTTG R: AAAGGTCACAAGTTTGTACAGTATGC	7	252-266	77.8	74.8
TKY019	AB048330	4.23	55	F: CTTCTGCTGATTCCTGAATG R: GGATCTCCTTAAATGGAACA	6	144-160	76.8	74.3
ABGe151	FM177589	10.78	58	F: CTCACTCTGGGCCCACTATC R: CGGAGTGAGAAGACAGTCCAG	8	194-212	74.7	68.8
LEX054	AF075656	16.95	55	F: TGCATGAGCCAATTCCTTAT R: TGGACAGATGACAGCAGTTC	7	164-180	71.6	63.9
ABGe152	FM177590	21.93	58	F: CCCTAGGTCCCCCACTTTAG R: CCATCCCTTCAGGAATACCAC	9	132-150	82.2	79.9
SG07	U90589	26.36	58	F: GAATTTGAATGTATCTATTCTGAATG R: GTGAGTTTTCAAGCTGGCATATTC	5	133-141	57.8	54.1
HMS46	U89814	26.46	58	F: GTCTCAGCCAAAAGGTATTCAAGC R: TGGCACAATATAGTTCACCTGG	5	122-134	71.4	62.9
ABGe153	FM177591	31.83	58	F: TTGGACACAAAAAGGTAGGC R: TTCCTTAGTTGGATATAGACACACAC	9	220-252	76.6	76.8
ABGe154	FM177592	35.51	58	F: TGTGGCTAATGCCCACTAC R: GCACCCAAGTACAAGACTGTG	6	144-160	54.0	47.8
COR096	AF154949	37.31	58	F: CCCCTCTTTTGCTTGAGAAT R: GCGTGTATGTGAGGATTGAAG	6	307-319	56.3	58.6
HTG028		38.64	58	F: AATCAACTAATATTAGGCCTCCT R: GAATACAGTTCTAGGGGCGT	5	156-160	49.3	42.1
ABGe155	FM177593	40.63	58	F: GGTCAGAAGACAGTCAAGAGTCC R: CCTCTCAGGCCTCTTACCAC	8	112-136	63.2	59.5
TKY545	AB103763	43.79	58	F: GCAGCTTCCCTCTGTCCAC R: TGACCTACGGCTTTGGTTTT	4	106-114	63.9	53.5
ABGe156	FM177594	48.41	58	F: TTAGTCATTGTCTCAAGACCTAAACAG R: ATTGTTAATCTTGGGCTAAGGATG	7	146-168	74.7	70.0
TKY741	AB103959	51.61	58	F: CCTTCCTTCTCCTAACTCAGTCC R: TGGAAACCAGGAATAGGTGTG	8	119-137	76.5	71.8
TKY322	AB034630	54.22	60	F: TGCAAACACTTGTGAACTGC R: AACCTAGTGTAATTGCTACC	7	115-137	70.0	62.4
HTG017		57.92	58	F: GCTATCCCTCCTGAGTCTTA R: AGGTAATTTGAAATAAAATACAC	4	155-161	67.6	54.2
TKY101		63.53	55	F: TCTGAAATACCGTGTGCCT R: TTCTGCCTCCCTCCAACTTT	8	197-217	82.2	74.2

Supplementary Table 2 continued

Marker	Acc. No.	Mb	Ta (°C)	Forward / Reversed Primer Sequence (5'-3')	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
TKY017	AB048328	66.81	58	F: CAACTGTATGTTGACAGCACA R: CGGCCATATTAGGTTTATCTG	4	120-130	55.9	47.7
TKY016	AB048327	66.84	60	F: GGTTATGGTTTGGTATCTGTC R: AAAACAATGGCTTCCTGGTCA	5	112-128	71.8	66.4
ABGe157	FM177595	70.99	58	F: GAGGGAGTCATTCCTGTACCC R: CCTCAGCCATGAATCTACCAG	10	166-184	88.3	77.2
HLM003	U36495	74.94	58	F: GAAGGTAGAAAAGGAGGGCTAGAAC R: TCTAGAGGACCATTCTCTGGGCTGTG	5	117-129	77.0	64.7
UCD387	U67404	75.25	58	F: ACCCCCGCCCCAGCAC R: TGCCCCGTCATTCTGC	6	76-86	64.8	50.7
ABGe158	FM177596	78.86	58	F: GCACCTGCCCTCAATACTTC R: CAGCGCAGTGAATCATTTTG	4	208-216	44.6	39.6
ABGe159	FM177597	82.25	58	F: TCGGCTCTTTTCTTCTATTTGC R: TCGGGCTCTGAATGAGAAAC	8	214-230	82.6	73.9

Mb: Location on the horse genome assembly EquCab2 in megabases
Ta: Annealing temperature in °C
HET: Observed heterozygosity
PIC: Polymorphism information content
Markers in bold indicate the microsatellites which were used in a previous whole genome scan for osteochondrosis in Hanoverian warmblood horses (Dierks et al., 2007).

CHAPTER 6

Mapping of a quantitative trait locus on equine chromosome 21 responsible for osteochondrosis in hock joints of Hanoverian warmblood horses

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6 Mapping of a quantitative trait locus on equine chromosome 21 responsible for osteochondrosis in hock joints of Hanoverian warmblood horses

6.1 Abstract

Objective: The aim of this study was to refine the map position of a quantitative trait locus (QTL) for osteochondrosis (OC) and osteochondrosis dissecans (OCD) in hock joints on equine chromosome (ECA) 21 using a very dense set of microsatellites.

Animals: Fourteen paternal half-sib families comprising 211 Hanoverian warmblood horses.

Procedures: A total of 22 highly polymorphic microsatellites were genotyped for 211 horses. Multipoint non-parametric linkage analysis was performed using an affected half-sib design. The Zmean and LOD score test statistics were used to test for the proportion of alleles shared by affected individuals identical by descent for the considered marker loci.

Results: The chromosome-wide linkage analysis revealed a QTL for osteochondrosis (OC and OCD) in hock joints between 5.45 and 17.14 Mb.

Conclusions and Clinical Relevance: This chromosome scan is an important step towards the identification of genes responsible for osteochondrosis in horses. The identification of contributing QTL is a strategy to elucidate the genetic mechanisms that underlie the disease. In this study the role of a potential candidate gene responsible for this pathologic alteration is also discussed.

Abbreviations

OC Osteochondrosis

OCD Osteochondrosis dissecans

QTL Quantitative trait loci

ECA Equine chromosome

HET Observed heterozygosity

PIC Polymorphism information content

PIK3R1 Phosphoinositide-3-kinase regulatory subunit 1

6.2 Introduction

Osteochondrosis is an orthopaedic disease which mainly affects young horses[1-3] and hereditary factors play a central role in its pathogenesis[4-8]. Cartilage flaps and joint mice or free joint bodies (chips) and joint effusions are the most often observed signs of OC. Osteochondral fragments like joint mice are a specific advanced stage of OC and are characteristic signs of OCD[9]. A whole genome scan in Hanoverian warmblood horses revealed a putative QTL for osteochondrosis on ECA21 and on further seven different horse chromosomes (ECA2, 3, 4, 5, 15, 16 and 19) QTL for osteochondrosis were found[10]. The traits analyzed were OC (fetlock and/or hock joints affected), OCD (fetlock and/or hock joints affected), fetlock OC, fetlock OCD, hock OC and hock OCD. QTL for fetlock OC and/or fetlock OCD were located on five different chromosomes (ECA2, 3, 4, 5, 16) and four chromosomes (ECA2, 4, 5, and 16) harboured QTL for hock OC or hock OCD as well. Only on three chromosomes there were QTL either significant for fetlock osteochondrosis (ECA3) or hock osteochondrosis (ECA15, 21). Chromosome-wide significant markers on ECA21 were located at 16.0 (*UMNe229*) and 24.5 cM (*HTG010*) for hock OC and at 0 (*SGCV16*) and 24.5 cM for hock OCD.

The aim of this study was to employ newly developed microsatellite markers in order to confirm and refine the QTL on ECA21, especially between 0 and 24.5 cM as this region was not well covered by markers and the chromosome-wide markers were at the significance threshold for significant test statistics. In this study the markers with relative marker positions in cM were relocated based on the horse genome assembly EquCab2.

6.3 Materials and Methods

From a large sample of Hanoverian warmblood horses, 14 paternal half-sib families were chosen and 211 horses were genotyped (Table 1). A more detailed description for these families can be found elsewhere[10].

In the previous whole genome scan, horse chromosome 21 was covered by seven microsatellites. For the present study horse linkage maps were screened for suitable markers. In addition to previously published microsatellites, microsatellites had to be newly developed in order to achieve a uniform coverage of ECA21 (Table 2). For that purpose the sequences were built with variations of di-, tri- and tetra-repeat motifs, with a minimum length of 15 repeats and a maximum length of 30 repeats, and were aligned with the horse genome assembly EquCab2[11] using the SSAHA2 package[a]. Alignment results that obtained a maximum score per length (100% identity) were selected for primer design. Equine PCR primers were designed using the Primer3 program[b] after masking repetitive elements with the RepeatMasker program[c]. A total of twelve polymorphic microsatellites (*ABGe160*-*ABGe170*, *ABGe261*) was newly developed and four polymorphic microsatellites (*TKY806*, *LEX060*, *TKY296*, *TKY623*) were selected from horse linkage maps to supplement the marker set. The final marker set for ECA21 consisted of 22 microsatellites because only six of the seven previously genotyped markers on ECA21 were employed in the present study. The marker *UMNe229* from the previous scan was discarded due to a low PIC.

For genotyping of microsatellites, 1.35 µg genomic DNA was isolated from 75 µl EDTA blood using the QIAamp® 96 Spin Blood Kit[d]. PCR reactions for genotyping of microsatellite markers were performed in 12-µl reaction volumes using 10 ng DNA, 1.2 µl 10x incubation buffer containing 15 mM $MgCl_2$, 0.5 µl DMSO, 0.15 µl each dNTP (100 µM each) and 0.5 U *Taq* polymerase[e]. The primer amount ranged from 2.0 pmol to 10.0 pmol. PCR amplification was carried out with variable annealing temperature (Ta) between 50°C and 62°C with the following standard program: 94°C for 4 min, followed by 36 cycles at 94°C for 30 sec, optimum Ta for 1 min, 72°C for 30 sec, and finally storing at 4°C for 10 min. The analysis of PCR products was performed as previously described by Dierks[10]. Mendelian inheritance and correctness of marker transmission in the pedigrees genotyped was confirmed using the Pedstats software[12]. Multipoint non-parametric linkage analysis (NPL) was performed using the Merlin software[13, f]. The Zmean and LOD score test statistics were used to test for the proportion of alleles shared by affected individuals identical by descent (IBD) for the considered marker loci[14-16]. Tests for chromosome-wide

linkage were performed using a permutation approach as described by Dierks et al.[10].

6.4 Results

The position of the chromosome-wide significant markers *SGCV16*, *UMNe229* and *HTG010* in the previously performed whole genome scan was at 1.93 Mb, 11.54 Mb and 17.14 Mb on EquCab2. The increase of the marker density to a total of 22 microsatellites on ECA21, with an average spacing of 2.45 Mb, made it possible to refine the QTL for osteochondrosis of hock joints in Hanoverian warmblood horses. In the region of ECA21 from 0 to 22.00 Mb the average distance among markers was 1.25 Mb. The average PIC of the microsatellites used in this study was 65.0% with a minimum of 40.7% and a maximum of 82.4%, while the mean HET was 72.3% ranging between 47.4% and 94.7% (Table 2).

The non-parametric multipoint linkage analysis showed chromosome-wide significant Zmeans and LOD scores in the region between 5.45 and 17.14 Mb on ECA21 for the traits hock OC and hock OCD (Table 3). The highest Zmeans and LOD scores reached the marker *ABGe162* with values of 2.44 and 0.80 for hock OC and corresponding chromosome-wide significant error probabilities of 0.007 and 0.03, and Zmeans and LOD scores of 2.45 and 1.17 for hock OCD with chromosome-wide significant error probabilities of 0.007 and 0.01 at 5.45 Mb (Table 3). The four microsatellites proximally to *ABGe162* were not significant due to recombination events. Thus the dense spacing of markers allowed the delineation of this QTL at 5.45-17.14 Mb on ECA21.

6.5 Discussion

This study presents an important step towards the identification of genes responsible for equine osteochondrosis in hock joints in the QTL region on ECA21. Due to their high content of polymorphisms microsatellites are well suited for delimiting QTL regions. Delineation of QTL is an important step to get a clear picture from the size of a QTL and based on this knowledge the QTL region can be searched for positional candidate genes. Markers useful for a genetic test for OC may be primarily

developed in these candidate genes. The Equine Articular Cartilage cDNA Library may be helpful to select candidate genes for QTL regions. About 13,964 equine articular ESTs (expressed sequence tag) can be found at the NCBI nucleotide database[g], from which 17 ESTs representing 12 different genes are located in the QTL region between 4.94 and 18.0 Mb on ECA21. Furthermore, single nucleotide polymorphisms (SNPs) can be searched in public databases to identify SNPs in candidate genes within candidate genes of this QTL region.

In the QTL region at 5.67 Mb there is a gene which encodes for the phosphoinositide-3-kinase regulatory subunit 1. *PIK3R1* is a candidate gene for osteoporosis[17] and involved in osteoblast differentiation[18] and in the osteoblastic responses to stress[19]. Previous studies demonstrated an involvement of *PIK3R1* on molecular mechanisms of bone repair[20] and emerging evidence supports the p85alpha regulatory subunit gene, *PIK3R1*, as a novel oncogene[21]. Therefore, this gene seems to be a suitable functional and positional candidate for hock osteochondrosis in Hanoverian warmblood horses. However, further sequence and SNP detection analyses are necessary to identify informative SNPs in this candidate gene and then to verify a possible association with equine osteochondrosis.

Footnotes

a. Sequence Search and Alignment by Hashing Algorithm combined with the cross-match sequence alignment program developed by Phil Green at the University of Washington, version 1.0.1, The Wellcome Trust Sanger Institute, UK, 2007
b. Web site: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
c. Web site: http://www.repeatmasker.org/
d. Qiagen, Hilden, Germany
e. Qbiogene, Heidelberg, Germany
f. Multipoint Engine for Rapid Likelihood Inference, version 1.1.2
g. Web site: http://www.ncbi.nlm.nih.gov/sites/entrez

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Table 1 Number of families analysed, their sizes and prevalences of osteochondrosis (OC), osteochondrosis dissecans (OCD), osteochondrosis in fetlock (OC-F) and hock (OC-H) joints and osteochondrosis dissecans in fetlock (OCD-F) and hock (OCD-H) joints by family and in total.

Half-sib family	Number of progeny	Male	Female	Prevalences in % for					
				OC	OCD	OC-F	OCD-F	OC-H	OCD-H
1	4	1	3	100.0	100.0	50.0	50.0	75.5	75.0
2	9	4	5	44.4	33.3	11.1	0.0	33.3	33.3
3	7	5	2	71.4	42.9	71.4	42.9	28.6	14.3
4	15	9	6	53.3	33.3	26.7	6.7	26.7	26.7
5	8	1	7	100.0	25.0	75.0	12.5	37.5	12.5
6	4	2	2	100.0	25.0	50.0	25.0	50.0	0.0
7	5	3	2	100.0	40.0	80.0	40.0	20.0	0.0
8	6	2	4	100.0	66.7	83.3	50.0	66.7	33.3
9	20	12	8	75.0	60.0	50.0	20.0	50.0	45.0
10	8	4	4	62.5	25.0	50.0	12.5	12.5	12.5
11	5	3	2	40.0	20.0	20.0	0.0	20.0	20.0
12	5	2	3	100.0	80.0	100.0	80.0	0.0	0.0
13	5	3	2	40.0	40.0	40.0	40.0	0.0	0.0
14	3	1	2	100.0	66.7	100.0	66.7	0.0	0.0
Total	104	52	52	73.1	45.2	51.9	25.0	32.7	24.0

Table 2 Characteristics and map positions on ECA21of the microsatellites used in this study.

Marker	Accession Number	cM*	Mb[ǂ]	Ta[§] (°C)	Forward/Reversed Primer Sequence (5'-3')	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
SGCV16	U90594	0.0	1.93	55	F: AATTCTCAAATGGTTCAGTGA R: CTCCCTCCCTTCCTTCTA	6	146-188	69.0	66.0
ABGe160	FM177704		2.99	62	F: AGACCCAAAGGGAAGCTAGG R: GGGTTGGCTTACAACTCTGC	6	118-130	76.0	75.9
ABGe161	FM177705		3.30	62	F: TGCTTGCTGGAATTCAGTTTC R: GCTGATCACAGAACCCTACCC	10	106-134	57.5	56.6
ABGe261	FM179765		4.94	58	F: TTGGCAAAATGTTGGATAAATG R: GAATACAGGGGCTTTTTCTGC	7	340-360	88.7	63.2
ABGe162	FM177706		5.45	50	F: ATTGCATACCTGTCCCTCAG R: AAAAAGGAAATGGAAAAGGAAG	6	251-271	69.0	62.0
ABGe163	FM177707		5.72	60	F: TTGGGGACTGAAATCGAAAC R: AAGTGTGGTGAAGCCATCAAG	10	208-234	85.3	76.6
ABGe164	FM177708		5.96	58	F: AATGTCCCATGGCTTTCAAC R: CTCTGGTTCAGCTCCCTCTG	7	208-228	61.7	57.3
TKY806	AB104024	3.0	6.99	58	F: TGGAACTGTGATGATGTTGC R: TCTTTCTTCCCTTCCGAGAG	9	159-181	81.2	77.5
ABGe165	FM177709		10.12	58	F: TTATGTCAGTCAGCACAGAAACAC R: TGATATCAGGCAGAATGAGTGG	9	88-114	77.0	77.5
ABGe166	FM177710		11.06	62	F: CCTCCAGGCAGATGATGAAC R: TGAAGCAAGAGCCTCAAAGAG	7	156-178	94.7	62.7
ABGe167	FM177711		15.11	62	F: CCAAAATAATCAACCAGTTTAAAAG R: TGTTTGTTTATGCGATATCAGTG	5	135-143	62.4	55.5
HTG010	AF169294	24.5	17.14	55	F: CAATTCCCGCCCCACCCCCGGCA R: TTTTTATTCTGATCTGTCACATTT	10	93-113	85.6	82.4
ABGe168	FM177712		18.00	50	F: CTCCGCCTTTTCATTGTTG R: CCAAAACAACCCCACTCTTC	7	261-275	76.7	70.9
ABGe169	FM177713		18.66	58	F: GGTGCAACTCCCATTTGTTC R: GCCCTTCATACACCATCTCC	6	208-220	60.5	53.8
COR073	AF142610	27.7	20.25	58	F: GCCAAGACATGGAAACAATC R: GTTCTCAAGGTGCATCCCTA	7	180-198	79.0	77.5
LEX060	AF075667	27.7	20.30	58	F: TTGCAGAAGGAGCCAATC R: AAGGCATTCGGAAATCTAAAT	6	143-159	80.1	77.1
COR068	AF142605	27.7	22.00	60	F: AACCAATTGTGAGATTTTTGCT R: GGCTAGTCCTGGATCATGTG	6	146-156	75.2	71.9
LEX031	AF075633	40.0	36.13	58	F: CCCATTAAGAACTTTTCATCCTG R: GGCAAGCCCCACAAAATTAT	4	252-258	59.4	41.3

Table 2 continued

Marker	Accession Number	cM*	Mb‡	Ta§ (°C)	Forward/Reversed Primer Sequence (5'-3')	Alleles (n)	Allele size (bp)	HET (%)	PIC (%)
ABGe170	FM177714		39.14	58	F: TTCCTTGCTCCTCTTCATGC R: CAACCTCATTGCCATCCTTC	3	245-249	47.4	40.7
TKY296	AB034605	52.0	44.53	55	F: CTCTCACTTCCAAGACACTC R: ATCAAACGTACAGGAAGAGC	11	169-191	81.6	78.6
LEX037	AF075639	47.0	47.82	55	F: GGATTCCTCAACCTCCTAAA R: AGGGATAAGTGACCACCAC	3	193-199	51.3	41.0
TKY623	AB103841	75.0	53.46	58	F: CAGTGTGGGTGGGCTTTATC R: ACCACTAGGGTGTGCATGTG	7	257-275	72.5	65.8

* The map positions in cM were taken from the linkage map of Swinburne et al.[22]. Some microsatellites were not mapped on this linkage map, for these markers the linkage map of Penedo et al. [23] was used.
‡ Location on the horse genome assembly 2.0 in megabases
§ Annealing temperature in °C

Table 3 Multipoint non-parametric chromosome-wide test statistics (Zmean and LOD score) with their chromosome-wide significant error probabilities (P_Z, P_L) and their map positions on ECA21 of the horse genome assembly 2.0 for hock OC (OC-H) and hock OCD (OCD-H) in Hanoverian warmblood horses.

