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Molecular genetic analyses for left-sided displacement of the abomasum in German Holstein cattle

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For Jens

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List of abbreviations

ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2 gene
bp	Base pair
BTA	*Bos taurus* chromosome
cDNA	Complementary DNA
cM	Centi Morgan
DA	Displacement of the abomasum
DGAT1	Diacylglycerol O-acyltransferase 1 gene
DNA	Deoxyribonucleic acid
EBV	Estimated breeding values
FP	Milk fat percentage
FY	Milk fat yield
GH	German Holstein
GWAS	Genome-wide association study
INS	Insertion
kb	Kilobases
LDA	Left-sided displacement of the abomasum
LPL	Length of productive life
Mb	Megabases
MLN	Motilin gene
mRNA	Messenger RNA
MY	Milk yield
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
PP	Milk protein percentage
PY	Milk protein yield
QTL	Quantitative trait loci
OMIA	Online Mendelian Inheritance in Animals database
RNA	Ribonucleic acid
SCS	Somatic cell score
SNP	Single nucleotide polymorphism
UTR	Untranslated region
WT	Wild type

Preamble

This postdoctoral thesis provides an overview of different studies, which were performed to analyse the molecular genetic aspects of left-sided displacement of the abomasum (LDA) in German Holstein cattle. LDA is economically important and frequently observed in many dairy cattle breeds. With an estimated heritability of up to 50%, genetics play a major role in the predisposition of a cow for this disease. The intention of the present work therefore was to detect genomic regions and causal genes influencing the affection risk or the course of the disease. This facilitates the development of a genetic test and gives insights into the etiopathogenesis of LDA.

The introduction (chapter I) gives an overview on the current progress made in mapping the bovine genome and modern SNP genotyping arrays. Furthermore, a survey is given on LDA including is genetics and analogies in other species.
A list of publications, which are part of this work, is supplied in chapter II.
Chapter III shows the results of each of these publications and in chapter IV, these results are discussed. The methodical details are available in the original manuscripts.
The two following chapters, chapters V and VI, provide English and German summaries of the main issues of this work.
Chapter VII provides the literature cited in this work. The own contributions to the scientific work are characterized in chapter VIII.

I Introduction

1.1 Mapping the bovine genome

Knowledge of structure and genetic variants in the bovine genome are of immense importance in cattle breeding to advance genetic selection for milk performance and meat production. At present, two main versions of the bovine genome exist, which are currently updated: the UMD 3.1 *bos taurus* assembly and the Baylor Btau 4.6.1/bosTau7 assembly. The UMD 3.1 *bos taurus* assembly was created in 2009 by the Center for Bioinformatics and Computational Biology, University of Maryland, based on original sequences generated by the Baylor College of Medicine (Zimin et al. 2009). For this purpose, 35 million sequence blocks were arranged and provide a 9.5-fold coverage of the bovine genome. The complete UMD 3.1 *bos taurus* genome comprises 2.67 billion base pairs and 19,994 coding genes, 3,825 non-coding genes and 797 pseudo genes. It further contains 11,890 structural variants as for example copy number variants or inversions, and 9,247,534 smaller sequence variants as single nucleotide polymorphisms (SNPs), small insertions or deletions, or somatic mutations (http://www.ensembl.org/Bos_taurus/ Info/Annotation/#assembly). Baylor Btau 4.6.1/bosTau7 was generated by the Human Genome Sequencing Center at the Baylor College of Medicine (Bovine Genome Sequencing and Analysis Consortium et al. 2009) and contains 2.98 billion base pairs. This genome is based on one cow and one bull of the Hereford cattle breed. It provides a 7.1-fold coverage of the genome. BAC (bacterial artificial chromosome) sequences as well as WGS (whole genome shotgun) sequences were used to build this genome, wherefore it is called a mixed assembly.

One main aspect in molecular genetic research is the detection of associations between sequence variants and hereditary phenotypes. These sequence variants are predominantly SNPs, which occur every 100 to 300 base pairs on the average. To date, databases contain a large number of polymorphisms, which are publicly available (Bovine Genome Sequencing and Analysis Consortium et al. 2009, Bovine HapMap Consortium et al. 2009). This facilitates more efficient and comprehensive association analyses for genetic diseases or desired traits. The NCBI dbSNP database for short genetic variants presently contains a total of 13,704,221 different SNPs, of which 4,803,553 are located intragenic (http://www.ncbi.nlm.nih.gov/SNP/ snp_summary.cgi). According to the Online Mendelian Inheritance in Animals (OMIA) database, 400 genetic disorders are currently known in cattle, 81 of which are mendelian disorders with already known key mutation (http://omia.angis.org.au/home/).

1.1.1 SNP genotyping on microarrays

Traditionally, the flow path of detection causal mutations for hereditary phenotypes starts with an initial linkage analysis. However, genome-wide linkage analyses usually result in concrete, but comparatively big quantitative trait loci (QTL). These locations have to be narrowed down by genome-wide association studies (GWAS) using a dense set of markers. For this purpose, the SNP-microarray technology is employed using so-called SNP chips. These microarrays facilitate simultaneous genotyping of each sample for all markers contained on the chip and therefore provide a highly efficient method of analysis. Microarray technology has proven suitable not only for investigation of monogenetically inherited traits (Charlier et al. 2008), but also for mapping genetic heterogenous diseases and quantitative traits in cattle (Kim et al. 2011, Jiang et al. 2010).

At present, SNP-microarrays are available mainly of the two manufacturers Illumina (Illumina, San Diego, CA, USA) and Affymetrix (Affymetrix, Santa Clara, CA, USA). Illumina provides the Bovine SNP 50 beadchip, containing more than 54,000 genome-wide equidistantly distributed SNPs, as the basis product. These markers were approved in 19 different cattle breeds. In addition, they merchandise the Bovine HD beadchip, which provides more than 777,000 genome-wide equidistantly distributed markers and therefore a much higher resolution. The markers were verified for 20 taurin cattle breeds, three zebu breeds, and four hybrids.

Affymetrix also offers two SNP chips with differing marker density. The Targeted Genotyping Bovine 25K SNP Panel contains about 25,000 SNP genome-wide equidistantly distributed markers, while the Axiom Genome-Wide BOS 1 Array contains more than 640,000 SNPs equidistantly distributed over the bovine genome. The markers of this array were validated in 20 different cattle breeds.

The Illumina Bovine SNP 50 beadchip is presently also used to facilitate genomic selection in German Holstein dairy cattle (Thaller 2009). However, selection in this breed mainly focuses on milk performance, somatic cell scores, fertility, calving ease, and length of functional longevity to increase the economic profit. In the past, selection for these desired traits has been accomplished through a total merit index. Due to the more complicated collection of individual disease data, many frequent diseases in dairy cattle have not been considered yet.

1.2 Left-sided displacement of the abomasum

Health problems in dairy cattle affect the animal well-being and cause considerable economic losses for the farmers. The first case report for a displaced abomasum was published in 1898 by Carougeau and Prestat, but till the nineteen-fifties it was no common health issue in cattle. At that time, new breeding goals were defined for many dual- and multi-purpose breeds, which since then were continuously specialized onto one purpose as milk production or muscular mass. Today, displacement of the abomasum (DA) is an economically important and frequently observed disease in dairy cows. The abomasum can be displaced to the left or to the right side. However, in 85% to 95% of all cases, left-sided displacement of the abomasum (LDA) is observed (Constable et al. 1992), but genetic correlations between both forms are at r_g=0.65 (Ricken et al. 2004). The disease is mainly seen in dairy cows of the German Holstein or Holstein Friesian breed or their crosses and was furthermore observed in Ayrshire, Brown-Swiss, Guernsey, and Jersey cattle, while German Fleckvieh dairy cattle shows a significantly less affection risk (Doll et al. 2009). A particular disposition of high-performance dairy cows for LDA is discussed controversially. No obvious genetic correlations were detected between DA and 305-days milk performance traits (Wolf et al. 2001, Hamann et al. 2004). However, Ricken et al. (2004) found positive genetic correlations between LDA and milk-fat yield, milk-protein yield, and milk yield, while fat and protein content showed negative correlations with LDA. They also observed an increased prevalence of LDA, but not right-sided DA, in German Holstein cows intensely selected for milk performance.