Position in Mb	Marker	OC-H				OCD-H			
		Zmean	P_Z	LOD	P_L	Zmean	P_Z	LOD	P_L
5.45	*ABGe162*	2.44	0.007	0.80	0.03	2.45	0.007	1.17	0.010
5.72	*ABGe163*	1.75	0.04	0.49	0.07	1.76	0.04	0.39	0.09
5.96	*ABGe164*	1.75	0.04	0.49	0.07	1.76	0.04	0.39	0.09
6.99	*TKY806*	1.75	0.04	0.49	0.07	1.76	0.04	0.39	0.09
10.12	*ABGe165*	1.75	0.04	0.49	0.07	1.76	0.04	0.39	0.09
11.06	*ABGe166*	1.75	0.04	0.49	0.07	1.75	0.04	0.39	0.09
15.11	*ABGe167*	1.76	0.04	0.50	0.07	1.76	0.04	0.39	0.09
17.14	*HTG010*	1.76	0.04	0.49	0.07	1.76	0.04	0.39	0.09

CHAPTER 7

Confirmation of quantitative trait loci for osteochondrosis in Hanoverian warmblood horses through the performance of a whole genome single nucleotide polymorphism assay

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7 Confirmation of quantitative trait loci for osteochondrosis in Hanoverian warmblood horses through the performance of a whole genome single nucleotide polymorphism assay

7.1 Abstract

Osteochondrosis is an inherited developmental disease in young horses most frequently observed in thoroughbreds, trotters, warmblood and coldblood horses. Quantitative trait loci (QTL) for equine osteochondrosis have been identified in Hanoverian warmblood horses employing a whole genome scan and chromosome scans using dense microsatellite marker sets. The aim of this study was to confirm and refine QTL on ECA5, 16, 18 and 21 employing the Illumina Equine SNP50 Beadchip. Using haplotype association and variance analyses, the QTL for fetlock OCD could be confirmed and delimited to intervals at 76.79 - 81.10 Mb on ECA5. On ECA16, the QTL for hock OCD was refined to an interval at 17.57 - 20.1 Mb. A further QTL for hock OCD was confirmed at 9.37 - 16.18 Mb on ECA21. On ECA18, significant SNP effects were found for OC in fetlock and/or hock joints in the interval from 79.36 to 80.84 Mb. Multiple analyses of variance using the SNP genotypes as explanatory variables revealed an explained phenotypic variance of 14.91% for fetlock OCD on ECA5. For hock OCD, the proportion of phenotypic variance explained by the SNP genotypes on ECA16 was 17.60%, on ECA21 18.29%. The SNP genotypes from ECA16 and ECA21 together explained 29.37% of the phenotypic variance. On ECA18, phenotypic variance explained by the SNP genotypes was 14.59% for OC in fetlock and/or hock joints. This report is a further step towards unravelling the genes underlying QTL for equine osteochondrosis and towards the development of a marker test for osteochondrosis in Hanoverian warmblood horses.

7.2 Introduction

Osteochondrosis (OC) is a developmental orthopaedic disorder which is commonly diagnosed in young horses (Arnan and Hertsch 2005; Stock et al. 2005a, Wittwer et

al. 2006). Fetlock, hock and stifle joints are mainly affected. Although many factors including genetics, nutrition, hormonal disturbances, trauma and ischemia are supposed to play roles in the OC development, the disease mechanisms are not fully understood. A disturbance in the process of enchondral ossification of growing cartilage of the growth plates (Van de Lest et al. 1999) leads to the signs of OC including subchondral bone cysts, wear lines, cartilage flaps, osseous fragments and synovitis (Jeffcott and Henson, 1998; Trotter and McIlwraith, 1981). Osteochondrosis dissecans (OCD) is an advanced stage of OC which is characterized by the presence of osteochondral fragments (joint mice, chips, corpora libera). Available epidemiological data indicate that OC is present in warmblood, coldblood, thoroughbred and trotter horse populations between 10 and 25% across a range of different breeds (Grøndahl and Dolvik 1993; KWPN 1994; Philipsson et al. 1993; Stock et al. 2005a; Wittwer et al. 2006).

Whole genome scans in Hanoverian warmblood and South German coldblood revealed quantitative trait loci (QTL) for OC (Dierks et al. 2007; Wittwer et al. 2007). The traits analyzed were OC (fetlock and/or hock joints affected), OCD (fetlock and/or hock joints affected), fetlock OC, fetlock OCD, hock OC and hock OCD. QTL for the different traits in Hanoverian warmblood horses were located on ECA2, 3, 4, 5, 15, 16, 18, 19 and 21 (Dierks et al. 2007, Lampe et al. 2009a). QTL on ECA2, 4, 5, 16, 18 and 21 were refined and narrowed down using newly developed microsatellites with marker distances < 1Mb. On ECA5 the QTL for fetlock OCD could be delimited to an interval at 76.69 to 92.77 Mb (Lampe et al. 2009b). On ECA16, QTL for hock OCD were at 3.44 to 6.63 Mb and at 17.60 to 45.18 Mb. A QTL for fetlock OC could be refined to an interval at 6.55 to 24.26 Mb on ECA16 (Lampe et al. 2009c). Significant effects for the trait OC in fetlock and/or hock joints on ECA18 were detected from 74.94 to 82.25 Mb Lampe et al. 2009a). On ECA21, the QTL could be narrowed down to an interval at 5.45 to 17.14 Mb (Felicetti et al. 2009).

The objective of this study was a subsequent association analysis of single nucleotide polymorphisms (SNPs) in the different QTL regions using the Equine SNP50 BeadChip (Illumina, San Diego, CA, USA) in order to validate the results of the linkage analysis.

7.3 Material and Methods

Pedigree structure and phenotypic traits

From a large sample of Hanoverian warmblood horses, 154 foals were chosen descending from 52 different stallions. Ninety animals were affected by osteochondrosis in fetlock and/or hock joints and 64 were free from signs of OC in each limb joint. Out of these 154 foals, 39 horses were also included in all the previous linkage analyses (Dierks et al. 2007, Lampe et al. 2009a, 2009b, 2009c, Felicetti et al. 2009). Diagnosis of osteochondrosis was done following the recording and evaluation scheme developed for warmblood horses (Kroll et al. 2001). The sagittal ridge of the 3rd metacarpal/metatarsal bone of fetlock joints, the intermediate ridge of the distal tibia, the lateral trochlea of the talus and the medial malleolus of the tibia were considered as predilection sites for OC. Signs consistent with osteochondrosis were irregular bone trabeculation with variable radiolucency, irregular bone margin, new bone formation or osseous fragments when these changes were located at these predilection sites. Horses showing radiographic changes of osteochondrosis with or without osseous fragments at the predilection sites of the fetlock and/or hock joints were classified as affected by osteochondrosis (OC) and those horses exhibiting radiodense bodies as signs for osteochondral fragments at the above mentioned predilection sites were treated as affected by osteochondrosis dissecans (OCD). Horses with pathological changes in fetlock or hock joints other than osteochondrosis were not employed in our study. Animals without any signs of radiographic changes at all joints examined (fetlock, hock and stifle) were considered as free from OC, and only these horses were included as controls.

Genotyping of SNPs

Genomic DNA was extracted from EDTA blood samples of 154 Hanoverian warmblood horses through a standard ethanol fractionation with concentrated sodiumchloride (6M NaCl) and sodium dodecyl sulphate (10% SDS). Concentration of extracted DNA was determined using the Nanodrop ND-1000 (Peqlab

Biotechnology, Erlangen, Germany). DNA concentration of samples was adjusted between 30 and 80 ng/µl.

Genotyping was performed using the Illumina Equine SNP50 BeadChip containing 57165 SNP markers using standard procedures as recommended by the manufacturer. Raw data were analyzed using the genotyping module version 3.2.32 of the BeadStudio program (Illumina). In order to assign the genotypes a cluster file was generated using the BeadStudio software and the genotyping module version 3.2.32. The clusters were validated for five parameters consecutively for the purpose of identifying unreliable assays. In order to ensure correct genotypings three samples were replicated.

Data analysis

A case-control association analysis based on χ^2-tests for genotypes, alleles and trend of the alleles was performed using the CASECONTROL procedure of SAS/Genetics (Statistical Analysis System, version 9.2, SAS Institute Inc., Cary, NC, USA, 2009). The ALLELE procedure of SAS/Genetics was used for estimation of allele frequencies and tests for Hardy-Weinberg equilibrium of genotype frequencies. Statistical calculation of pairwise LD was performed and pictured using HAPLOVIEW 4.0 (http://www.broad.mit.edu/mpg/haploview/) (Barrett et al. 2005). We used the Tagger algorithm $r^2 \geq 0.8$ (de Bakker et al. 2005) to detect SNPs with strong LD among alleles. Subsequently, the association of haplotypes with OC was tested using the HAPLOTYPE procedure and the proportion of explained phenotypic variance of the trait was estimated by a multiple analyses of variance using the procedure GLM of SAS. Different analyses were performed for the same phenotypic traits as in the whole genome scan.

In the analysis only these SNPs were regarded which are located in the QTL regions or nearby.

7.4 Results

Hardy-Weinberg equilibrium and minor allele frequencies

For subsequent association analysis on ECA5, 345 SNPs were considered from which 326 SNPs are located in the QTL. Out of these SNPs, 24 were not in Hardy-Weinberg equilibrium (HWE). On ECA16, 80 SNPs were genotyped with 58 SNPs being located in the proximal QTL region for hock OCD, ten SNPs of them were not in Hardy-Weinberg equilibrium. For the distal QTL region significant for hock OCD, 599 SNPs were genotyped with 567 SNPs in the QTL and 31 SNPs not being in HWE. Additional 378 SNPs were considered from which 355 SNPs are located in the QTL for fetlock OC. 26 SNPs of them were not in HWE. On ECA18, 186 SNPs were considered from which 153 SNPs are located in the QTL region for OC. The genotypic distribution of 168 SNPs were in Hardy-Weinberg equilibrium and only 18 SNP markers were not in Hardy-Weinberg equilibrium. Furthermore, 242 SNPs were regarded for the QTL significant for hock OCD on ECA21 with 212 SNPs being located in the QTL and 9 SNPs not being in HWE.

The results of the tests for HWE, the observed heterozygosity (HET), polymorphism information content (PIC), and minor allele frequencies (MAF) for the SNPs which reached an error probability of <0.05 of χ^2-tests for the distribution of genotypes and/or alleles are shown in Table 1.

Association analysis

We detected five SNPs in the QTL region on ECA5 associated with fetlock OCD in χ^2-tests for the distribution of genotypes and alleles, additional eight SNPs reached significant results in allele or genotype test statistics.

On ECA16, we detected one SNP in the proximal QTL region associated with hock OCD in χ^2-tests for the distribution of genotypes and alleles, and one SNP with significant results in allele or genotype test statistics. In the distal QTL for hock OCD seven SNPs were significant in allele and genotype test statistics and 27 SNPs were significant in only one of the test statistics. In the QTL for fetlock OC on ECA16 there were two SNPs associated with fetlock OC in both test statistics, 21 SNPs reached significant results in allele or genotype test statistics.

We detected nine SNPs in the QTL region on ECA18 associated with osteochondrosis in χ^2-tests for the distribution of genotypes and alleles, additional 15 SNPs reached significant results in allele or genotype test statistics.
On ECA21, three SNPs were significant in both test statistics, additional nine SNPs were significantly associated with hock OCD in only one test statistic (Table 2).

Haplotype association and variance analysis
The genotypes significant in χ^2-test statistics were tested in haplotype and multiple analyses of variance using the HAPLOTYPE and GLM procedures of SAS.
On ECA5, the proportion of phenotypic variance for fetlock OCD explained by genotypes of the markers BIEC2-919955, BIEC2-922891 and BIEC2-922937 was 14.91% with an error probability of 0.018. The marker-trait association including these three SNPs was significant (χ^2=16.09; p=0.0133). In total, six different haplotypes of these markers had a frequency of at least 1%. These haplotypes spanned the region from 76.79 Mb (BIEC2-919955) to 81.10 Mb (BIEC2-922937). Three of these haplotypes (A-G-A, A-G-G, C-A-G) were significantly associated with the affection status and occurred with a frequency of 10.85%, 1.00% and 6.38% in our sample. The A-G-A haplotype was found with 15.2% in the sample of unaffected animals and with 0% in affected animals. The A-G-G haplotype could be clearly assigned to the affected animals (4.3%) because none of the unaffected horses showed this individual haplotype. The third associated haplotype (C-A-G) was present in 1.9% of the affected animals and occurred with 9.7% in the sample of controls (Table 3).
The proportion of phenotypic variance for hock OCD explained by genotypes of the markers BIEC2-330698, BIEC2-330714 and BIEC2-332027 on ECA16 was 17.6% with an error probability of 0.0073.These SNPs were located between 17.57 Mb and 20.18 Mb. The calculation of marker-trait association, including these three SNPs was also significant (χ^2= 17.41, p=0.0149). All haplotypes had a frequency of at least 1% and two of these haplotypes (A-C-G and G-C-A) which occurred with frequencies of 28.1% and 12.0%, were significantly associated with the affection status. The A-C-G haplotype was present in 20.8% of the controls and in 37.4% of the cases. The G-

C-A haplotype could be clearly assigned to the unaffected animals (16.5%) because none of the affected horses showed this individual haplotype (Table 4). For the proximal QTL for hock OCD as well as for the QTL for fetlock OC on ECA16 we were not able to identify any significantly associated haplotype.

The proportion of phenotypic variance for OC explained by genotypes of the markers BIEC2-421484, BIEC2-421493, BIEC2-421739 and BIEC2-421806 on ECA18 was 14.59% with an error probability of 0.0048.

The marker-trait association including these four SNPs was significant (χ^2=22.36; p=0.0337). In total, six different haplotypes of these markers had a frequency of at least 1%. One of these haplotypes (C-A-C-A) was significantly associated with the affection status and occurred with a frequency of 50.8% (62.3%) in our sample. Thereof 61.6% of the affected horses and 37.4% of the controls shared this haplotype spanning the region from 79.36 Mb to 80.84 Mb (Table 5).

On ECA21, the proportion of phenotypic variance for hock OCD explained by genotypes of the markers BIEC2-550500, BIEC2-551657and BIEC2-554071 was 18.3% with an error probability of 0.0046.These SNPs were located between 17.57 Mb and 20.18 Mb. The calculation of marker-trait association, including these three SNPs was also significant (χ^2= 24.93, p=0.0008). All haplotypes had a frequency of at least 1% and six of these haplotypes (A-A-A, A-G-A, A-G-C, G-A-A, G-G-A and G-G-C) which occurred with frequencies of 20.7%, 3.9%, 5.0%, 14.5%, 1.8% and 6.2% were significantly associated with the affection status. The A-A-A haplotype was present in 25.4% of the controls and in 6.2% of the cases. The A-G-A haplotype could be clearly assigned to the unaffected animals (8.0%) because none of the affected horses showed this individual haplotype. The A-G-C haplotype was present in 1.5% of the controls and in 10.6% of the affected animals, the G-A-A haplotype was found in 11.1% of the controls and in 23.1% of the affected animals. While the G-G-A haplotype was clearly assigned to the affected horses with 4.6%, the G-G-C haplotype was visible in 2.9% of the unaffected and in 9.8% of the affected animals (Table 6).

For hock OCD, a combination of the significantly associated haplotypes from ECA16 and ECA21 revealed an explained phenotypic variance of 29.37% (p=<0.0012).

7.5 Discussion

The aim of this study was to confirm and refine the already known QTL for different phenotypic traits for osteochondrosis in Hanoverian warmblood horses. The analysis of QTL regions on ECA5, 16, 18 and ECA21 using the Equine SNP50 BeadChip revealed a large number of significantly associated SNPs distributed over the QTL regions. Most of the SNPs which reached significant results in χ^2 test statistics for genotypic and/or allelic distribution were located in intergenic regions or in intronic sequences. Therefore, the detection of the causal mutation for OC could not be expected based on SNP analysis. Further statistical calculations were necessary to refine the QTL regions. Using multiple analyses of variance, we detected four SNPs on ECA5 for fetlock OCD, three SNPs on ECA16 for hock OCD, furthermore four SNPs on ECA18 for OC in fetlock and/or hock joints and three SNPs on ECA21 for hock OCD which explained best the proportion of phenotypic variance for the respective trait with values of 14.91%, 17.60%, 14.59% and 18.29%. These markers were further tested for marker-trait association and we could show haplotypes in all QTL, except the QTL for fetlock OC and the proximal QTL for hock OCD on ECA16, significantly associated with the different phenotypic traits of OC. The fact that the development of OC is influenced by many different genes may explain the relatively low proportion of phenotypic variance for the respective traits when regarding only one QTL.

Due to the haplotypes we were able to further decrease the QTL regions on ECA5 from 76.69-92.77 Mb to an interval spanning from 76.68 to 81.10 Mb. The QTL for hock OCD on ECA16 was former located between 17.60 and 45.18 Mb and could be delimited to an interval between 17.57 and 20.18 Mb. On ECA18, the QTL for OC in fetlock and/or hock joints could be reduced to a 1.5Mb-interval between 79.36 and 80.84 Mb and the QTL for hock OCD on ECA21 could be decreased to 9.37-16.18 Mb. These enormous delimitations of the QTL further limit the range of genes being located in the respective regions, and therefore the assortment of potential candidate genes (Table 7). Besides the already known QTL, we could identify further significant regions for the different phenotypic traits, which can possibly increase the explained phenotypic variance for the respective traits.

7.6 References

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Table 1 Observed heterozygosity (HET), polymorphism information content (PIC) Hardy-Weinberg equilibrium (HWE) and minor allele frequency (MAF) for the respective trait of SNPs which reached an error probability of <0.05 in $\chi 2$-tests for the distribution of genotypes and/or alleles in the QTL on ECA5, ECA16, ECA18 and ECA21.