Cows affected by LDA are usually between three and seven years of age (Dirksen 1961, Martin 1972). For LDA in German Holsteins, prevalences were estimated between 1.2% and 2.6% in German Holsteins (Wolf et al. 2001, Hamann et al. 2004). In US Holsteins they were estimated even higher at up to 5.5% (Detilleux et al. 1997).

LDA usually takes place most often in cows around parturition or within the first four weeks after giving birth (Dirksen 1961, Wolf et al. 2001). In the course of the disease the abomasum starts bloating and displaces from the abdominal floor to the left abdominal wall. The condition usually requires veterinary treatment and is often treated surgically. In the first 60 days of lactation following LDA affection, the amount of lactated milk is reduced by 557 kg compared to healthy cows (Detilleux et al. 1997). Even if the cows completely convalesce after veterinary treatment, they are culled in many cases due to significantly reduced milk, milk-fat and milk-protein yields as well as conception problems (Geishauser et al. 1998a, Wolf et al. 2001, Hamann et al. 2004). On the average, about one half of all cows affected by LDA were culled within the first year after surgery (Ricken et al. 2005, Wolf et al. 2001).

The etiopathology of LDA is generally accepted to be multifactorial. In addition to genetic factors, various environmental effects such as twin births, housing system, stress, concomitant diseases, endotoxins, and feeding factors (Dirksen 1961, Coppock 1974, Stöber et al. 1981, Wolf et al 2001, Ricken 2003, Zebeli et al. 2011) were found to be significantly related with the prevalence of LDA. A high body condition score or even obesity at calving is also regarded as a risk factor for LDA, as incipient lactation causes a high lipomobilization. This might lead to a reduced availability of cholesterol and in succession to a rise of endotoxin concentration, which again promotes an LDA (Fürll and Krüger 1999).

1.2.1 Genetics of LDA

A genetic component predisposing a cow for LDA is not in question nowadays. It was first hypothesized by Robertson (1964), who suggested an innately enlarged lesser omentum as the primary cause for the disease. Bloodlines of certain sires were repeatedly described to considerably increase the occurrence of LDA in their progeny (Martin 1972, Jubb et al. 1991). Heritabilities were estimated mostly between h^2=0.15 to 0.3 for the DA in general (Uribe et al. 1995, Geishauser et al. 1996, Ricken et al. 2004, Zwald et al. 2004a) and up to h^2=0.53 for LDA (Hamann et al. 2004) (Table 1).

Table 1. Heritabilities estimated for all cases of displacement of the abomasum (DA) or left-sided displacement of the abomasum (LDA) in different studies. The number of cows, breed, and methods of analysis are given.

Study	Heritability	Number of cows Breed	Analysis method
Lyons et al. 1991	0.09 ±0.04 (DA, CD) 0.16 ±0.07 (DA, PD)	9,187 cows (CD) 2,898 cows (PD) Holstein Friesian	Linear model
Uribe et al. 1995	0.28 (DA, AL)	2,941 cows * Canadian Holstein	Threshold model
Geishauser et al. 1996	0.24 (DA)	5,252 cows German Black-Pied	Regression analysis
Wolf et al. 2001	0.038 ±0.01 (DA) 0.043 ±0.012 (LDA)	9,315 cows German Holstein	Linear model
Wolf et al. 2001	0.36 (DA) 0.51 (LDA)	9,315 cows German Holstein	Threshold model
Zwald et al. 2004a	0.18 ±0.01 (DA, FL) 0.15 ±0.006 (DA, AL)	75,252 US dairy cattle	Threshold model
Zwald et al. 2004b	0.14 ±0.03 (DA, FL)	161,622 cows US dairy cattle	Threshold model
Ricken et al. 2004	0.034 ±0.014 (DA) 0.017 ±0.013 (LDA)	3,706 cows German Holstein	Linear model
Ricken et al. 2004	0.18 (DA) 0.11 (LDA)	3,706 cows German Holstein	Threshold model
Hamann et al. 2004	0.052 ±0.012 (LDA)	9,315 cows German Holstein	Linear model
Hamann et al. 2004	0.53 (LDA)	9,315 cows German Holstein	Threshold model
Appuhamy et al. 2009	0.03 (DA)	90,237 cows Holstein Friesian	Linear model

* Selected of a total material of 7,416 cows
FL First lactation
AL All lactations
CD Complete data
PD Producer data only (records are more complete and therefore less biased)

Factors influencing the affection risk of a cow for LDA are important for the genetic analysis of this disease, as they can be used for candidate gene selection. Mutations within these genes might cause altered enzymes, ionic channels, or proteins in general and account for the different affection risk for LDA between cows.

A main predisposing factor for LDA is assumed to be a disturbed motility of the abomasum, sometimes to the point of gastroparesis, with insufficient transport of gas. This leads to bloating of the abomasum. This condition was previously shown to be influenced by insulin, metabolic alkalosis, potassium, calcium metabolism, and alterations affecting the enteric as well as parasympathetic nervous system (van Winden and Kuiper 2003, Doll et al. 2009, Türck and Leonhard-Marek 2010, Zurr and Leonhard-Marek 2012). Enteric neurons in the abomasal wall of cows with a DA showed a decreased sensitivity to the neurotransmitter acetylcholine in vitro and the abomasal hypomotility preceding LDA was discussed to be associated with malfunctions at the level of the intrinsic nervous system combined with impaired cholinergic muscle responses (Geishauser et al. 1998b). Nitric oxide was discussed as a further reason for the abomasal atony. Nitric oxide is synthesized from the amino acid arginine. Therefore the concentration of arginine was quantified in the abomasum, but a correlation with a DA could not be substantiated (Geishauser and Gronostay 1998). The total innervation density within the abomasal wall was measured by the detection of neurofilament 200 (Sickinger et al. 2008) and obvious differences were found between German Holsteins and the infrequently LDA-affected German Fleckvieh cattle.

The neurotransmitter substance P (SP), which stimulates gastric motility, and the vasoactive intestinal polypeptide (VIP), which inhibits gastric motility, may also play a role in the development of LDA. Differences in the levels of SP and VIP within the abomasal wall were detected between cattle breeds (Sickinger et al. 2008). The stimulatory SP was significantly less concentrated in the abomasal corpus of German Holstein compared with German Fleckvieh cattle, whereas the inhibitory VIP was markedly increased in the abomasal antrum. These findings may explain the high susceptibility of German Holstein cows to LDA compared with other breeds.

To date, with exception of the present work, only one molecular genetic analysis for DA has been carried out. It indicated a SNP within the second exon of leptin (LEP) gene (Chebel et al. 2008), which was moderately associated (P=0.02) with the development of DA in dairy cows. Though the SNP allele associated with higher incidence of DA is also associated with other diseases and overall morbidity risk and may therefore be a causal factor for postparturient diseases in general (Chebel et al. 2008), leptin is an appropriate functional candidate gene for DA. The leptin protein is secreted by adipocytes and acts as a regulator of body weight. It is capable of decreasing food intake and mutations within this gene are known to play roles in obesity and type 2 diabetes mellitus.