SNP name	ECA-position on EquCab2 in bp	Location	HET	PIC	P (HWE)	MAF	
						cases	controls
BIEC2-920030	05-77076395	HS2ST1	47.4	36.6	0.8320	0.4595 [a]	0.3750 [a]
BIEC2-922934	05-81097874	intergenic	20.8	16.9	0.1502	0.1622 [a]	0.0703 [a]
BIEC2-922937	05-81099966	intergenic	21.4	17.3	0.1364	0.1757 [a]	0.0703 [a]
BIEC2-923002	05-81177328	intergenic	39.0	29.4	0.2772	0.3243 [a]	0.1875 [a]
BIEC2-923008	05-81189433	intergenic	50.0	35.5	0.2940	0.2838 [a]	0.4219 [a]
BIEC2-926382	05-86909932	intergenic	37.7	31.4	0.6546	0.1892 [a]	0.3281 [a]
BIEC2-926384	05-86909992	intergenic	37.7	31.4	0.6546	0.1892 [a]	0.3281 [a]
BIEC2-927105	05-88196782	intergenic	38.6	31.0	0.9335	0.3649 [a]	0.2266 [a]
BIEC2-929536	05-92328534	intergenic	45.0	34.7	0.9347	0.4054 [a]	0.2823 [a]
BIEC2-929562	05-92398909	intergenic	40.9	29.6	0.1038	0.3108 [a]	0.1953 [a]
BIEC2-929591	05-92415071	intergenic	29.2	23.8	0.4759	0.2568 [a]	0.1328 [a]
BIEC2-929595	05-92415275	intergenic	29.2	23.8	0.4759	0.2568 [a]	0.1328 [a]
BIEC2-929674	05-92466718	intergenic	46.8	36.9	0.6052	0.4865 [a]	0.3594 [a]
BIEC2-929787	05-92814419	RPE65	44.2	34.7	0.8736	0.2568 [a]	0.4063 [a]
BIEC2-328174	16-3753939	intergenic	50.0	37.5	0.9962	0.3971 [c]	0.4766 [c]
BIEC2-328894	16-6800733	IRAK2	11.0	9.9	0.4685	0.1176 [c]	0.0391 [c]
BIEC2-329542	16-10590554	intergenic	26.6	21.1	0.1793	0.0965 [b]	0.1875 [b]
BIEC2-329552	16-10603575	intergenic	26.1	20.9	0.1987	0.0893 [b]	0.1875 [b]
BIEC2-329553	16-10603830	intergenic	26.6	21.1	0.1793	0.0965 [b]	0.1875 [b]
BIEC2-329564	16-10705361	intergenic	33.1	30.1	0.2095	0.1842 [b]	0.2969 [b]

Table 1 continued

SNP name	ECA-position on EquCab2 in bp	Location	HET	PIC	P (HWE)	MAF	
						cases	controls
BIEC2-329757	16-11882163	intergenic	50.3	37.4	0.8932	0.4375 [b]	0.4766 [b]
BIEC2-329983	16-13367022	intergenic	48.7	34.6	0.2434	0.3070 [b]	0.3594 [b]
BIEC2-329992	16-13447232	intergenic	51.6	37.5	0.6796	0.4912 [b]	0.4921 [b]
BIEC2-330058	16-13933281	intergenic	50.0	37.5	0.9896	0.4123 [b]	0.5431 [b]
BIEC2-330359	16-15547790	intergenic	33.8	29.0	0.6314	0.2456 [b]	0.2344 [b]
BIEC2-330561	16-16984946	PDZRN3	45.5	37.5	0.2593	0.4912 [b]	0.4609 [b]
BIEC2-330572	16-16993987	PDZRN3	40.9	32.0	0.7688	0.2719 [b]	0.2578 [b]
BIEC2-330578	16-17001997	PDZRN3	37.0	30.1	0.9541	0.3529 [c]	0.2109 [c]
BIEC2-330594	16-17006523	PDZRN3	35.7	29.2	0.9336	0.3235 [c]	0.2188 [c]
BIEC2-330600	16-17017547	PDZRN3	32.5	26.5	0.6645	0.2941 [c]	0.1641 [c]
BIEC2-330615	16-17094667	PDZRN3	33.8	27.0	0.5342	0.2941 [c]	0.1719 [c]
BIEC2-330628	16-17098315	PDZRN3	33.8	27.0	0.5342	0.2941 [c]	0.1719 [c]
BIEC2-330677	16-17421511	PPP4R2	37.7	31.1	0.7981	0.3676 [c]	0.2344 [c]
BIEC2-330691	16-17546589	GLT8D4	33.8	28.0	0.9727	0.3529 [c]	0.1875 [c]
BIEC2-330698	16-17565806	intergenic	45.4	34.3	0.6791	0.4559 [c]	0.2937 [c]
BIEC2-330708	16-17670327	SHQ1	36.8	35.9	0.0083	0.4412 [c]	0.3438 [c]
BIEC2-330714	16-17698340	SHQ1	38.3	30.5	0.7882	0.2632 [b] 0.1618 [c]	0.2578 [b] 0.2578 [c]
BIEC2-330720	16-17754789	SHQ1	46.8	36.5	0.7278	0.4853 [c]	0.3516 [c]
BIEC2-330725	16-17763943	intergenic	45.5	34.0	0.5546	0.4412 [c]	0.2656 [c]
BIEC2-330739	16-17790340	intergenic	46.1	34.6	0.6581	0.4265 [c]	0.2813 [c]
BIEC2-330748	16-17885080	intergenic	35.3	29.1	0.9976	0.3529 [c]	0.1953 [c]
BIEC2-330760	16-18040578	intergenic	35.1	29.0	0.9834	0.3529 [c]	0.1953 [c]
BIEC2-331723	16-19782127	intergenic	37.5	27.5	0.0815	0.2411 [b]	0.1984 [b]

Table 1 continued

SNP name	ECA-position on EquCab2 in bp	Location	HET	PIC	P (HWE)	MAF	
						cases	controls
BIEC2-331775	16-19839423	intergenic	34.0	25.4	0.0912	0.2321 [b]	0.1641 [b]
BIEC2-332027	16-20179799	intergenic	32.0	27.9	0.5962	0.1061 [c]	0.2500 [c]
BIEC2-332303	16-20652633	FRMD4B	29.2	23.8	0.4759	0.2368 [b]	0.1250 [b]
BIEC2-332371	16-20755410	FRMD4B	29.9	22.9	0.0933	0.2105 [b]	0.1094 [b]
BIEC2-333011	16-21612414	intergenic	18.2	16.0	0.6726	0.0294 [c]	0.1094 [c]
BIEC2-333414	16-22005338	intergenic	31.8	27.3	0.7827	0.1930 [b] 0.1912 [c]	0.1953 [b] 0.1953 [c]
BIEC2-333671	16-22468598	intergenic	46.8	36.5	0.7278	0.4211 [b]	0.3750 [b]
BIEC2-334005	16-23311651	intergenic	16.9	16.2	0.7903	0.1471 [c]	0.0625 [c]
BIEC2-334347	16-23706842	intergenic	43.5	37.0	0.1594	0.4649 [b]	0.3906 [b]
BIEC2-334359	16-23709798	intergenic	43.5	37.0	0.1594	0.4649 [b]	0.3906 [b]
BIEC2-334540	16-23886963	intergenic	49.4	37.2	0.9932	0.4474 [b]	0.4531 [b]
BIEC2-334746	16-24120843	intergenic	42.9	36.4	0.1961	0.3158 [b]	0.4688 [b]
BIEC2-334795	16-24192718	intergenic	30.1	23.6	0.2176	0.2281 [b]	0.1250 [b]
BIEC2-335076	16-24760301	ADAMTS9	42.9	33.7	0.9883	0.3971 [c]	0.2891 [c]
BIEC2-335182	16-24827127	intergenic	43.5	33.0	0.5764	0.3824 [c]	0.2813 [c]
BIEC2-335655	16-25602224	intergenic	42.2	33.8	0.7864	0.3824 [c]	0.2656 [c]
BIEC2-336573	16-27097038	intergenic	22.7	21.1	0.5030	0.0735 [c]	0.1797 [c]
BIEC2-338439	16-30953707	intergenic	39.0	35.0	0.0893	0.2647 [c]	0.4141 [c]
BIEC2-338511	16-31029398	ASB14	37.0	32.7	0.2180	0.1765 [c]	0.3516 [c]
BIEC2-338512	16-31029554	ASB14	35.1	32.2	0.1098	0.1765 [c]	0.3359 [c]
BIEC2-338514	16-31031690	ASB14	35.1	32.2	0.1098	0.1765 [c]	0.3359 [c]
BIEC2-338575	16-31097426	intergenic	33.8	30.7	0.1832	0.1471 [c]	0.2969 [c]
BIEC2-338592	16-31115480	intergenic	27.3	25.9	0.1807	0.1029 [c]	0.2422 [c]
BIEC2-338862	16-31750953	intergenic	46.1	34.5	0.5419	0.3529 [c]	0.2813 [c]

Table 1 continued

SNP name	ECA-position on EquCab2 in bp	Location	HET	PIC	P (HWE)	MAF	
						cases	controls
BIEC2-341699	16-38714465	intergenic	24.7	23.5	0.2499	0.2206 [c]	0.1172 [c]
BIEC2-344527	16-43202177	intergenic	33.8	29.8	0.3515	0.2941 [c]	0.1719 [c]
BIEC2-346535	16-45983092	intergenic	40.3	32.8	0.7403	0.3235 [c]	0.2344 [c]
BIEC2-420030	18-075879418	intergenic	53.7	36.5	0.1564	0.4375 [d]	0.3525 [d]
BIEC2-420031	18-075879559	intergenic	52.7	36.4	0.2063	0.4302 [d]	0.3417 [d]
BIEC2-420826	18-077593952	ICA1L	38.3	30.9	0.9557	0.2611 [d]	0.2500 [d]
BIEC2-421484	18-079364656	PARD3B	26.7	25.2	0.2365	0.1379 [d]	0.2318 [d]
BIEC2-421493	18-079398715	PARD3B	31.2	27.5	0.5085	0.1667 [d]	0.2656 [d]
BIEC2-421715	18-080387076	intergenic	37.7	34.0	0.1015	0.3278 [d]	0.3047 [d]
BIEC2-421739	18-080534779	Intergenic	18.7	18.9	0.1548	0.0930 [d]	0.1563 [d]
BIEC2-421798	18-080793438	ADAM23	45.5	35.9	0.6916	0.4000 [d]	0.3438 [d]
BIEC2-421806	18-080836383	intergenic	27.3	24.7	0.4804	0.1389 [d]	0.2266 [d]
BIEC2-421818	18-080972147	FASTKD2	46.8	36.8	0.6417	0.4278 [d]	0.3984 [d]
BIEC2-421879	18-081340848	intergenic	43.5	35.1	0.6153	0.4000 [d]	0.2734 [d]
BIEC2-421929	18-081510682	intergenic	45.5	36.4	0.5359	0.4444 [d]	0.3281 [d]
BIEC2-421931	18-081581136	intergenic	46.8	37.4	0.4624	0.4889 [d]	0.3906 [d]
BIEC2-421944	18-081703071	intergenic	46.8	36.9	0.6052	0.4722 [d]	0.3516 [d]
BIEC2-421970	18-081775453	intergenic	50.0	37.5	0.9962	0.4722 [d]	0.4375 [d]
BIEC2-421975	18-081798786	intergenic	43.5	33.3	0.6908	0.2556 [d]	0.3672 [d]
BIEC2-421977	18-081817905	intergenic	50.7	37.2	0.7374	0.4889 [d]	0.3750 [d]
BIEC2-421989	18-081855169	intergenic	43.5	33.3	0.6908	0.2556 [d]	0.3672 [d]
BIEC2-422004	18-081986880	intergenic	43.5	33.3	0.6908	0.2556 [d]	0.3672 [d]
BIEC2-422014	18-082077699	intergenic	44.2	33.7	0.7181	0.2667 [d]	0.3750
BIEC2-422024	18-082195702	PIP5K3	44.2	33.7	0.7181	0.2667 [d]	0.3750 [d]

Table 1 continued

SNP name	ECA-position on EquCab2 in bp	Location	HET	PIC	P (HWE)	MAF cases	MAF controls
BIEC2-422025	18-082198807	PIP5K3	44.8	33.8	0.6341	0.2667 [d]	0.3750 [d]
BIEC2-422062	18-082495575	intergenic	46.1	36.8	0.5110	0.4778 [d]	0.3359 [d]
BIEC2-548383	21-5996157	intergenic	11.7	12.4	0.1401	0.0294 [c]	0.0625 [c]
BIEC2-548855	21-6623818	intergenic	49.7	36.8	0.7978	0.4853 [c]	0.3492 [c]
BIEC2-550182	21-8966664	intergenic	11.7	10.4	0.4411	0.1176 [c]	0.0313 [c]
BIEC2-550500	21-9371289	intergenic	57.1	37.2	0.0489	0.4118 [c]	0.3906 [c]
BIEC2-550542	21-9485226	intergenic	24.0	22.5	0.3782	0.0735 [c]	0.1719 [c]
BIEC2-551657	21-11006036	intergenic	25.3	22.5	0.7964	0.2500 [c]	0.1250 [c]
BIEC2-552154	21-11939082	intergenic	50.7	37.1	0.7063	0.3235 [c]	0.4688 [c]
BIEC2-553100	21-13131951	intergenic	32.5	26.5	0.6645	0.0882 [c]	0.1953 [c]
BIEC2-554033	21-15907810	intergenic	46.1	36.7	0.5458	0.2794 [c]	0.4219 [c]
BIEC2-554035	21-15923414	intergenic	46.1	36.7	0.5458	0.2794 [c]	0.4219 [c]
BIEC2-554052	21-16133211	intergenic	42.9	37.5	0.0763	0.3676 [c]	0.4766 [c]
BIEC2-554058	21-16145605	intergenic	44.8	37.5	0.2014	0.3529 [c]	0.5000 [c]
BIEC2-554066	21-16153112	intergenic	42.9	37.5	0.0763	0.3676 [c]	0.4766 [c]

[a] cases and controls refer to fetlock OCD
[b] cases and controls refer to fetlock OC
[c] cases and controls refer to hock OCD
[d] cases and controls refer to fetlock and /or hock OC

Table 2 SNPs with error probabilities <0.05 in χ2-tests for the distribution of genotypes and/or alleles on ECA5, 16, 18 and 21.

SNP name	Map info	χ2 allele	P allele	χ2 genotype	P genotype	χ2 trend	P trend
ECA5 fetlock OCD							
BIEC2-920030	05-77076395	1.39	0.239	5.84	0.054	1.42	0.233
BIEC2-922934	05-81097874	4.25	0.039	4.80	0.028	4.80	0.028
BIEC2-922937	05-81099966	5.36	0.021	6.11	0.013	6.11	0.013
BIEC2-923002	05-81177328	4.85	0.028	5.54	0.063	5.34	0.021
BIEC2-923008	05-81189433	3.83	0.050	6.97	0.031	4.17	0.041
BIEC2-926382	05-86909932	4.52	0.034	4.72	0.094	4.26	0.039
BIEC2-926384	05-86909992	4.52	0.034	4.72	0.094	4.26	0.039
BIEC2-927105	05-88196782	4.48	0.034	5.52	0.063	4.90	0.027
BIEC2-929536	05-92328534	3.19	0.074	6.61	0.037	3.14	0.076
BIEC2-929562	05-92398909	3.45	0.063	4.07	0.130	4.05	0.044
BIEC2-929591	05-92415071	4.92	0.027	5.88	0.053	5.36	0.021
BIEC2-929595	05-92415275	4.92	0.027	5.88	0.053	5.36	0.021
BIEC2-929674	05-92466718	4.59	0.032	4.71	0.095	4.67	0.031
BIEC2-929787	05-92814419	4.60	0.032	4.54	0.103	4.31	0.038
ECA16 fetlock OC							
BIEC2-329542	16-10590554	4.04	0.045	4.56	0.102	4.50	0.034
BIEC2-329552	16-10603575	4.74	0.029	5.28	0.071	5.24	0.022
BIEC2-329553	16-10603830	4.04	0.045	4.56	0.102	4.50	0.034
BIEC2-329564	16-10705361	4.15	0.042	4.01	0.135	4.01	0.045
BIEC2-329757	16-11882163	1.77	0.184	10.98	0.004	1.83	0.176

Table 2 continued

SNP name	Map info	χ2 allele	P allele	χ2 genotype	P genotype	χ2 trend	P trend
BIEC2-329983	16-13367022	0.74	0.389	7.21	0.027	0.76	0.384
BIEC2-329992	16-13447232	0.01	0.990	5.98	0.050	0.01	0.989
BIEC2-330058	16-13933281	4.37	0.036	7.80	0.020	4.01	0.045
BIEC2-330359	16-15547790	0.04	0.838	6.07	0.048	0.04	0.838
BIEC2-330561	16-16984946	0.22	0.638	6.89	0.032	0.21	0.647
BIEC2-330572	16-16993987	0.06	0.804	6.03	0.049	0.07	0.80
BIEC2-330714	16-17698340	0.01	0.925	6.65	0.036	0.01	0.923
BIEC2-331723	16-19782127	0.63	0.427	6.06	0.048	0.73	0.394
BIEC2-331775	16-19839423	1.76	0.185	6.38	0.041	2.04	0.153
BIEC2-332303	16-20652633	5.16	0.023	6.07	0.048	5.41	0.020
BIEC2-332371	16-20755410	4.66	0.031	5.44	0.066	5.32	0.021
BIEC2-333414	16-22005338	0.01	0.964	9.19	0.010	0.01	0.965
BIEC2-333671	16-22468598	0.53	0.465	12.69	0.002	0.55	0.457
BIEC2-334347	16-23706842	5.07	0.024	4.83	0.090	4.53	0.033
BIEC2-334359	16-23709798	5.07	0.024	4.83	0.090	4.53	0.033
BIEC2-334540	16-23886963	0.01	0.928	7.10	0.029	0.01	0.929
BIEC2-334746	16-24120843	5.89	0.015	5.71	0.058	5.19	0.023
BIEC2-334795	16-24192718	4.47	0.035	5.34	0.069	4.93	0.026
ECA16 hock OCD							
BIEC2-328174	16-3753939	2.84	0.092	9.19	0.010	2.91	0.088
BIEC2-328894	16-6800733	4.43	0.035	4.77	0.029	4.77	0.029
BIEC2-330578	16-17001997	4.65	0.031	6.07	0.048	4.56	0.033
BIEC2-330594	16-17006523	2.57	0.109	5.91	0.052	2.48	0.115

Table 2 continued

SNP name	Map info	χ2 allele	P allele	χ2 genotype	P genotype	χ2 trend	P trend
BIEC2-330600	16-17017547	4.54	0.033	5.11	0.078	4.62	0.032
BIEC2-330615	16-17094667	3.94	0.047	4.41	0.110	4.06	0.044
BIEC2-330628	16-17098315	3.94	0.047	4.41	0.110	4.06	0.044
BIEC2-330677	16-17421511	3.91	0.048	4.10	0.129	3.85	0.050
BIEC2-330691	16-17546589	6.57	0.010	6.54	0.038	6.53	0.011
BIEC2-330698	16-17565806	5.11	0.024	5.55	0.062	5.09	0.024
BIEC2-330708	16-17670327	8.44	0.004	7.02	0.030	7.02	0.008
BIEC2-330714	16-17698340	2.35	0.125	7.91	0.019	2.65	0.103
BIEC2-330720	16-17754789	4.89	0.027	5.37	0.068	4.76	0.029
BIEC2-330725	16-17763943	6.22	0.013	6.89	0.032	6.67	0.010
BIEC2-330739	16-17790340	4.23	0.040	4.64	0.098	4.38	0.036
BIEC2-330748	16-17885080	5.88	0.015	5.76	0.056	5.62	0.018
BIEC2-330760	16-18040578	5.88	0.015	5.76	0.056	5.62	0.018
BIEC2-332027	16-20179799	5.62	0.018	5.05	0.080	4.69	0.030
BIEC2-333011	16-21612414	3.79	0.052	4.16	0.041	4.16	0.041
BIEC2-333414	16-22005338	0.01	0.944	6.12	0.047	0.01	0.948
BIEC2-334005	16-23311651	3.81	0.051	4.24	0.040	4.24	0.040
BIEC2-335076	16-24760301	2.36	0.125	7.70	0.021	2.30	0.130
BIEC2-335182	16-24827127	2.10	0.147	8.26	0.016	2.18	0.140
BIEC2-335655	16-25602224	2.85	0.091	7.17	0.028	2.74	0.098
BIEC2-336573	16-27097038	4.09	0.043	3.80	0.150	3.77	0.052
BIEC2-338439	16-30953707	4.29	0.038	4.26	0.119	3.92	0.048
BIEC2-338511	16-31029398	6.60	0.010	6.44	0.040	6.38	0.012
BIEC2-338512	16-31029554	5.59	0.018	5.26	0.072	5.25	0.022