1.2.2 Analogies to LDA in other species

It is difficult to compare LDA with clinical pictures in other animal species or human, because most of the species normally medicinally examined are no ruminants and therefore possess no abomasum. Nevertheless, it is possible to draw an analogy between these species, as the abomasum corresponds to the acid-containing stomach of the non-ruminants (Nickel et al. 2004).

That way, bloating and rotation of the stomach were described in human (Tsai and Tseng 2009), dogs (Monnet 2003), cats (Bredal et al. 1996), pigs (Bethlehem and Hilvering 1987), guinea pigs (Willemse 1975) and horses (Hudson and Merritt 2008). However, though these disease patterns often show a genetic disposition, no genetic or molecular genetic analyses have been reported to date.

II List of publications which are parts of this work

Publication 1

S. Mömke, H. Scholz, K. Doll, J. Rehage, O. Distl (2008)

Mapping Quantitative Trait Loci for Left-sided Displaced Abomasum (LDA) in German Holstein Dairy Cows. Journal of Dairy Science 91: 4383-4392.

Publication 2

S. Mömke, W. Brade, O. Distl (2011)

Co-segregation of quantitative trait loci (QTL) for milk production traits and length of productive life with QTL for left-sided displacement of the abomasum in German Holstein dairy cows. Livestock science 140: 149-154.

Publication 3

S. Mömke, M. Sickinger, J. Rehage, K. Doll, O. Distl (2012)

Transcription factor binding site polymorphism in the *motilin* gene associated with left-sided displacement of the abomasum in German Holstein cattle. PLoS ONE 4: e35562.

Publication 4

S. Mömke, M. Sickinger, P. Lichtner, K. Doll, J. Rehage, O. Distl (2013)

Genome-wide association analysis identifies loci for left-sided displacement of the abomasum in German Holstein cattle. In press: Journal of Dairy Science.

Publication 5

S. Lehner, C. Dierks, J. Rehage, O. Distl (2013)

A genome-wide association study for left-sided displacement of the abomasum using the Illumina bovine high-density bead chip. Prepared: PLoS ONE.

Publication 6

S. Lehner, C. Dierks, J. Rehage, O. Distl (2013)

Whole genome sequencing of German Holstein cows to detect polymorphisms for left-sided displacement of the abomasum. Prepared: Journal of Heredity.

III Results

3.1 Linkage analysis for detection of quantitative trait loci for left-sided displacement of the abomasum

S. Mömke, H. Scholz, K. Doll, J. Rehage, O. Distl (2008) Mapping Quantitative Trait Loci for Left-sided Displaced Abomasum (LDA) in German Holstein Dairy Cows. Journal of Dairy Science 91: 4383-4392.

In this study we performed a whole genome scan employing an affected paternal half-sib design to detect quantitative trait loci (QTL) for left-sided displaced abomasum (LDA) in German Holsteins. A total of 360 animals from 14 paternal half-sib families were genotyped for a total of 306 polymorphic microsatellite markers.

Of the 360 individuals used for the linkage analysis, 328 were securely affected by LDA, since they had been examined at the Clinic for Cattle, University for Veterinary Medicine Hannover and at the Clinic for Ruminants, Justus-Liebig-University for Veterinary Medicine Giessen, respectively, and their affection status had been verified during LDA-surgery. Furthermore, we included 24 cows which had completed more than seven lactations and were never affected by LDA into the paternal half-sib families. The paternal half-sib families consisted of 12 to 58 siblings of progeny tested artificial insemination sires. The average family size was 25.1 cows. The pedigrees of seven and four sires could be traced back to each one common grandsire, respectively. The remaining three families were not closely related to the other families. The 306 microsatellite markers were highly polymorphic and had a mean number of 7.6 alleles. The average polymorphism information content (PIC) in our data was 56.7 %, and the mean heterozygosity was 61.6 %.

For the first whole genome scan, 221 microsatellite markers were equally distributed over all 29 bovine autosomes with an average distance of 13.7 cM. The number of markers per chromosome ranged from four on *bos taurus* chromosome (BTA) 27 and 28, to ten on BTA5. Marker density was highest on BTA29 with an average distance of 9.96 cM, and lowest on BTA27 with an average distance of 17.8 cM. After including 85 additional markers on 14 chromosomes for fine mapping, the average marker distance was 7.9 cM on these chromosomes.

We identified five QTL on BTA1, 3, 21, 23, and 24 significantly linked with LDA across all families used for analysis. The region of linkage on BTA1 spanned from 23.9 to 77.7 cM. The

linked region on BTA3 extended between 2.2 and 27.7 cM. For BTA21, the QTL was located between 62.7 and 82.0 cM. On BTA23, the QTL extended between the proximal end of the chromosome (0 cM) and 9.6 cM, and on BTA24, the QTL ranged from 68.5 cM to the distal end of the chromosome. QTL containing genome-wide significant markers were found on BTA1 from 54.6 cM to 58.3 cM and on BTA3 at 5.9 cM.

Subsequent to this conjoined analysis across all families, we performed separate analyses by compacting those families to the five grandsire families. Thus, we detected eleven QTL, which co-segregated in grand-sire families, but were not significant in the across-family analysis. These regions showed significant chromosome-wide linkage and were located on BTA15 in two grandsire families and on BTA5, 6, 10, 12, 16, 17, 19, 23, and 26 in each one grandsire family. The QTL on BTA26 even reached genome-wide significance.

This study was the first report on QTL for LDA and a first step towards identifying polymorphisms for this disease.

3.2 Co-segregation of QTL for milk production traits and length of productive life with QTL for left-sided displacement of the abomasum

S. Mömke, W. Brade, O. Distl (2011) Co-segregation of quantitative trait loci (QTL) for milk production traits and length of productive life with QTL for left-sided displacement of the abomasum in German Holstein dairy cows. Livestock science 140: 149-154.

Left-sided displacement of the abomasum (LDA) is a commonly observed disease in Holstein dairy cows and has previously been shown to be genetically correlated with several milk performance traits and length of productive life (LPL) (Wolf et al. 2001, Hamann et al. 2004, Ricken et al. 2004, 2005). The objective of this study was to detect joint QTL among milk production traits and LDA using a paternal half-sib design including only LDA affected daughters. Joint QTL for LDA and milk production traits in LDA-affected daughters were assumed to indicate genetic correlations among these traits due to linkage disequilibrium or even pleiotropic genes. For the study, we used genotyping data of 14 paternal half-sib families comprising 328 LDA-affected German Holstein cows and eight sires using 302 microsatellites distributed over all 29 autosomes. For each individual cow and its sire, estimated breeding values (EBVs) were provided for the traits MY, FY, FP, PY, PP, SCS, and LPL by VIT (Vereinigte Informationssysteme Tierhaltung), Verden/Aller, Germany. The data was obtained from the official release of the September 2007 genetic evaluation. EBVs were based on animal model evaluations for the whole GH cattle population (http://www.vit.de/?id=zuchtwertschaetzung&L=1). The LDA affection status of each cow in the present study had been ascertained at university cattle clinics during LDA surgery.

Linkage analyses were performed for milk performance traits, somatic cell score (SCS), and LPL. In total, we identified seven genome-wide and further 30 chromosome-wide significant QTL for milk performance traits, SCS, and LPL. Genome-wide QTL were detected for FP on BTA15 and 23, for PP on BTA11 and 23, and for SCS on BTA15, 16, and 19. In total, we found three QTL for MY, five for FY, five for FP, two for PY, eight for PP, ten for SCS, and four for LPL. Of all these QTL, 21 had been reported before for the specific trait. Seven QTL showed overlap with the previously reported QTL for LDA. All four QTL for LPL on BTA1, 21, 23, and 24, two of the QTL for FY on BTA1 and 24, and one QTL for PP on BTA23 shared positions with QTL previously identified for LDA. Twelve further ones shared position with or were adjacent to family-specific QTL for LDA. These QTL were detected for MY, FP, PY, PP, and SCS.