Table 2 continued

SNP name	Map info	χ2 allele	P allele	χ2 genotype	P genotype	χ2 trend	P trend
BIEC2-338514	16-31031690	5.59	0.018	5.26	0.072	5.25	0.022
BIEC2-338575	16-31097426	5.39	0.020	5.27	0.072	5.08	0.024
BIEC2-338592	16-31115480	5.51	0.019	5.42	0.067	5.40	0.020
BIEC2-338862	16-31750953	1.07	0.300	6.87	0.032	1.20	0.273
BIEC2-340215	16-34168194	4.32	0.038	5.18	0.075	3.31	0.069
BIEC2-341699	16-38714465	3.66	0.056	6.16	0.046	3.47	0.062
BIEC2-344527	16-43202177	3.94	0.047	3.96	0.138	3.61	0.057
BIEC2-346535	16-45983092	1.81	0.178	9.23	0.010	1.80	0.180
ECA18 OC							
BIEC2-420030	18-075879418	2.17	0.141	7.31	0.026	2.45	0.117
BIEC2-420031	18-075879559	2.32	0.128	8.91	0.012	2.59	0.107
BIEC2-420646	18-077130425	1.75	0.186	5.90	0.052	1.78	0.182
BIEC2-420826	18-077593952	0.05	0.826	6.95	0.031	0.05	0.825
BIEC2-421484	18-079364656	4.97	0.026	6.36	0.042	4.53	0.033
BIEC2-421493	18-079398715	4.45	0.035	11.89	0.003	4.22	0.040
BIEC2-421715	18-080387076	0.18	0.668	6.13	0.047	0.16	0.687
BIEC2-421739	18-080534779	2.78	0.096	3.14	0.208	2.49	0.115
BIEC2-421798	18-080793438	1.01	0.315	8.91	0.012	0.98	0.323
BIEC2-421806	18-080836383	3.98	0.046	3.78	0.151	3.76	0.052
BIEC2-421818	18-080972147	0.27	0.607	6.48	0.039	0.26	0.613
BIEC2-421879	18-081340848	5.29	0.022	5.50	0.064	5.08	0.024
BIEC2-421929	18-081510682	4.23	0.040	4.06	0.131	4.03	0.045
BIEC2-421931	18-081581136	4.37	0.037	4.92	0.086	4.13	0.042
BIEC2-421944	18-081703071	4.46	0.035	4.92	0.086	4.29	0.038

Table 2 continued

SNP name	Map info	χ2 allele	P allele	χ2 genotype	P genotype	χ2 trend	P trend
BIEC2-421970	18-081775453	2.44	0.118	9.52	0.009	2.44	0.118
BIEC2-421975	18-081798786	4.42	0.035	6.60	0.037	4.57	0.033
BIEC2-421977	18-081817905	3.93	0.047	6.41	0.041	4.04	0.044
BIEC2-421989	18-081855169	4.42	0.035	6.60	0.037	4.57	0.033
BIEC2-422004	18-081986880	4.42	0.035	6.60	0.037	4.57	0.033
BIEC2-422014	18-082077699	4.09	0.043	7.23	0.027	4.21	0.040
BIEC2-422024	18-082195702	4.09	0.043	7.23	0.027	4.21	0.040
BIEC2-422025	18-082198807	3.66	0.056	6.48	0.039	3.81	0.051
BIEC2-422062	18-082495575	6.18	0.013	5.98	0.050	5.87	0.015
ECA21 hock OCD							
BIEC2-548383	21-5996157	1.00	0.316	6.36	0.042	0.87	0.352
BIEC2-548855	21-6623818	3.42	0.065	6.37	0.041	3.31	0.069
BIEC2-550182	21-8966664	5.77	0.016	6.17	0.013	6.17	0.013
BIEC2-550500	21-9371289	6.98	0.008	8.90	0.012	8.64	0.003
BIEC2-550542	21-9485226	3.62	0.057	4.98	0.083	3.29	0.070
BIEC2-551657	21-11006036	4.96	0.026	7.22	0.027	4.88	0.027
BIEC2-552154	21-11939082	3.85	0.050	3.91	0.142	3.87	0.049
BIEC2-553100	21-13131951	3.82	0.051	4.30	0.116	4.30	0.038
BIEC2-554033	21-15907810	3.86	0.050	3.45	0.178	3.36	0.067
BIEC2-554035	21-15923414	3.86	0.050	3.45	0.178	3.36	0.067
BIEC2-554052	21-16133211	4.33	0.038	4.09	0.130	3.80	0.051
BIEC2-554058	21-16145605	3.88	0.049	4.91	0.086	3.55	0.059
BIEC2-554066	21-16153112	4.33	0.038	4.09	0.130	3.80	0.051

Table 3 Haplotypes with at least 1% in the total sample of 154 Hanoverian warmblood horses, their frequencies, standard error, haplotype frequencies of cases and controls, their association with fetlock OCD on ECA5

SNP				Frequency	Standard	Frequency (%)			
1	2	3	Haplotype	total (%)	error	controls	cases	χ2	P
A	A	A	A-A-A	45.53	0.028	44.43	51.85	1.25	0.2631
A	A	G	A-A-G	30.27	0.025	25.49	35.69	2.42	0.1200
A	**G**	**A**	**A-G-A**	**10.85**	**0.020**	**15.24**	**0.00**	**11.54**	**0.0007**
A	**G**	**G**	**A-G-G**	**1.00**	**0.005**	**0.00**	**4.34**	**9.96**	**0.0016**
C	A	A	C-A-A	5.25	0.016	4.96	4.53	0.10	0.7532
C	**A**	**G**	**C-A-G**	**6.38**	**0.016**	**9.67**	**1.56**	**4.85**	**0.0276**

1: BIEC2-919955

2: BIEC2-922891

3: BIEC2-922937

Table 4 Haplotypes with at least 1% in the total sample of 154 Hanoverian warmblood horses, their frequencies, standard error, haplotype frequencies of cases and controls, their association with hock OCD on ECA16

SNP				Frequency	Standard	Frequency (%)			
1	2	3	Haplotype	total (%)	error	controls	cases	χ2	P
A	A	A	A-A-A	1.31	0.009	1.89	0.00	1.24	0.2659
A	A	G	A-A-G	1.02	0.008	1.69	0.00	1.27	0.2600
A	C	A	A-C-A	4.74	0.011	5.12	8.22	1.87	0.1719
A	**C**	**G**	**A-C-G**	**28.09**	**0.025**	**20.82**	**37.36**	**6.24**	**0.0125**
G	A	A	G-A-A	2.14	0.007	1.54	2.06	0.22	0.6408
G	A	G	G-A-G	17.98	0.022	20.65	14.12	1.31	0.2528
G	**C**	**A**	**G-C-A**	**11.98**	**0.019**	**16.45**	**0.00**	**11.65**	**0.0006**
G	C	G	G-C-G	32.74	0.027	31.83	38.22	0.98	0.3234

1: BIEC2-330698
2: BIEC2-330714
3: BIEC2-332027

Table 5 Haplotypes with at least 1% in the total sample of 154 Hanoverian warmblood horses, their frequencies, standard error, haplotype frequencies of cases and controls, their association with OC on ECA18

SNP					Frequency	Standard	Frequency (%)			
1	2	3	4	Haplotype	total (%)	error	controls	cases	$\chi 2$	P
A	C	A	A	A-C-A-A	1.56	0.0047	2.61	0.35	2.63	0.1046
A	C	C	A	A-C-C-A	16.00	0.0209	19.70	13.66	2.03	0.1541
C	A	A	A	C-A-A-A	15.25	0.0205	18.35	12.23	2.22	0.1365
C	**A**	**C**	**A**	**C-A-C-A**	**50.81**	**0.0285**	**37.37**	**61.66**	**17.73**	**<0.0001**
C	A	C	G	C-A-C-G	11.78	0.0184	15.20	8.86	2.92	0.0875
C	C	C	A	C-C-C-A	3.20	0.0100	4.26	0.90	3.54	0.0600

1: BIEC2-421484

2: BIEC2-421493

3: BIEC2-421806

4: BIEC2-421739

Table 6 Haplotypes with at least 1% in the total sample of 154 Hanoverian warmblood horses, their frequencies, standard error, haplotype frequencies of cases and controls, their association with hock OCD on ECA21

SNP				Frequency	Standard	Frequency (%)			
1	2	3	Haplotype	total (%)	error	controls	cases	χ2	P
A	**A**	**A**	**A-A-A**	**20.69**	**0.024**	**25.39**	**6.17**	**10.46**	**0.0012**
A	A	C	A-A-C	24.49	0.025	26.00	24.39	0.16	0.6901
A	**G**	**A**	**A-G-A**	**3.90**	**0.012**	**8.01**	**0.00**	**8.51**	**0.0035**
A	**G**	**C**	**A-G-C**	**5.00**	**0.011**	**1.54**	**10.62**	**7.75**	**0.0054**
G	**A**	**A**	**G-A-A**	**14.45**	**0.021**	**11.12**	**23.10**	**5.26**	**0.0218**
G	A	C	G-A-C	23.53	0.023	24.99	21.35	0.33	0.5648
G	**G**	**A**	**G-G-A**	**1.77**	**0.007**	**0.00**	**4.56**	**5.32**	**0.0210**
G	**G**	**C**	**G-G-C**	**6.17**	**0.012**	**2.94**	**9.82**	**3.87**	**0.0491**

1: BIEC2-550500
2: BIEC2-551657
3: BIEC2-554071

Table 7 All annotated genes located in the QTL for osteochondrosis in Hanoverian warmblood horses with their respective positions in base pairs (bp) on equine chromosomes (ECA) and the detected QTL region. Potential functional candidate genes are marked in bold.

ECA	QTL region (Mb)	Gene name	Position of gene (bp)	Full gene name
5	76.79 – 81.10	*HS2ST1*	76.959.579 - 77.123.440	*heparan sulfate 2-O-sulfotransferase 1*
		SEP-15	77.124.128 - 77.160.069	*15 kDa selenoprotein*
		SH3GLB1	77.240.598 - 77.277.567	*SH3-domain GRB2-like endophilin B1*
		CLCA3	77.308.114 - 77.328.364	*chloride channel calcium activated 3*
		CLCA1	77.472.150 - 77.500.196	*chloride channel calcium activated 1*
		CLCA2	77.507.513 - 77.552.085	*chloride channel calcium activated 2*
		ODF2L	77.574.621 - 77.602.815	*outer dense fiber of sperm tails 2-like*
		COL24A1	**77.783.455 - 78.131.454**	***collagen, type XXIV, alpha 1***
		ZNHIT6	78.153.175 - 78.205.451	*zinc finger, HIT type 6*
		CYR61	**78.262.823 - 78.264.924**	***cysteine-rich, angiogenic inducer, 61***
		DDAH1	**78.365.481 - 78.486.705**	***dimethylarginine dimethylaminohydrolase 1***
		BCL10	78.526.534 - 78.536.091	*B-cell CLL/lymphoma 10*
		SYDE2	78.586.016 - 78.628.704	*synapse defective 1, Rho GTPase, homolog 2 (C. elegans)*
		WDR63	78.652.892 - 78.722.397	*WD repeat domain 63*
		MCOLN3	78.753.553 - 78.780.546	*mucolipin 3*
		MCOLN2	78.791.187 - 78.838.921	*mucolipin 2*
		EDG7	**78.895.543 - 78.943.911**	***endothelial cell differentiation gene 7***
		SSX2IP	79.076.965 - 79.106.787	*synovial sarcoma, X breakpoint 2 interacting protein*

Table 7 continued

ECA	QTL region (Mb)	Gene name	Position of gene (bp)	Full gene name
5	76.79 – 81.10	*CTBS*	79.167.030 - 79.185.065	*chitobiase, di-N-acetyl-*
		SPATA1	79.186.712 - 79.222.754	*spermatogenesis associated 1*
		BXDC5	79.244.253 - 79.265.687	*brix domain containing 5*
		DNASE2B	79.314.548 - 79.328.676	*deoxyribonuclease II beta*
		SAMD13	79.375.341 - 79.411.429	*sterile alpha motif domain containing 13*
		PRKACB	79.479.582 - 79.529.127	*protein kinase, cAMP dependent, catalytic, beta*
		TTLL7	79.667.289 - 79.743.271	*tubulin tyrosine ligase-like family, member 7*
		LPHN2	81.489.753 - 81.573.049	*latrophilin 2*
16	17.57 – 20.10	*SHQ1*	17.586.324 - 17.755.976	*SHQ1 homolog (S. cerevisiae)*
		RYBP	17.925.952 - 17.995.521	*RING1 and YY1 binding protein*
		PROK2	18.505.112 - 18.514.447	*prokineticin 2*
		EIF4E3	18.569.283 - 18.591.172	*eukaryotic translation initiation factor 4E family member 3*
		FOXP1	18.770.909 - 19.256.184	*forkhead box P1*
18	79.36 – 80.84	*PARD3B*	79.106.315 - 80.034.010	*par-3 partitioning defective 3 homolog B (C. elegans)*
		NRP2	**80.076.435 - 80.186.347**	***neuropilin 2***
		NDUFS1	80.452.383 - 80.476.152	*NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)*
		EEF1B2	80.481.339 - 80.482.639	*eukaryotic translation elongation factor 1 beta 2*
		GPR1	80.490.238 - 80.491.305	*G protein-coupled receptor 1*
		ADAM23	80.681.827 - 80.829.663	*ADAM metallopeptidase domain 23*

Table 7 continued

ECA	QTL region (Mb)	Gene name	Position of gene (bp)	Full gene name
21	9.37 – 16.18	*IPO11*	10.561.205 - 10.774.653	*importin 11*
		DIMT1L	10.917.390 - 10.927.440	*DIM1 dimethyladenosine transferase 1-like (S. cerevisiae)*
		KIF2A	10.929.739 - 10.958.463	*kinesin heavy chain member 2A*
		ZSWIM6	11.635.169 - 11.706.080	*zinc finger, SWIM-type containing 6*
		NDUFA12L	11.972.502 - 12.118.184	*NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2*
		ERCC8	12.174.516 - 12.218.077	*excision repair cross-complementing rodent repair deficiency, complementation group 8*
		ELOVL7	12.296.700 - 12.331.617	*ELOVL family member 7, elongation of long chain fatty acids (yeast)*
		DEPDC1B	12.367.100 - 12.456.155	*DEP domain containing 1B*
		RAB3C	13.999.408 - 14.263.410	*RAB3C, member RAS oncogene family*
		PLK2	14.348.187 - 14.354.106	*polo-like kinase 2 (Drosophila)*
		GPBP1	15.346.075 - 15.391.230	*GC-rich promoter binding protein 1*
		MIER3	15.604.653 - 15.631.548	*mesoderm induction early response 1, family member 3*
		MAP3K1	15.665.635 - 15.696.787	*mitogen-activated protein kinase kinase kinase 1*

CHAPTER 8

A whole genome scan to map quantitative trait loci for osteochondrosis in Hanoverian warmblood horses using a dense single nucleotide polymorphism map

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8 A whole genoms scan to map quantitative trait loci for osteochondrosis in Hanoverian warmblood horses using a dense single nucleotide polymorphism map

8.1 Abstract

Osteochondrosis is an inherited developmental disease in young horses most frequently observed in thoroughbreds, trotters, warmblood and coldblood horses. Quantitative trait loci (QTL) for equine osteochondrosis have been identified in Hanoverian warmblood horses employing a whole genome linkage analysis with microsatellites. The aim of this study was to perform a whole genome scan with single nucleotide polymorphisms employing the Illumina Equine SNP50 Beadchip. From a large sample of Hanoverian warmblood horses, 154 unrelated animals were chosen and six different phenotypic traits were distinguished. For the trait OC in fetlock and/or hock joints we detected six potential QTL on ECA1, 4, 14, 16, 18 and 30 and four QTL for OCD in fetlock and/or hock joints on ECA1, 3, 30 and X. For the trait OC in fetlock joints we were able to identify five different potential QTL on ECA2, 16, 26, 30 and X, for the trait OCD in fetlock joints five potential QTL were observed on ECA2, 3, 13, 22 and 30. For hock OC five QTL were discovered being significant on ECA1, 4, 14, 29 and 30, for hock OCD three potential QTL were identified on ECA1, 6 and 30. This report is a further step towards unravelling the genes underlying QTL for equine osteochondrosis and towards the development of a marker test for osteochondrosis in Hanoverian warmblood horses.

8.2 Introduction

Osteochondrosis (OC) is an inherited developmental orthopaedic disorder in young horses (Grøndahl and Dolvik, 1993; Philipsson et al., 1993; KWPN, 1994; Stock et al., 2005; Wittwer et al., 2006; Ytrehus et al., 2007). Abnormal chondrocyte differentiation and maturation causes altered enchondral ossification of the joints (Jeffcott and Henson, 1998). Articulations most commonly affected in horses are fetlock, hock and stifle joints. Osteochondrotic lesions can be identified as

subchondral bone cysts, fissures, cartilage flaps and osteochondrosis dissecans (OCD) (Van Weeren, 2005).

Whole genome scans in Hanoverian warmblood and South German coldblood revealed quantitative trait loci (QTL) for OC (Dierks et al. 2007; Wittwer et al. 2007). The traits analyzed were OC (fetlock and/or hock joints affected), OCD (fetlock and/or hock joints affected), fetlock OC, fetlock OCD, hock OC and hock OCD. QTL for the different traits in Hanoverian warmblood horses were located on ECA2, 3, 4, 5, 15, 16, 19 and 21, another QTL could be detected on ECA18 (Lampe et al. 2009a). QTL on ECA5, 16 and 21 were refined and narrowed down using newly developed microsatellites with marker distances <1Mb (Lampe et al. 2009b, 2009c, Felicetti et al. 2009).

Genotyping arrays containing SNP markers were successfully used in human (Winkelmann et al., 2007) and dogs for mapping mendelian traits and genes causing a disease (Karlsson et al., 2007). With the completion of the equine genome sequence assembly, single nucleotide polymorphism (SNP) assays spanning the whole equine genome and research work on large-scale identification, validation and analysis of genotypic variation in horses has become possible.

The objective of this study was a genome-wide association analysis of Single nucleotide polymorphisms (SNPs) using the Equine SNP50 BeadChip (Illumina, San Diego, CA, USA) in order to validate the results of the linkage analysis and to detect new QTL for osteochondrosis in Hanoverian warmblood horses.

8.3 Material and Methods

Pedigree structure and phenotypic traits

From a large sample of Hanoverian warmblood horses, 154 foals were chosen descending from 52 different stallions. 39 of these animals were also included in all the previous linkage analyses (Dierks et al. 2007, Lampe et al. 2009a, 2009b, 2009c, Felicetti et al. 2009). 90 animals were affected by osteochondrosis in fetlock and/or hock joints and 64 were free from signs of OC in each limb joint. 43 of the 90 affected animals were only affected in fetlock joints, 33 horses were only affected in hock joints. 14 horses were affected in both fetlock and hock joints. Out of this sample, 86

horses were male and 68 animals were female. Diagnosis of osteochondrosis was done following the recording and evaluation scheme developed for warmblood horses (Kroll et al. 2001). The sagittal ridge of the 3rd metacarpal/metatarsal bone of fetlock joints, the intermediate ridge of the distal tibia, the lateral trochlea of the talus and the medial malleolus of the tibia were considered as predilection sites for OC. Signs consistent with osteochondrosis were irregular bone trabeculation with variable radiolucency, irregular bone margin, new bone formation or osseous fragments when these changes were located at these predilection sites. Horses showing radiographic changes of osteochondrosis with or without osseous fragments at the predilection sites of the fetlock and/or hock joints were classified as affected by osteochondrosis (OC) and those horses exhibiting radiodense bodies as signs for osteochondral fragments at the above mentioned predilection sites were treated as affected by osteochondrosis dissecans (OCD). Horses with pathological changes in fetlock or hock joints other than osteochondrosis were not employed in our study. Animals without any signs of radiographic changes at all joints examined (fetlock, hock and stifle) were considered as free from OC, and only these horses were included as controls.

Genotyping of SNPs

Genomic DNA was extracted from EDTA blood samples of 154 Hanoverian warmblood horses through a standard ethanol fractionation with concentrated sodiumchloride (6M NaCl) and sodium dodecyl sulphate (10% SDS). Concentration of extracted DNA was determined using the Nanodrop ND-1000 (Peqlab Biotechnology GmbH, Erlangen, Germany). DNA concentration of samples was adjusted between 30 and 80 ng/µl.

Genotyping was performed with the Illumina Equine SNP50 BeadChip containing 54,602 SNP markers using standard procedures as recommended by the manufacturer. Raw data were analyzed using the genotyping module version 3.2.32 of the BeadStudio program (Illumina). In order to assign the genotypes we generated a cluster file with the help of the BeadStudio software and the genotyping module version 3.2.32. The clusters were validated for five parameters consecutively for the

purpose of identifying unreliable assays. In order to evaluate mistakes three samples were replicated.

Data analysis

For genome-wide mapping we performed a case-control association analysis on all SNPs with a minor allele frequency (MAF) >1% and a call rate >90% by using the software package PLINK version 1.05 (http://pngu.mgh.harvard.edu/purcell/plink/; Purcell et al. 2007). Genome-wide significance was ascertained through adaptive permutation testing using a maximum of 5,000,000 permutations.

Additionally, the MIXED procedure of the Statistical Analysis System (SAS) version 9.2 (SAS Inc., Cary, NC, 2009) was used for analysis in order to correct for the effect of mean relatedness of each horses with all other horses.

8.4 Results

Due to missingness test no SNP was excluded, 3,582 SNPs did not reach a sufficient MAF, so 51,020 SNPs were left for the association analysis.

We defined potential QTL as genomic regions with not less than 3 highly significant neighbouring markers ($-\log_{10}$ (P) >2.5) for the same trait. On ECA1 we were able to detect a new QTL for OC and OCD in fetlock and/or hock joints, as well as for OC and OCD in hock joints in a region between 43.1 and 44.6 Mb and a QTL for OC in fetlock joints at 43.1 to 43.3 Mb. On ECA2 a QTL for fetlock OC and fetlock OCD could be identified at 104.1 to 104.4 Mb. On ECA3 the association analysis showed significant results for OCD in fetlock joints at 27.8 to 27.9 Mb and for OCD in fetlock and/or hock joints at 64.2 to 64.6 Mb. On ECA4 a QTL for OC in fetlock and/or hock joints and for OC in hock joints could be identified at 41.2 to 41.3 Mb. On ECA6 the association analysis showed significant effects for hock OCD at 47.2 to 47.3 Mb. On ECA13 we could observe significant association for fetlock OCD in the region between 15.3 and 15.4 Mb. On ECA14 one QTL for OC in fetlock and/or hock joints could be observed at 57.7 to 57.8 Mb and a second QTL for hock OC was identified at 58.6 to 58.7 Mb. On ECA16 a QTL for fetlock OC was visible at 81.1 Mb and a QTL for OC in fetlock and/or hock joints could be detected at 82.3 to 82.6 Mb.

Another QTL for OC in fetlock and/or hock joints was discovered on ECA18 between 36.4 and 38.7 Mb. On ECA22 the association analysis revealed a QTL for fetlock OCD between 42.6 and 42.9 Mb. On ECA26 there was significant association at 27.6 to 28.0 Mb for fetlock OC. Furthermore, ECA29 contains a new QTL for hock OC in a region from 16.8 to 17.1 Mb. On ECA30 QTL analyses discovered a QTL for hock OC between 8.6 and 8.7 Mb and QTL for all traits except hock OC at 12.1 to 12.2 Mb. On equine chromosome X, a QTL for OCD in fetlock and/or hock joints, as well as a QTL for fetlock OC were identified at 55.5 Mb (Table 1, Figure 1-6).
Additionally, for a great number of single SNPs, which are distributed over the complete genome highly significant associations could be identified.

8.5 Discussion

To the authors' knowledge, the current study is the first to report the use of a SNP microarray in the investigation of complex inherited diseases.
The aim of our study was to reveal SNP markers associated with different traits of osteochondrosis in Hanoverian warmblood horses. Considering the marker density used, it is unlikely that any of the SNPs found associated with osteochondrosis represent causal mutations. However, we discovered new potential QTL on ten different chromosomes, which suggest that several genes are involved in the development osteochondrosis. Furthermore the results indicate that the genetic influences on the development of fetlock and hock OC are mostly due to different gene loci. Identified QTL were compared to annotated genes of the second horse assembly (Table 2). The genomic regions contain positional candidate genes with potential influence on the development of OC. Those genes have to code for hormones, enzymes, metabolic factors and/or their receptors which are involved in the complex of cartilage maturation, differentiation and vascularisation during enchondral ossification or in growth processes. Besides a variety of positional candidate genes with in some extent unknown function, *COL28A1* on ECA4, *WWTR1* on ECA16, *NR4A2, ACVR1 and ACVR1C* on ECA18 seem to be suitable functional candidate genes due to their function. The *collagen type XXVIII, alpha 1 (COL28A1)* belongs to the class of collagens, which is the main component of

cartilage. *WW domain containing transcription regulator 1 (WWTR1)* coactivates RUNX2-dependent gene transcription while repressing PPARG-dependent gene transcription, which drive mesenchymal stem cells to differentiate into either osteoblasts or adipocytes. By modulating *WWTR1* expression in model cell lines, mouse embryonic fibroblasts, and primary mesenchymal stem cells in culture and in zebrafish in vivo, Hong et al. (2005) observed alterations in osteogenic versus adipogenic potential. The *nuclear receptor subfamily 4, group A, member 2 (NR4A2)* is emerging as a key regulator of cytokine and growth factor action in chronic inflammatory diseases. Mix et al. (2007) propose a protective function for this gene in cartilage homeostasis by selectively repressing MMP gene expression during inflammation. The *activin A receptor, type I (ACVR1)* and the *activin A receptor, type IC (ACVR1C)* are dimeric growth and differentiation factors which belong to the transforming growth factor-beta (TGF-beta) superfamily of structurally related signaling proteins. Mutations in the *ACVR1* gene are associated with fibrodysplasia ossificans progressive.

The Equine 50SNP Beadchip revealed other potential chromosomal regions of interest than did the whole genome scan with microsatellites (Dierks et al. 2007). The differences in QTL discoveries among the two methods are likely related to the sample composition and the insufficient marker density in the former whole genome scan so that the power to detect QTL strongly diminishes. Nevertheless, it was possible to confirm the former QTL through highly associated haplotypes (Lampe et al. 2009d).

In the future, use of much denser marker sets will help clarifying the relevance of the numerous single significant peaks and possibly allows moving from the marker associations towards revealing causal mutations underlying equine osteochondrosis.

8.6 References

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Table 1 Genomic regions with highly significant neighbouring SNPs ($-\log_{10}$ (P) > 2.5) for osteochondrosis (OC) and osteochondrosis dissecans (OCD) in fetlock and/or hock joints, fetlock OC (OC-F), fetlock OCD (OCD-F), hock OC (OC-H) and hock OCD (OCD-H)

ECA	SNP name	Position on EquCab2 in bp	$-\log_{10}$ (P) OC	$-\log_{10}$ (P) OCD	$-\log_{10}$ (P) OC-F	$-\log_{10}$ (P) OCD-F	$-\log_{10}$ (P) OC-H	$-\log_{10}$ (P) OCD-H
1	BIEC2-20123	43130411	3.25	3.65	2.63		4.22	3.96
	BIEC2-20129	43155466	3.44	3.86	2.68		4.49	4.26
	BIEC2-20133	43250448	3.24	3.72	2.61		4.15	3.96
	BIEC2-20135	43314847	3.34	3.78	2.63		4.42	4.22
	BIEC2-20143	43358920	3.25	3.65	2.63		4.21	3.96
	BIEC2-20208	43852372					3.21	2.73
	BIEC2-20277	43955641					3.49	3.29
	BIEC2-20528	44337341					2.94	
	BIEC2-20684	44631979					3.27	3.86
	BIEC2-20693	44645964	2.66	2.58			4.05	3.57
	BIEC2-20711	44669679	2.66	2.58			4.05	3.57
	BIEC2-20718	44675827	3.11	3.17			4.73	4.26
2	BIEC2-504586	104132151				3.31		
	BIEC2-504592	104214898				2.65		
	BIEC2-504593	104230418			2.54	3.65		
	BIEC2-504623	104410695			2.73	3.24		
	BIEC2-504629	104457996			2.73	3.24		
	BIEC2-504630	104481141			2.73	3.24		
3	BIEC2-775047	27815405				2.91		
	BIEC2-775049	27854550				2.65		
	BIEC2-775055	27900045				2.69		
3	BIEC2-786718	64213851		3.06				
	BIEC2-786980	64616371		4.76				
	BIEC2-786999	64632362		3.00				

Table 1 continued

ECA	SNP name	Position on EquCab2 in bp	$-\log_{10}$ (P) OC	$-\log_{10}$ (P) OCD	$-\log_{10}$ (P) OC-F	$-\log_{10}$ (P) OCD-F	$-\log_{10}$ (P) OC-H	$-\log_{10}$ (P) OCD-H
4	BIEC2-860632	41202168	2.80	2.58			3.78	2.64
	BIEC2-860635	41206554	2.84				3.67	
	BIEC2-860673	41310083					2.80	
	BIEC2-860690	41365096	2.74				3.30	
6	BIEC2-953013	47274114						2.59
	BIEC2-953030	47351781						2.76
	BIEC2-953043	47367411						2.92
	BIEC2-953049	47375886						2.76
	BIEC2-953053	47384220						2.76
13	BIEC2-212701	15329164				3.10		
	BIEC2-212705	15333180				3.04		
	BIEC2-212721	15418840				3.12		
14	BIEC2-259867	57700667	3.08					
	BIEC2-259869	57701162	2.58					
	BIEC2-259878	57826754	2.85					
14	BIEC2-259991	58688195					2.61	
	BIEC2-260004	58715759					2.77	
	BIEC2-260007	58716055					2.84	
16	BIEC2-364637	81123678			2.86			
	BIEC2-364642	81124134			2.86			
	BIEC2-364644	81125923			2.64			
	BIEC2-364650	81153503			3.83	2.84		
16	BIEC2-364908	82398594	2.95					
	BIEC2-364943	82647591	2.54					
	BIEC2-364945	82667438	2.53					
	BIEC2-364993	82803587	2.66					
18	BIEC2-410927	36408881	4.17	3.19	2.69		2.84	
	BIEC2-411132	38240449	2.82					
	BIEC2-411133	38240578	2.82					
	BIEC2-411136	38244258	2.82					
	BIEC2-411142	38345198	2.89					

Table 1 continued

ECA	SNP name	Position on EquCab2 in bp	$-log_{10}$ (P) OC	$-log_{10}$ (P) OCD	$-log_{10}$ (P) OC-F	$-log_{10}$ (P) OCD-F	$-log_{10}$ (P) OC-H	$-log_{10}$ (P) OCD-H
18	BIEC2-411181	38738316	2.82					
22	BIEC2-599102	42632808				2.82		
	BIEC2-599165	42680141				2.82		
	BIEC2-599208	42690881				3.13		
	BIEC2-599209	42690906				2.62		
	BIEC2-599412	42900035				2.95		
26	BIEC2-692043	27609841			3.04			
	BIEC2-692085	27710659			2.93			
	BIEC2-692087	27714188			3.68			
	BIEC2-692173	28029040			2.79			
	BIEC2-692176	28031982			2.79			
	BIEC2-692181	28038716			2.62			
29	BIEC2-755460	16809771					2.56	2.55
	BIEC2-755465	16812990					2.56	2.55
	BIEC2-755491	16943810					2.52	
	BIEC2-755521	17158761					2.77	
30	BIEC2-817757	8655041					3.05	
	BIEC2-817839	8703905					3.09	
	BIEC2-817840	8704075					4.05	
30	BIEC2-820456	12182691	3.93	4.22	3.65	2.57	2.74	3.48
	BIEC2-820475	12226055	3.97	4.00	4.67	3.68		2.89
	BIEC2-820482	12239797	3.96	4.14	4.67	3.99		2.84
X	BIEC2-1125200	55573885		2.69	2.63			
	BIEC2-1125201	55574156		3.49				
	BIEC2-1125202	55574309		2.71	2.52			
	BIEC2-1125203	55574350		2.71	2.52			

Table 2 All annotated genes located in the potential QTL for the different traits of osteochondrosis in Hanoverian warmblood horses with their respective positions in base pairs (bp) on equine chromosomes (ECA) and the detected QTL region. Potential functional candidate genes are marked in bold.

ECA	QTL region (Mb)	Gene name	Position of candidate gene (bp)	Full gene name
1	43.1 – 44.6	LOC100034103	43,245,495 – 43,249,033	mannose-binding lectin
		LOC100071940	43,657,579 – 43,657,923	similar to IMP1 inner mitochondrial membrane peptidase-like
		LOC100071973	44,373,407 – 44,145,692	similar to protocadherin 15
2	104.1 – 104.4	-		
3	27.8 – 27.9	-		
	64.2 – 64.6	RUFY3	64.195.136 - 64.254.480	RUN and FYVE domain containing 3
		UTP3	64.279.194 - 64.280.627	UTP3, small subunit (SSU) processome component, homolog (S. cerevisiae)
		IGJ	64.304.110 - 64.312.973	immunoglobulin joining chain
		ENAM	64.322.635 - 64.331.616	enamelin
		AMBN	64.353.595 - 64.365.691	ameloblastin (enamel matrix protein)
		AMTN	64.402.546 - 64.415.583	amelotin
		PROL1	64.586.063 - 64.591.124	proline rich, lacrimal 1
4	41.2 – 41.3	**COL28A1**	41.122.490 - 41.298.272	**collagen, type XXVIII, alpha 1**
		RPA3	41.382.400 - 41.386.772	replication protein A3
6	47.2 – 47.3	PDE3A	47.028.357 - 47.310.624	phosphodiesterase 3A, cGMP-inhibited
		SLCO1C1	47.333.344 - 47.432.071	solute carrier organic anion transporter family, member 1C1

Table 2 continued

ECA	QTL region (Mb)	Gene name	Position of candidate gene (bp)	Full gene name
13	15.3 – 15.4	-		
14	57.7 – 57.8	KCNN2	57.817.614 - 57.949.852	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
	58.6 – 58.8	YTHDC2	58.564.474 - 58.638.210	YTH domain containing 2
		MCC	58.662.614 - 59.065.164	mutated in colorectal cancers
16	81.1	-		
	82.3 – 82.8	**WWTR1**	**82.236.057 - 82.353.127**	**WW domain containing transcription regulator 1**
		COMMD2	82.413.725 - 82.421.373	COMM domain containing 2
		RNF13	82.504.534 - 82.635.720	ring finger protein 13
		PFN2	82.641.496 - 82.643.565	profilin 2
18	36.4 – 38.7	**NR4A2**	**37.540.296 - 37.548.445**	**nuclear receptor subfamily 4, group A, member 2**
		GPD2	37.677.926 - 37.776.266	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)
		GALNT5	38.305.373 - 38.350.504	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)
		ERMN	38.358.862 - 38.363.678	ermin, ERM-like protein
		PSCDBP	38.415.741 - 38.441.154	pleckstrin homology, Sec7 and coiled/coil domains, binding protein
		ACVR1C	**38.515.698 - 38.555.776**	**activin A receptor, type IC**
		ACVR1	**38.687.297 - 38.741.250**	**activin A receptor, type I**
22	42.6 – 42.9	CBLN4	42.875.914 - 42.881.190	cerebellin 4 precursor

Table 2 continued

ECA	QTL region (Mb)	Gene name	Position of candidate gene (bp)	Full gene name
26	27.6 – 28.0	KRTAP8-1	27.896.836 - 27.897.027	keratin associated protein 8-1
		KRTAP11-1	27.953.688 - 27.954.149	keratin associated protein 11-1
29	16.8 – 17.1	ARL5B	16.961.924 - 16.983.746	ADP-ribosylation factor-like 5B
		NSUN6	17.011.865 - 17.076.400	NOL1/NOP2/Sun domain family, member 6
		CACNB2	17.080.443 - 17.257.624	calcium channel, voltage-dependent, beta 2 subunit
30	8.6 – 8.8	CNIH3	8.615.526 - 8.720.875	cornichon homolog 3 (Drosophila)
		WDR26	8.861.158 - 8.896.765	WD repeat domain 26
	12.1 – 12.2	-		
X	55.5	-		

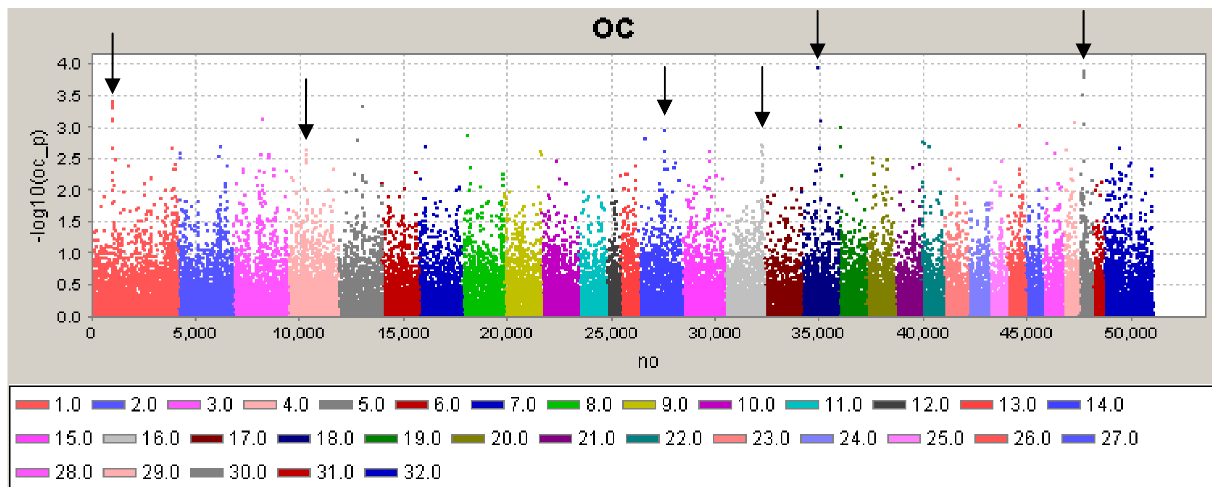

Figure 1 Results of the association analysis of all SNPs on the Illumina Equine SNP50 BeadChip with osteochondrosis in fetlock and/or hock joints (OC). QTL are marked with arrows.

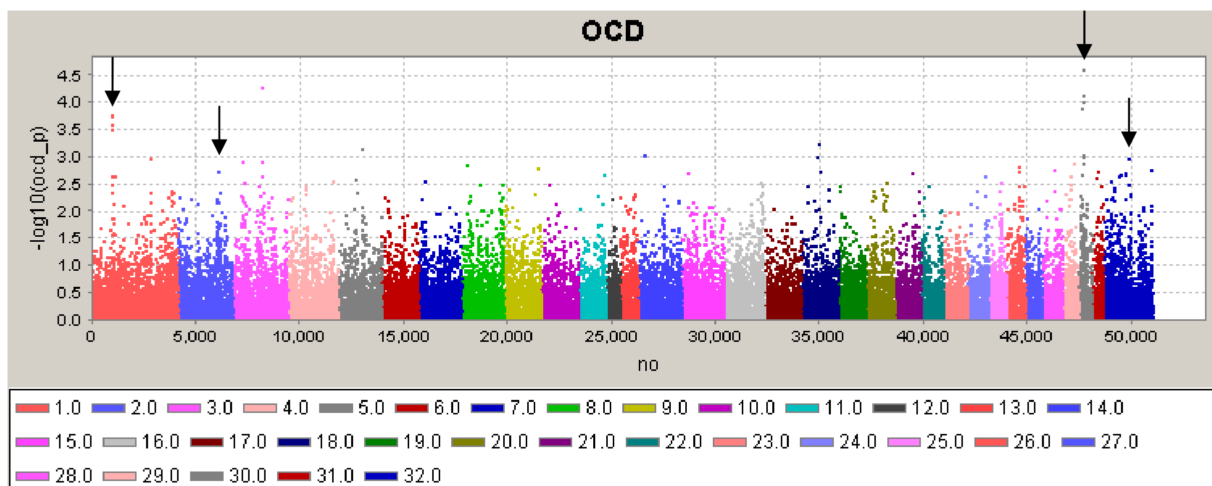

Figure 2 Results of the association analysis of all SNPs on the Illumina Equine SNP50 BeadChip with osteochondrosis dissecans in fetlock and/or hock joints (OCD). QTL are marked with arrows.

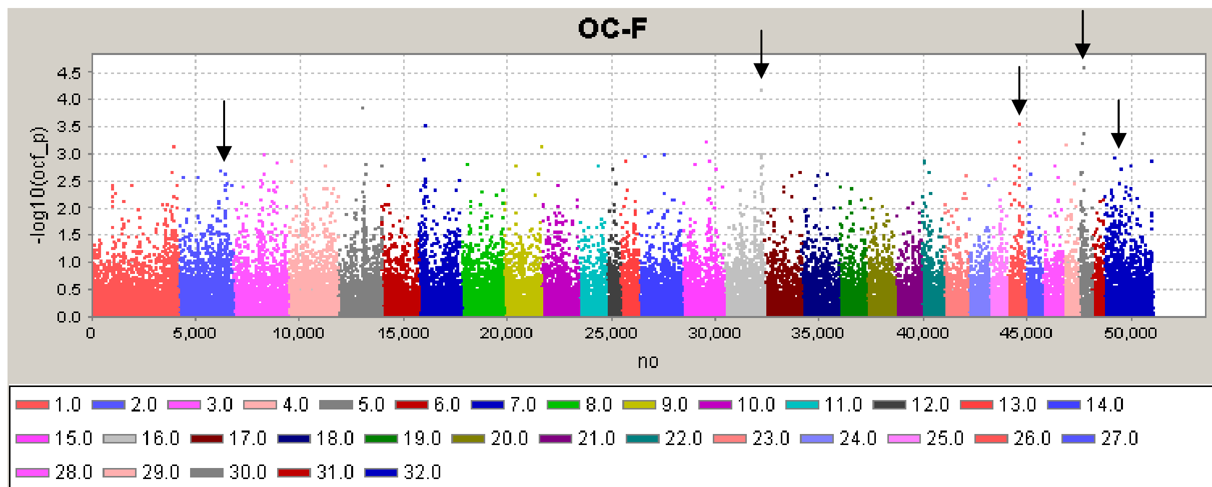

Figure 3 Results of the association analysis of all SNPs on the Illumina Equine SNP50 BeadChip with osteochondrosis in fetlock joints (OC-F). QTL are marked with arrows.

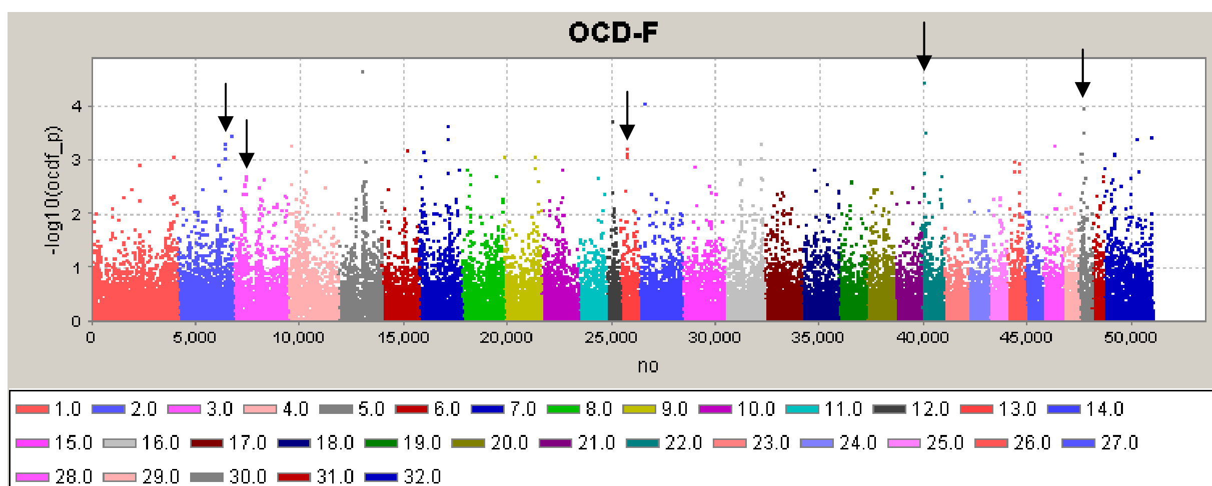

Figure 4 Results of the association analysis of all SNPs on the Illumina Equine SNP50 BeadChip with osteochondrosis dissecans in fetlock joints (OCD-F). QTL are marked with arrows.

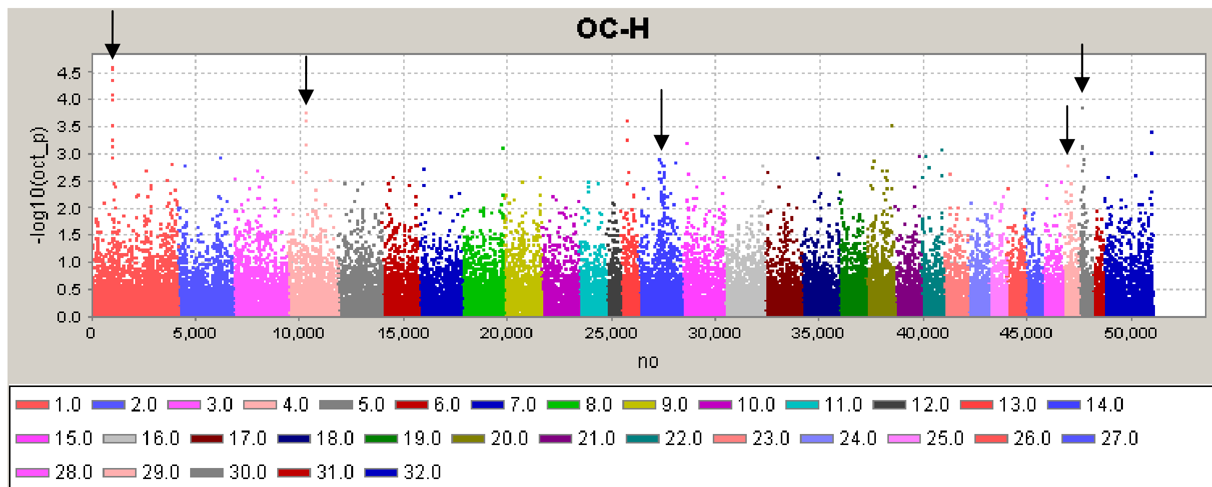

Figure 5 Results of the association analysis of all SNPs on the Illumina Equine SNP50 BeadChip with osteochondrosis in hock joints (OC-H). QTL are marked with arrows.

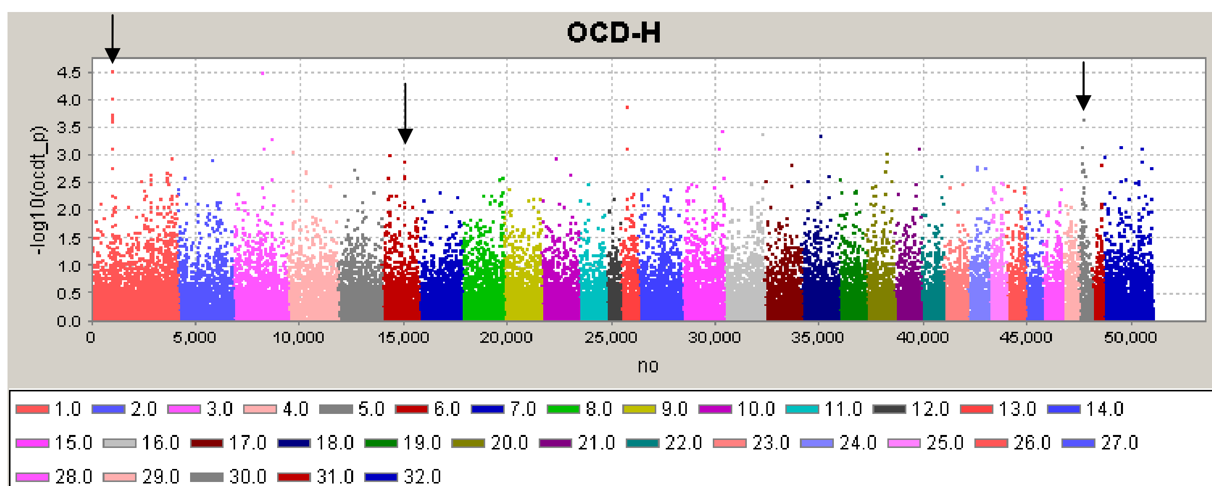

Figure 6 Results of the association analysis of all SNPs on the Illumina Equine SNP50 BeadChip with osteochondrosis dissecans in hock joints (OCD-H). QTL are marked with arrows.

CHAPTER 9

General discussion

9 General discussion

A whole genome scan for the identification of chromosomal locations of quantitative trait loci (QTL) for equine osteochondrosis (OC) and osteochondrosis dissecans (OCD) was performed in order to identify positional candidate genes (Dierks et al. 2007). The high number of QTL on different chromosomes found for OC and OCD in this study suggests that several genes are possibly involved in the development of OC and OCD but there is no hint on the type of gene action and in which way the different genes may interact with each other.

Potential candidate genes have to code for hormones, enzymes, metabolic factors and/or their receptors involved in the complex of cartilage differentiation and maturation during enchondral ossification, in growth processes, or vascularisation. Genes causing osteoarthritis in other species can also be used as candidate genes for the molecular genetic analysis of OC in horses. Studies on the variation in gene expression of key chondrogenic genes and genes associated with cartilage pathology between normal and OC chondrocytes may also help to identify candidate genes and their potential role in the pathogenesis of osteochondrosis.

Furthermore, the Equine Articular Cartilage cDNA Library can be helpful to select genes which are at least expressed in cartilage. At the moment, 13,964 equine articular ESTs can be found in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez).

Mandatory requirements for evaluating responsible genes for OC were further refinement of the QTL with a higher marker density. The lack of published microsatellites in the era before the horse genome assembly only enabled linkage studies with marker distances of about 20 to 40 cM so that it was not possible to determine exact QTL positions, let alone to detect QTL in regions with a low marker density or insufficient informative markers. A proper knowledge of the QTL helps clarifying how many positional candidate genes have to be tested for linkage disequilibrium with OC and which of these candidate genes may be responsible for OC.

Genome-wide significant QTL on equine chromosome (ECA) 5 and 16 and additionally the QTL on ECA21 were chosen for fine mapping. The release of the horse genome assembly EquCab2 made it possible to identify new microsatellites so that the average marker distances in the QTL regions could be decreased to less

than one Mb. All QTL could be successfully confirmed. On ECA5 the QTL for fetlock OC and fetlock OCD could be delimited to a region between 76.69 Mb and 92.77 Mb by the use of 49 microsatellites in total.

A QTL for hock OCD on ECA16 extended from 17.60 to 45.18 Mb and a QTL for fetlock OC could be narrowed down to an interval between 6.55 and 24.26 Mb by genotyping 56 microsatellites and 15 single nucleotide polymorphisms (SNPs).

On ECA21, 22 microsatellites were used to refine the QTL for hock OC and hock OCD to a region between 5.45 and 17.14 Mb.

In South German coldblood horses a linkage study for osteochondrosis revealed a QTL on ECA18 (Wittwer et al. 2007). This gave reason for further investigation of ECA18 in Hanoverian warmblood horses. Due to a much more evenly and densely distributed marker set of 27 microsatellites, it was possible to identify a new QTL for OC in fetlock and/or hock joints between 74.94 and 82.25 Mb.

On all mentioned chromosomes a partial consistence of the QTL for hock OC and hock OCD respectively fetlock OC and fetlock OCD leads to the assumption that the same genes may play a role in the development of this disease and that OCD is an aggravated form of OC. However, the genetic influences on the development of fetlock OC and hock OC seem to be due to different loci as the hock QTL do not map at the fetlock QTL. This seems most likely as the genetic correlations between fetlock OC and hock OC were close to zero in trotter horses (Grøndahl and Dolvik 1993), or even negative in Hanoverian warmblood horses (Stock et al. 2005).

Due to their locations in the vicinity of the identified QTL and additionally their indicated role, an amount of putative candidate genes could be identified. The QTL region on ECA5 includes *collagen type XXIV, alpha 1 (COL24A1)* at 78.1 Mb. *COL24A1* is a marker for embryonic bone formation and may play a role in regulation of type I collagen fibrillogenesis (Koch et al. 2003). Furthermore, Matsuo et al. (2008) found out that *COL24A1* is not only expressed in the forming skeleton of the mouse embryo, but also transcribed in the trabecular bone and periosteum of the newborn mouse. Due to its function in bone formation *COL24A1* seems to be a suitable functional candidate gene for osteochondrosis.

In the hock QTL on ECA16 candidate genes could be several hyaluronoglucosaminidase genes, *HYAL1, HYAL2* and *HYAL3* at 36.9 Mb. Hyaluronidases intracellularly degrade hyaluronan, one of the major glycosaminoglycans of the extracellular matrix. Hyaluronan is an important integral

structural component of articular cartilage and other tissues and acts as a lubricant in joints.

The QTL region on ECA18 includes a gene which encodes the parathyroid hormone 2 receptor (*PTH2R*). The exact function of *PTH2R* is unknown but as parathyroid hormone is a key regulator of calcium metabolism, this gene possibly plays a role in the development of osteochondrosis.

On ECA21 at 5.67 Mb, the *PIK3R1* gene encodes for the phosphoinositide-3-kinase regulatory subunit 1. *PIK3R1* is a candidate gene for osteoporosis (Huang et al. 2008) and involved in osteoblast differentiation (Zhang et al. 2007) and in the osteoblastic responses to stress (Hamamura et al. 2008). Previous studies demonstrate an involvement of *PIK3R1* on molecular mechanisms of bone repair (Li et al. 2007). This gene also seems to be one of the functional candidate genes for osteochondrosis. Further studies are necessary to evaluate their actual influence on the development of osteochondrosis, and if so, to detect functional causative mutations in this genes.

In the course of horse genome sequencing, a SNP collection was compiled which built the basis of a SNP microarray containing ~57,000 SNPs. This array is commercially available since 2008 and offers the opportunity to genotype very fast a large number of SNPs capturing a large variation of the horse genome. A whole genome association analysis was performed using the Illumina Equine SNP50 Beadchip in order to confirm and further refine the QTL and to identify new genomic regions harbouring genes responsible for osteochondrosis in Hanoverian warmblood horses.

The analysis revealed a large number of associated SNPs distributed over the already known QTL regions. Most of the SNPs were located in intergenic regions; therefore, the detection of the causal mutations for OC could not be expected based on SNP analysis. Further statistical calculations were necessary to refine the QTL regions, so we performed haplotype association and variance analyses. It was possible to show haplotypes in the QTL regions significantly associated with the different phenotypic traits of OC which could highly delimit the genomic regions harbouring QTL for osteochondrosis. Furthermore, SNPs which were included in the haplotypes explained best the proportion of phenotypic variance for the respective trait with values of about 15 to 18%. The hypothesis that the development of OC is

influenced by many different genes may explain the relatively low proportion of phenotypic variance for the respective traits.
Besides the already known QTL, we could identify further significant regions for the different phenotypic traits on ten different chromosomes, which can possibly increase the explained phenotypic variance for the respective traits. The differences in QTL discoveries among the two methods are likely related to the sample composition and the insufficient marker density in the former whole genome scan so that the power to detect QTL strongly diminishes. It seems that the previously identified QTL are family dependent, as many OC predisposing alleles segregate in different families and possibly only a few OC-alleles are distributed generally in Hanoverian warmblood horses. Using the SNP microarray, common variants will be detected and family specific alleles are at lower chance to be identified.
The present study was able to narrow down QTL regions in the horse genome. Further studies including more horses are necessary to validate the results, i.e. to verify the significant associated SNPs, also in other breeds than the Hanoverian warmblood horse. To make use of the genotypic information of highly associated SNPs without knowing the causal mutations, marker assisted selection (MAS) can be beneficial. The main advantage of the use of genotypic information is that it is available very early in life. Identification of parents carrying OC predisposing alleles can substantially improve selection for progeny with a reduced risk for OC. This is not only desirable from an economic point of view; it is also desirable from an animal welfare point of view. Selection of horses with regard to OC is currently based on radiological examination. The success of this selection mode was found to be very limited. Summing up, integrating MAS into breeding programs can be a valuable tool to lower the prevalence of OC in horses.

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CHAPTER 10

Summary

10 Summary

Virginie Lampe (2009)

Fine mapping of quantitative trait loci (QTL) for osteochondrosis in Hanoverian warmblood horses

The objective of this study was to refine quantitative trait loci (QTL) for osteochondrosis (OC) in Hanoverian warmblood horses in order to identify candidate genes with influence on the development of OC.
From a high number of QTL identified in a previous whole genome scan, genome-wide significant QTL located on ECA5 and ECA16 and a chromosome-wide significant QTL located on ECA21 were chosen for further fine mapping.
The release of the horse genome assembly EquCab2 made it possible to develop new microsatellites which enabled a decrease of the marker distances in QTL regions of less than 1 Mb. Microsatellites were genotyped for 104 progeny of 14 paternal half-sib families of Hanoverian warmblood horses, their dams and eight sires. Data was analysed using non-parametric linkage analysis based on identical by descent (IBD) mapping. Traits used were OC (fetlock and/or hock joints affected), OCD (fetlock and/or hock joints affected), fetlock OC, fetlock OCD, hock OC and hock OCD.
On ECA5, a total of 49 new microsatellites were used, which could narrow down the QTL for fetlock OCD to an interval at 76.69 to 92.77 Mb. For fetlock OC, the QTL could be refined to a region from 79.65 to 89.31 Mb.
On ECA16, altogether 56 microsatellites and 15 SNPs were genotyped in order to refine the putative QTL. The analysis revealed a delimitation of the QTL for hock OCD to an interval between 17.60 and 45.80 Mb. The QTL for fetlock OC could be narrowed down to a region from 6.55 to 24.26 Mb. Furthermore, the analysis revealed a QTL for OC in fetlock and/or hock joints at 14.38 Mb and in the region from 12.10 to 24.26 Mb for OCD in fetlock and/or hock joints.
The refinement of the QTL on ECA21 using 22 microsatellites allowed a verification of the QTL for hock OC and hock OCD at 5.45 to 17.14 Mb.

On ECA18, a new QTL for osteochondrosis in fetlock and/or hock joints could be detected between 74.94 and 82.25 Mb after increasing the marker density from seven microsatellites in the whole genome scan to a total of 27 markers.

Due to the availability of an equine SNP chip, which assays ~50,000 SNPs simultaneously, a whole genome association analysis was performed with 154 unrelated Hanoverian warmblood horses.

Focussing on the refined QTL on ECA5, 16, 18 and 21, single marker and haplotype association was performed for SNPs being located in the QTL and nearby. Haplotype association of significantly associated SNPs could narrow down the genomic regions to intervals between 76.79 and 81.10 Mb on ECA5, between 17.57 and 20.1 Mb on ECA16 for the QTL for hock OCD, between 79.36 and 80.84 Mb on ECA18 and between 8.97 and 16.18 Mb on ECA21.

Furthermore, six QTL on ECA1, 4, 14, 16, 18 and 30 were detected for the trait OC in fetlock and/or hock joints and four QTL for OCD in fetlock and/or hock joints on ECA1, 3, 30 and X. For the trait OC in fetlock joints, five different QTL on ECA2, 16, 26, 30 and X were identified, for the trait OCD in fetlock joints five QTL were observed on ECA2, 3, 13, 22 and 30. For hock OC five QTL were discovered being significant on ECA1, 4, 14, 29 and 30, for hock OCD three QTL were identified on ECA1, 6 and 30.

The refinement and identification of QTL was a first step towards the discovery of genes responsible for equine osteochondrosis. It is unlikely that any of the SNP found associated with osteochondrosis represent causal mutations, as most of them are located in intergenic regions. In order to develop a marker test, significant associated SNPs should be verified in a large sample of horses.

CHAPTER 11

Zusammenfassung

11 Erweiterte Zusammenfassung

Feinkartierung von quantitativen Merkmalsgenorten (QTL) für Osteochondrose beim Hannoverschen Warmblutpferd

Virginie Lampe

Einleitung

In den letzten Jahren sind beim Pferd Probleme mit Gelenkerkrankungen im Zusammenhang mit dem Komplex der Osteochondrose verstärkt in den Blickpunkt des Interesses gerückt, da sie Leistungseinbußen und teil hohe wirtschaftliche Verluste nach sich ziehen. Die Osteochondrose (OC) entwickelt sich bei Fohlen als Folge einer gestörten Differenzierung und Reifung der Knorpelzellen an besonders dafür disponierten Gelenken und Gelenkflächen. Bei Pferden sind besonders Fessel-, Sprung- und Kniegelenke von OC betroffen. Röntgenologisch stellt sich die OC als eine degenerative Gelenkerkrankung in Form von Konturveränderungen, Rauhigkeiten oder osteochondralen Fragmenten dar. Die Osteochondrosis dissecans (OCD) ist eine spezielle Form der Osteochondrose, bei der sich ein Knochen- oder Knorpelfragment bildet, das sich im Gelenk frei bewegt oder mit dem Knochen verbunden bleibt. Schwerwiegende osteochondrotische Veränderungen im Zusammenhang mit klinischen Symptomen wie Gelenkschwellung, Lahmheit und Schmerzen können langfristig zum Verlust des Tieres führen.

Osteochondrose kommt beim Vollblut, Warmblut, Trabern und Kaltblut mit einer Häufigkeit von bis zu 30% vor. Für die Entstehung der Osteochondrose spielen ernährungs-, haltungs-, management- und genetisch-bedingte Faktoren eine Rolle. Die genetische Komponente hat einen maßgeblichen Einfluss auf die Entwicklung der OC und stellt von allen diskutierten Einflüssen den wichtigsten dar.

In einer früheren Studie beim Hannoverschen Warmblut wurden bereits Quantitative Trait Loci (QTL) für Osteochondrose identifiziert. Diese QTL lagen auf den Pferdechromosomen 2, 3, 4, 5, 15, 16, 19 und 21. Das Ziel dieser Dissertation ist es, einen Teil der mit OC gekoppelten Genomregionen mit einer großen Anzahl neu entwickelter Marker weiter einzugrenzen. Die genaue Kenntnis der QTL sollte somit die Identifizierung potentieller Kandidatengene erleichtern. Außerdem sollten die gefundenen QTL mit einer genomweiten Assoziationsstudie von SNPs (Single

Nucleotide Polymorphisms) verifiziert und unter Umständen neue QTL aufgedeckt werden.

Feinkartierung der Quantitative Trait Loci auf den Chromosomen 5, 16, 18 und 21 des Pferdes

Material und Methoden

Pedigreematerial

Im Rahmen eines interdisziplinären Forschungsprojektes in Zusammenarbeit mit dem Hannoveraner Zuchtverband wurden Röntgenuntersuchungen an insgesamt 629 Hannoveraner Fohlen und deren Müttern der Fessel-, Sprung- und Kniegelenke durchgeführt. Ca. 68% der Fohlen wurden als Zweijährige nochmals geröntgt. 403 Fohlen (64,1%) zeigten weder Veränderungen im Sinne einer OC noch einer OCD. 118 Fohlen (18,8%) waren OCD positiv, 108 Fohlen (17,2%) OC positiv. Für den vorausgegangenen Genomscan wurden 14 väterliche Halbgeschwisterfamilien mit insgesamt 211 Pferden ausgewählt. Diese Gruppe setzte sich zusammen aus 104 Fohlen, 99 dazugehörige Mütter, sowie acht der 14 Hengste. Insbesondere wurden solche Familien verwendet, die eine hohe Frequenz von OC betroffener Halbgeschwister aufwiesen und in denen die gesamte Variationsbreite der OC-Befunde vertreten war. Es wurden nur Tiere als OC frei eingestuft, die sowohl beim ersten, als auch beim zweiten Röntgen keine Befunde aufwiesen. Die durchschnittliche Nachkommenanzahl in den ausgewählten Familien betrug 7,4. Das Verhältnis der Geschlechter war ausgewogen.
Für die Feinkartierung wurde das identische Familienmaterial verwendet, das bereits für den Genomscan eingesetzt wurde.

Identifizierung von Mikrosatelliten

Zur Identifizierung von bisher unveröffentlichten Mikrosatelliten wurde das gesamte Pferdegenom nach allen Variationen von Nukleotidwiederholungen durchsucht. Für gefundene Mikrosatelliten innerhalb der zu untersuchenden QTL wurden mit Hilfe der Programme Repeat Masker und Primer3 Primer entwickelt. Der durchschnittliche Markerabstand sollte 1 Mb nicht überschreiten. Alle Mikrosatellitenmarker wurden über PCR und Polyacrylamidgelelektrophorese ausgewertet.

Identifizierung von SNPs (Single Nucleotide Polymorphisms)

Für die Feinkartierung auf Pferdechromosom (ECA) 16 wurden neben neuentwickelten Mikrosatelliten auch Einzelbasenaustausche (SNPs) typisiert. Zur Identifizierung von SNPs wurden positionelle Kandidatengene für Osteochondrose auf ECA16 ausgewählt. Mit Hilfe von in Internetdatenbanken verfügbaren WGS und EST-Sequenzen wurden in den Kandidatengenen intronische Primer entwickelt. Die verwendeten Primer wurden mit Hilfe des Programms Primer3 nach Markierung repetitiver Bereiche mit dem Programm Repeat Masker entwickelt. Die SNPs wurden an den DNA-Sequenzen von acht Hannoveraner Hengsten aus den Halbgeschwisterfamilien identifiziert. Die PCR-Produkte wurden auf einem Kapillarsequenziergerät (Megabace 1000) sequenziert und mit Hilfe der Sequencher 4.7 Software auf Polymorphismen untersucht. So konnten 15 SNPs gefunden werden, die anschließend für die gesamten 14 Familien ebenfalls genotypisiert wurden.

Statistische Analyse

Eine nicht-parametrische Kopplungsanalyse wurde unter Verwendung der Software MERLIN (multipoint engine for rapid likelihood inference, Version 1.1.2) durchgeführt und basierte auf dem „identical-by-descent“ (IBD) Verfahren (Abecasis et al. 2002). In dem sogenannten „Pairs“ Modus (paarweiser Vergleich mit gleichmäßiger Gewichtung der betroffenen Tiere) wurden Markerallele chromosomenweit zwischen Paaren von Geschwistern bzw. Verwandten auf Kosegregation mit der phänotypischen Ausprägung der Erkrankung getestet. Darauf folgend wurde die einer Normalverteilung folgende Teststatistik für den Anteil von IBD-Markerallelen (Zmean) und ein daraus abgeleiteter LOD-Score berechnet. Als signifikant für die Kosegregation eines Markerallels mit dem Phänotyp der OC gelten Irrtumswahrscheinlichkeiten (p) von 0,05 oder kleiner. Für die Merkmale OC, OCD, OC im Fesselgelenk, OCD im Fesselgelenk, OC im Sprunggelenk und OCD im Sprunggelenk wurden getrennte Berechnungen durchgeführt.

Außerdem wurden die Genotypen der Marker mit den Prozeduren ALLELE, CASECONTROL und HAPLOTYPE von SAS/Genetics, Version 9.2 ausgewertet. Mittels X^2-Tests zwischen Genotypen, Allelen, Allelvorkommen und OC, sowie zwischen Haplotypen und OC wurde geprüft, ob für diese Tiere eine signifikante

Assoziation zwischen dem jeweiligen Phänotyp und dem Marker, bzw. Haplotyp besteht.

Ergebnisse für Chromosom 5

Zur Feinkartierung der genomischen Region auf ECA5 wurden insgesamt 49 Mikrosatelliten typisiert, die sich auf 21 Mb erstreckten. Dadurch konnte ein QTL für Fesselgelenks-OCD auf einen Bereich von 76.69 bis 92.77 Mb eingegrenzt werden. Dieser QTL erreichte in einem Intervall zwischen 78.03 and 90.23 Mb sogar genomweite Signifikanz. Einzelne Mikrosatelliten in diesem Bereich waren auch mit Fesselgelenks-OCD assoziiert. Innerhalb dieses QTL konnte das COL24A1-Gen als ein potentielles Kandidatengen identifiziert werden. COL24A1 spielt sowohl in der embryonalen Knochenentwicklung, als auch in der Kollagen-Fibrillogenese eine Rolle.

Ergebnisse für Chromosom 16

Auf ECA16 wurden insgesamt 56 Mikrosatelliten und 15 SNPs in einem Bereich von 0.49 bis 52.38 Mb genotypisiert. Die nicht-papametrische Kopplungsanalyse erbrachte QTL für OC bei 14.38 Mb und für OCD zwischen 12.10 und 24.26 Mb. Außerdem konnte der QTL für Fesselgelenks-OC auf einen Bereich von 6.55 bis 24.26 Mb und der QTL für Sprunggelenks-OCD auf 17.60 bis 45.80 Mb eingegrenzt werden. In diesem Intervall konnten auch mit Sprunggelenks-OCD assoziierte Mikrosatelliten und SNPs, sowie aus diesen Markern gebildete assoziierte Haplotypen identifiziert werden. Auch hier konnten potentielle Kandidatengene (HYAL1, HYAL2 und HYAL3) gefunden werden. Diese Gene kodieren für Hyaluronidasen, welche Hyaluronan degradieren, einer wichtigen strukturellen Komponente artikulären Knorpels.

Ergebnisse für Chromosom 18

Auf ECA18 wurde die Markerdichte von sieben Mikrosatelliten im Genomscan auf 27 Mikrosatelliten erhöht. Dadurch konnte ein bisher nicht bekannter QTL für OC in Fessel- und/oder Sprunggelenken in einer Region zwischen 74.94 und 82.25 Mb aufgedeckt werden. In diesem Bereich ist das PTH2R-Gen lokalisiert, welches für einen Parathyroidhormon-Rezeptor kodiert. Da Parathyroidhormon ein

Hauptregulator im Calcium-Metabolismus ist, scheint dieses Gen ein mögliches Kandidatengen für Osteochondrose zu sein.

Ergebnisse für Chromosom 21

In der genomischen OC-Region auf ECA21 wurden 22 Mikrosatelliten typisiert, was eine Eingrenzung des QTL für Sprunggelenks OC und Sprunggelenks-OCD auf einen Bereich von 5.45 bis 17.14 Mb erbrachte. Dabei wurde ein durchschnittlicher Markerabstand von 2.45 Mb auf dem gesamten Chromosom 21, und ein Markerabstand von 1.25 Mb im QTL erzielt. Im QTL konnte wiederum ein mögliches Kandidatengen bestimmt werden. Das PIK3R1-Gen ist zum Einen ein Kandidatengen für Osteoporose, zum Anderen beteiligt and der Differenzierung der Osteoblasten und an der Reaktion der Osteoblasten auf Stress. Außerdem konnte eine Beteiligung des Gens an der Knochenheilung nachgewiesen werden.

Diskussion

Die Entwicklung und Analyse von Mikrosatellitenmarkern ist für die Feinkartierung von QTL über Kopplungs- und Assoziationsstudien sehr hilfreich. Es konnten alle untersuchten QTL bestätigt und darüber hinaus die exakte Ausdehnung bestimmt werden. Außerdem konnte durch eine dichte und gleichmäßige Markerabdeckung ein neuer QTL gefunden werden.

Die Ergebnisse der Feinkartierung lassen vermuten, dass die Genorte auf den Chromosomen 5, 16, 18 und 21 Gene beherbergen, die an der Entstehung der Osteochondrose beteiligt sind. Des Weiteren legt die Tatsache, dass zahlreiche QTL identifiziert werden konnten, die Vermutung nahe, dass mehrere Gene an der Entstehung von Osteochondrose beteiligt sein müssen. Außerdem kann man davon ausgehen, dass die Erkrankung in den Fesselgelenken von anderen Genen determiniert wird, als OC/OCD in den Sprunggelenken, da sich QTL für die unterschiedlichen Lokalisationen selten decken. Zur weiteren Aufklärung müssen Kandidatengene auf Mutationen untersucht werden.

Genomweite Assoziationsanalyse für Osteochondrose mittels eines SNP-Chips

Material und Methoden

Pedigreematerial

Es wurden aus dem erwähnten Gesamtmaterial 154 Hannoveraner Warmblutpferde ausgewählt, die von insgesamt 52 verschiedenen Hengsten abstammen. 90 Tiere waren von OC betroffen, 43 davon ausschließlich in der Fessel, 33 Tiere nur im Sprunggelenk und 14 sowohl in Fessel- als auch in Sprunggelenken. Zusätzlich dienten 64 Tiere als gesunde Kontrollen. Von diesen Pferden waren 86 Tiere männlich und 68 Tiere weiblich. 39 dieser Pferde wurden bereits im Genomscan und den Feinkartierungen eingesetzt.

Analyse der SNPs

Mit einem seit 2008 kommerziell erhältlichen SNP-Chip wurden 54.602 einzelne SNPs in einem Arbeitsschritt durch ein automatisiertes Verfahren auf einer Illumina-Plattform genotypisiert. Diese SNPs wurden mit der Software PLINK Version 1.05 auf Assoziation mit den einzelnen Merkmalen der OC getestet. Außerdem wurden aus assoziierten SNPs im Bereich der bekannten QTL Haplotypen gebildet und diese mit der Prozedur HAPLOTYPE von SAS/Genetics, Version 9.2 auf Assoziation der Haplotypen mit OC untersucht. Des Weiteren wurde die Proportion der erklärten genotypischen Varianz der assoziierten Haplotypen mit der Prozedur GLM überprüft.

Ergebnisse

Von den 54.602 SNPs konnten 51.020 SNPs in die Analyse einfliessen. In den QTL auf den Chromosomen 5, 16, 18 und 21 wurden eine große Anzahl assoziierter SNPs gefunden. Daraus konstruierte Haplotypen konnten daraufhin die einzelnen QTL weiter eingrenzen. Der QTL auf ECA5 beschränkt sich nunmehr auf ein Intervall von 76.79 bis 81.10 Mb. Auf ECA16 wurde der QTL für Sprunggelenks-OCD zwischen 17.57 und 20.1 Mb eingegrenzt. Haplotypen auf ECA18 verringerten den QTL auf eine Region zwischen 79.36 and 80.84 Mb und auf ECA21 auf einen Bereich von 8.97 bis 16.18 Mb.

Es konnten außerdem neue QTL identifiziert werden, wobei dafür mindestens drei benachbarte SNPs hoch signifikant mit dem entsprechenden Merkmal assoziiert sein mussten. Für OC in Fessel- und/oder Sprunggelenken wurden fünf QTL auf ECA1, 4,

14, 16, 18 und 30, für OCD in Fessel- und/oder Sprunggelenken vier QTL auf ECA1, 3, 30 und X entdeckt. Für Fesselgelenks-OC wurden fünf QTL auf den Chromosomen 2, 16, 26, 30 und X und für Fesselgelenks-OCD fünf QTL auf ECA2, 3, 13, 22 und 30 nachgewiesen. Fünf QTL auf den Chromosomen 1, 4, 14, 29 und 30 für OC in Sprunggelenken, sowie drei signifikante genomische Regionen für Sprunggelenks-OCD auf ECA1, 6 und 30 konnten ebenfalls neu identifiziert werden.

Diskussion

Diese Studie ist die erste, die über die Untersuchung komplexer erblicher Erkrankungen beim Pferd mittels SNP-Microarray-Technologie berichtet. Mit dem neuerdings kommerziell erhältlichen SNP-Chip bietet sich nun auch für das Pferd eine einfache und kostengünstige Möglichkeit, genomweite Untersuchungen mit enorm hoher Auflösung durchzuführen. Allein durch diese enge Abdeckung des gesamten Pferdegenoms mit SNPs war es möglich, eine große Anzahl neuer QTL für Osteochondrose zu entdecken, was mit der Markerdichte des vorausgegangenen Genomscans nicht erzielt werden konnte. Außerdem wurden primär andere genomische Regionen identifiziert, was möglicherweise auf eine andere Zusammensetzung des Probandenmaterials zurückzuführen ist. Im Genomscan wurden 14 Familien typisiert, was annehmen lässt, dass die dadurch identifizierten QTL unter Umständen familiär bedingt sind. Nichtsdestotrotz konnten die durch den Genomscan ermittelten QTL mittels zusätzlicher Analysen verifiziert und weiter eingegrenzt werden. Es ist nicht davon auszugehen, dass es sich bei den typisierten SNPs um kausale Mutationen handeln könnte, da eine Vielzahl der SNPs lediglich intergenisch lokalisiert ist. Allerdings vereinfacht diese neue Technologie die Kandidatengenauswahl immens, da QTL auf eine begrenzte Ausdehnung reduziert werden können. Um einen Markertest entwickeln zu können, sollten diese Ergebnisse an einer größeren Gruppe an Pferden überprüft werden. Um den Schritt von assoziierten Markern zur Identifizierung kausaler Mutationen gehen zu können, sind weitere Untersuchungen notwenig, möglicherweise ist dazu eine neue Generation des SNP-Chips mit noch höherer Auflösung hilfreich.

CHAPTER 12

Appendix

12 Appendix

Laboratory paraphernalia

Equipment

Thermocycler

PTC-100™ Programmable Thermal Controller (MJ Research, Watertown, USA)

PTC-100™ Peltier Thermal Cycler (MJ Research, Watertown, USA)

PTC-200™ Peltier Thermal Cycler (MJ Research, Watertown, USA)

Biometra TProfessional Thermocycler (Biometra, Göttingen, Germany)

Automated sequencers

LI-COR Gene Read IR 4200 DNA Analyzer (LI-COR, Inc., Lincoln, NE, USA)

LI-COR Gene Read IR 4300 DNA Analyzer (LI-COR, Inc., Lincoln, NE, USA)

MegaBACE 500 (Amersham Biosciences, Freiburg)

Centrifuges

Sigma centrifuge 4-15 (Sigma Laborzentrifugen GmbH, Osterode)

Desk-centrifuge 5415D (Eppendorf, Hamburg)

Biofuge stratos (Heraeus, Osterode)

Megafuge 1. OR (Heraeus, Osterode)

Speed Vac® Plus (Savant Instruments, Farmingdale, NY, USA)

Agarose gel electrophoresis and pulsed field gel electrophoresis

Electrophoresis chambers	OWL Separation Systems, Portsmouth, NH, USA
	Biometra, Göttingen
	BioRad, München
Generators	2301 Macrodrive 1 (LKB, Bromma, Sweden)
	Power Pac 3,000 (BioRad, München)
Gel documentation system	BioDocAnalyze 312 nm, Göttingen

Pipettes

Multipette® plus (Eppendorf AG, Hamburg, Germany)

Pipetus®-akku (Hirschmann® Laborgeräte GmbH & Co.KG, Eberstadt, Germany)

Pipetman® (P2, P10, P20, P100, P200, P1000) (Gilson Medical Electronics S.A., Villiers-le-bel, France)
Pipettor, Multi 12 Channel (0.1 – 10 µl) (Micronic® systems, Lelystad, The Netherlands)
12 Channel Manual Pipettor (0.5 – 10 µL) (Matrix Technologies Corporation, Cheshire UK)
12 Channel Manual Pipettor (25 – 200 µL) (Matrix Technologies Corporation, Cheshire UK)
8-Channel, gel loading syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland)

Others
Milli-Q® biocel water purification system (Millipore GmbH, Eschborn, Germany)
Incubator VT 5042 (Heraeus, Osterode)
UV-Illuminator 312 nm (Bachhofer, Reutlingen)
IKA® MTS 2/4 digital (IKA®-Werke GmbH & Co. KG, Staufen)
Certomat® H (B. Braun, Melsungen AG, Melsungen)
Certomat® R (B. Braun, Melsungen AG, Melsungen)

Kits
Isolation of DNA
QIAamp 96 DNA Blood Kit (QIAGEN, Hilden, Germany)
NucleoSpin Kit 96 Blood Quick Pure Kit (Macherey-Nagel, Düren, Germany)

DNA purification
Montage PCR_{96} Cleanup Kit (Millipore GmbH, Eschborn, Germany)
MinElute® 96 UF Plate (QIAGEN, Hilden, Germany)
AutoSeq™ 96 Plate (GE Healthcare, Freiburg, Germany)

Sequencing
DYEnamic-ET-Terminator Cycle Sequencing Kit (GE Healthcare, Freiburg, Germany)

Genotyping
TaqMan® SNP genotyping assay (Applied Biosystems, Darmstadt)
SensiMix DNA Kit (Quantance Ltd., London, Great Britain)

Size standards

100 bp Ladder (New England Biolabs, Schwalbach Taunus)

1 kb Ladder (New England Biolabs, Schwalbach Taunus)

IRDye™ 700 or 800 (LI-COR, Inc., Lincoln, NE, USA)

Primers

Primers were produced by MWG-Biotech AG, Ebersberg, Germany and biomers.net GmbH, Ulm, Germany

Reagents and buffers

APS solution (10 %)

1 g APS

10 ml H_2O

Bromophenol blue solution

0.5 g bromophenol blue

10 ml 0.5 M EDTA solution

H_2O ad 50 ml

dNTP solution

100 µl dATP [100 mM]

100 µl dCTP [100 mM]

100 µl dGTP [100 mM]

100 µl dTTP [100 mM]

1600 µl H_2O

the concentration of each dNTP in the ready-to-use solution is 5 mM

Gel solution (6%)

12.75 ml Urea/TBE solution (6%)

2.25 ml Rotiphorese® Gel 40 (38% acrylamide and 2% bisacrylamide)

95 µl APS solution (10%)

9.5 µl TEMED

Loading buffer for agarose gels

EDTA, pH 8	100 mM
Ficoll 400	20% (w/v)
Bromophenol blue	0,25% (w/v)
Xylencyanol	0,25% (w/v)

Loading buffer for gel electrophoresis

2 ml	bromophenol blue solution
20 ml	formamide

TBE-buffer (1x)

100 ml TBE-buffer (10x)

900 ml H_2O

TBE-buffer (10x)

108 g Tris [121.14 M]

55 g boric acid [61.83 M]

7.44 g EDTA [372.24 M]

H_2O ad 1000 ml

pH 8.0

Urea/TBE solution (6 %)

425 g urea [60.06 M]

250 ml H_2O

100 ml TBE-buffer (10x)

solubilise in a water bath at 65°C

H_2O ad 850 ml

Chemicals

Agarose (Invitrogen, Paisley, UK)

Ammonium persulfate (APS) ≥ 98 % (Sigma-Aldrich Chemie GmbH, Taufkirchen)

Boric acid ≥ 99.8 %, p.a. (Carl Roth GmbH & Co, Karlsruhe)

Bromophenol blue (Merck KgaA, Darmstadt)

dATP, dCTP, dGTP, dTTP > 98% (Carl Roth GmbH & Co. KG, Karlsruhe)

DMSO ≥ 99.5 %, p.a. (Carl Roth GmbH & Co, Karlsruhe)
dNTP-Mix (Qbiogene GmbH, Heidelberg)
EDTA ≥ 99 %, p.a. (Carl Roth GmbH & Co, Karlsruhe)
Enhancer solution P 5x (peqlab Biotechnologie GmbH, Erlangen)
Ethidium bromide (Carl Roth GmbH & Co, Karlsruhe)
Ethyl alcohol (AppliChem, Darmstadt)
Formamide ≥ 99.5 %, p.a. (Carl Roth GmbH & Co, Karlsruhe)
Natriumdihydrogenphosphat (Biochrom AG, Berlin)
Paraffin (Merck KgaA, Darmstadt)
Rotiphorese®Gel40 (Carl Roth GmbH & Co, Karlsruhe)
Sephadex^{TM} G-50 Superfine (Amersham Biosciences, Freiburg)
TEMED 99 %, p.a. (Carl Roth GmbH & Co, Karlsruhe)
Tris PUFFERAN® ≥ 99.9 %, p.a. (Carl Roth GmbH & Co, Karlsruhe)
Urea ≥ 99.5 %, p.a. (Carl Roth GmbH & Co, Karlsruhe)
Water was taken from the water purification system Milli-Q®

Enzymes

PCR

Taq-DNA-Polymerase 5 U/µl (Qbiogene GmbH, Heidelberg)
Incubation Mix (10x) T.Pol with $MgCl_2$ [1.5mM] (Qbiogene, Heidelberg)
The polymerase was always used in the presence of Incubation Mix T. Pol 10x buffer

RFLP

The enzymes used have been produced by New England Biolabs, Frankfurt, Germany, Fermentas St. Leon-Rot, Germany or Roche, Mannheim, Germany
All enzymes were used with the adequate 10x enzyme buffer

Consumables

Thermo-fast 96 well plate, skirted (ABgene, Hamburg, Germany)
PCR-Plate PP, nature, 96x0.2ml, skirted, RNase-, DNA- und pyrogenfree (nerbe plus, Winsen/Luhe, Germany)
Adhesive PCR Film (nerbe plus, Winsen/Luhe, Germany)
Adhesive PCR Film (ABgene, Hamburg, Germany)
Combitips® plus (Eppendorf AG, Hamburg, Germany)

Pipette tips 0.1 – 10 µl (K138.1), 0.1 – 10 µl (A407.1), 5 – 200 µl (7058.1) (Carl Roth GmbH & Co, Karlsruhe, Germany)

Pipette tips 0.1 – 10 µl (7600) (Matrix Technologies Corporation, Lowell, USA)

Reaction tubes 1.5 ml and 2.0 ml (nerbe plus GmbH, Winsen/Luhe, Germany)

Reaction tubes 10 and 50 ml (Falcon) (Renner, Darmstadt, Germany)

Software

BLASTN, trace archive	http://www.ncbi.nlm.nih.gov
BLAT Search Genome	http://genome.ucsc.edu/cgi-bin/hgBlat
ENSEMBL Genome browser	http://www.ensembl.org/index.html
HORSEMAPdatabase	http://dga.jouy.inra.fr/cgibin/lgbc/loci_ micro.operl?BASE=horse
MERLIN software package version 1.0.1	http://www.sph.umich.edu/csg/abecasis/Merlin
Order of TaqMan® SNP genotyping assay	https://products.appliedbiosystems.com
Order of primers	MWG Biotech-AG, Ebersberg (https://ecom. mwgdna.com/register/index.tcl) biomers.net GmbH, Ulm (order@biomers.net)
Order of enzymes	http://www.neb.com/nebecomm/products/ categories.asp
PED5.0	Dr. H. Plendl et al. (2005) Institute for Human Genetics, Kiel
HaploPainter V.029.5	http://haplopainter.sourceforge.net/html/index.html
Haploview V.4.1	http://www.broad.mit.edu/mpg/haploview/
Primer design (Primer3)	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_ www.cgi
Repeat Masker	http://www.repeatmasker.genome. washington.edu/
Sequencher 4.7	GeneCodes, Ann Arbor, MI, USA
Spidey	http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/ Spidey/index.html
SUN Ultra Enterprise 450	Sun microsystems GmbH, Kirchheim-Heimstetten
SUN FIRE V490	Sun microsystems GmbH, Kirchheim-Heimstetten

Abbreviations of gene names

ACAN	aggrecan
ADAM17	metallopeptidase domain 17
ADAM23	metallopeptidase domain 23
ADAMTS-4	ADAM metallopeptidase with thrombospondin type 1 motif, 4
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9
AK5	adenylate kinase 5
ANKH	ankylosis, progressive homolog (mouse)
AOAH	acyloxyacyl hydrolase
AR	androgen receptor
ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3
ARL6IP5	ADP-ribosylation-like factor 6 interacting protein 5
ARPP-21	cyclic AMP-regulated phosphoprotein, 21 kD
ASB14	ankyrin repeat and SOCS box-containing 14
ASPN	asporin
ATP7B	ATPase, Cu++ transporting, beta polypeptide
BMP-2	bone morphogenetic protein 2
BMP-6	bone morphogenetic protein 6
BXDC5	brix domain containing 5
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit
CADPS	Ca++-dependent secretion activator
CALM1	calmodulin 1 (phosphorylase kinase, delta)
CART1	cartilage homeoprotein 1
CASR	calcium-sensing receptor
CCDC13	coiled-coil domain containing 13
CCL2	chemokine (C-C motif) ligand 2
CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
CHDH	choline dehydrogenase
CLCN7	chloride channel 7
COL1A1	collagen, type I, alpha 1
COL1A2	collagen, type I, alpha 2
COL3A1	collagen, type III, alpha 1

COL5A1	collagen, type V, alpha 1
COL5A2	collagen, type V, alpha 2
COL9A1	collagen, type IX, alpha 1
COL9A2	collagen, type IX, alpha 2
COL9A3	collagen, type IX, alpha 3
COL10A1	collagen, type X, alpha 1
COL11A1	collagen, type XI, alpha 1
COL11A2	collagen, type XI, alpha 2
COL24A1	collagen type XXIV, alpha 1
COL28A1	collagen type XXVIII, alpha 1
Coll-I	collagen type I
Coll-II	collagen type II
Coll-X	collagen type X
CRTAP	cartilage associated protein
CTH	cystathionase (cystathionine gamma-lyase)
CTSK	cathepsin K
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9
CYR61	cysteine-rich, angiogenic inducer, 61
DCAMKL3	doublecortin-like kinase 3
DDAH1	dimethylarginine dimethylaminohydrolase 1
DKK1	dickkopf homolog 1 (Xenopus laevis)
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4
DOCK3	dedicator of cytokinesis 3
ELTD1	EGF, latrophilin and seven transmembrane domain containing 1
ENTPD3	ectonucleoside triphosphate diphosphohydrolase 3
ESR1	estrogen receptor 1
ESR2	estrogen receptor 2 (ER beta)
FAM73A	family with sequence similarity 73, member A
FASTKD2	FAST kinase domains 2
FLNA	filamin A, alpha (actin binding protein 280)
FRMD4B	FERM domain containing 4B
FRZB	frizzled-related protein
FUBP1	far upstream element (FUSE) binding protein 1

GDF5	growth differentiation factor 5
GIPC2	PDZ domain protein GIPC2
Gli1	glioma-associated oncogene homolog 1 (zinc finger protein)
Gli3	glioma-associated oncogene homolog 3 (zinc finger protein)
GLT8D4	glycosyltransferase 8 domain containing 4
GRM7	glutamate receptor, metabotropic 7
HS2ST1	heparan sulfate 2-O sulfotransferase 1
HTRA1	HtrA serine peptidase 1
HYAL1	hyaluronoglucosaminidase 1
HYAL2	hyaluronoglucosaminidase 2
HYAL3	hyaluronoglucosaminidase 3
ICA1L	islet cell autoantigen 1,69kDa-like
IGF-I	insulin-like growth factor
IGSF10	immunoglobulin superfamily, member 10
Ihh	Indian hedgehog
IL2RB	interleukin 2 receptor, beta
IL2RG	interleukin 2 receptor, gamma (severe combined immunodeficiency)
IL17A	interleukin 17A
IL32	interleukin 32
ITGA1	integrin, alpha 1
ITGA9	integrin, alpha 9
KIF15	kinesin family member 15
LEP	leptin
LGALS3	lectin, galactoside-binding, soluble, 3
LRRC7	leucine rich repeat containing 7
LTBP3	latent transforming growth factor beta binding protein 3
LRCH1	leucine-rich repeats and calponin homology (CH) domain containing 1
LUM	lumican
MAP3K2	mitogen-activated protein kinase kinase kinase 2
MATN3	matrilin 3
MEFV	Mediterranean fever
MMP-1	matrix metalloproteinase 1

MMP-3	matrix metalloproteinase 3
MMP-13	matrix metalloproteinase 13
MYL3	myosin, light chain 3, alkali; ventricular, skeletal, slow
NEGR1	neuronal growth regulator 1
OSBPL10	oxysterol binding protein-like 10
P4HA3	prolyl 4-hydroxylase, alpha polypeptide III
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2
PARD3B	par-3 partitioning defective 3 homolog B (C. elegans)
PDZRN3	PDZ domain containing ring finger 3
PHEX	phosphate regulating endopeptidase homolog, X-linked
PIGK	phosphatidylinositol glycan, class K
PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2 (beta)
PIP5K3	phosphatidylinositol-3-phosphate/phosphatidylinositol 5-kinase, type III
POU1F1	pituitary specific transcription factor 1
PPP4R2	protein phosphatase 4, regulatory subunit 2
PRDX5	peroxiredoxin 5
PRG4	proteoglycan 4
PRKACB	protein kinase, cAMP-dependent, catalytic, beta
Ptc1	patched homolog 1
PTGER3	prostaglandin E receptor 3 (subtype EP3)
PTH	parathyroid hormone
PTHLH	parathyroid hormone-like hormone
PTH2R	parathyroid hormone 2 receptor
PTHR1	parathyroid hormone receptor 1
PTHrP	parathyroid-hormone related peptide
PTPRG	protein tyrosine phosphatase, receptor type, G
PXK	PX domain containing serine/threonine kinase
RAB3C	RAB3C, member RAS oncogene family
RARRES2	retinoic acid receptor responder (tazarotene induced) 2
SCAP	SREBF chaperone
SEP15	15 kDa selenoprotein
SFRS11	splicing factor, arginine/serine-rich 11
SH3GLB1	SH3-domain GRB2-like endophilin B1

SHQ1	SHQ1 homolog (S. cerevisiae)
SLC26A2	solute carrier family 26 (sulfate transporter), member 2
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1
Smo	Smoothened
ST6GALNAC3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3
SYNPR	synaptoporin
TGFß1	transforming growth factor beta 1
TIMP-1	TIMP metallopeptidase inhibitor 1
TIMP-2	TIMP metallopeptidase inhibitor 2
TIMP-3	TIMP metallopeptidase inhibitor 3
TMF1	TATA element modulatory factor 1
TNNI3K	TNNI3 interacting kinase
TNXB	tenascin XB
TP53	tumor protein p53
TRAPPC2	trafficking protein particle complex 2
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor
WDR63	WD repeat domain 63
WISP3	WNT1 inducible signaling pathway protein 3
WNT5A	wingless-type MMTV integration site family, member 5A
ZNHIT6	zinc finger, HIT type 6
ZRANB2	zinc finger, RAN-binding domain containing 2

CHAPTER 13

List of publications

13 List of publications

Journal articles

Dierks C, Löhring K, **Lampe V**, Wittwer C, Drögemüller C, Distl O (2007). Genome-wide search for markers associated with osteochondrosis in Hanoverian warmblood horses. Mammalian Genome 18. 739-747.

Lampe V, Dierks C, Distl O (2009). Refinement of a quantitative trait locus on equine chromosome 5 responsible for fetlock osteochondrosis in Hanoverian warmblood horses. Animal Genetics, in press.

Lampe V, Dierks C, Distl O (2009). Refinement of a quantitative gene locus on equine chromosome 16 responsible for osteochondrosis in Hanoverian warmblood horses. Animal Journal, in press.

Lampe V, Dierks C, Komm K, Distl O (2009). Identification of a new quantitative trait locus on equine chromosome 18 responsible for osteochondrosis in Hanoverian warmblood horses. Journal of Animal Sciene, in review.

Felicetti M, **Lampe V**, Ehrhardt S, Cappelli K, Supplizi AV, Silvestrelli M, Distl O (2009). Mapping of a quantitative trait locus on equine chromosome 21 responsible for osteochondrosis in hock joints of Hanoverian warmblood horses. Submitted for publication.

Oral presentations

Lampe V, Dierks C, Wittwer C, Distl O: SNP markers for osteochondrosis in horses. 58th Annual Meeting of the European Association for Animal Production, August 26-29 2007, Dublin, Ireland. Received Best Paper Award in Horse session.

Lampe V, Dierks C, Distl O: Insights into equine osteochondrosis based on genetic markers in Hanoverian warmblood horses. 31. International Conference on Animal Genetics, July 20-24 2008, Amsterdam, The Netherlands.

Lampe V, Dierks C, Komm K, Distl O: Molekulargenetische Analyse der Osteochondrose beim Hannoverschen Warmblutpferd. Göttinger Pferdetage, Feburary 25-26 2009, Göttingen, Germany.

CHAPTER 14

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14 Acknowledgements

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