Furthermore, polymorphisms located within the *DGAT1* (Diacylglycerol O-acyltransferase 1) and *ABCG2* (ATP-binding cassette, sub-family G (WHITE), member 2) genes, which had

previously been reported to be associated with milk performance traits (Cohen-Zinder et al. 2005, Grisart et al. 2002) were analysed for all cows. For the DGAT1 K232A polymorphism, the homozygous genotypes K/K and A/A had frequencies of 14.78% and 38.17%, and the heterozygous genotype A/K showed a frequency of 47.04%. Allele frequencies were 38.3% for allele K and 61.7% for allele A. The polymorphism was associated with milk yield, fat yield, fat percentage, and protein percentage, but showed no association with LDA. The highest associations of *DGAT1* genotypes were found with EBVs for FP and PP. Allele K was related with higher EBVs for FY, FP, and PP, and lower EBVs for MY. The *ABCG2*-SNP was monomorphic for the Y-allele in this study.

In conclusion, genetic correlations among LDA and LPL may arise from the joint QTL described. Therefore, selection for longevity should even lower LDA incidence in German Holsteins. A further co-incidence for LDA-QTL locations was found for fat yield and protein percentage. This study is a step towards better understanding of genetic correlations of LDA with milk performance traits and identification of possible side-effects due to selection for milk production in dairy cows.

3.3 A Mutation within a transcription factor binding site of the motilin gene is associated with left-sided displacement of the abomasum

S. Mömke, M. Sickinger, J. Rehage, K. Doll, O. Distl (2012) Transcription factor binding site polymorphism in the motilin gene associated with left-sided displacement of the abomasum in German Holstein cattle. PLoS ONE 4: e35562.

The bovine abomasum is the equivalent to the stomach in monogastric species (Nickel et al. 2004) and its pathologic displacement is usually preceded by bloating due to reduced gastrointestinal contractions. A QTL for LDA on proximal bovine chromosome 23, as well as its importance to gastrointestinal peristalsis indicated motilin (*MLN*) as positional and functional candidate gene. Genomic DNA sequence analysis of *MLN* revealed a total of 32 polymorphisms, of which 30 were SNPs and two were short tandem repeats. None of the identified SNPs was located within the coding sequence of *MLN*, but one was located within the promoter of *MLN* and another one affected a predicted NKX2-5 transcription factor binding site. All polymorphisms were tested for their information content and all informative polymorphisms were used for association analyses in a random sample of 1,136 German Holstein cows, with 601 of them affected by LDA and 535 unaffected. Seven SNPs showed significant allelic and genotypic associations with LDA. In order to correct for the data structure, we employed a logit model including a random sire effect besides the fixed genotypic SNP effects. This association analysis showed the most significant values for two SNPs FN298674:g.90T>C ($-\log_{10}P=3.8$) and FN298674:g.1891insG ($-\log_{10}P=3.7$). Located within the first non-coding exon of bovine *MLN,* FN298674:g.90T>C affects a NKX2-5 transcription factor binding site. FN298674:g.1891insG is located within the first intron.

The phenotypic variance explained after correction for the sire effect was at 3.1% for FN298674:g.90T>C and at 3.9% when FN298674:g.90T>C and FN298674:g.1891insG were combined. Of all cows carrying the homozygously mutated genotype (C/C) at FN298674:g.90T>C, 67.3% were affected by LDA. Of the cows homozygous for the wildtype allele (T/T), 44.0% were affected by LDA, and of the heterozygous (C/T) individuals, 49.6% were affected. Of all 601 LDA-affected animals, only 19.8% were homozygous for the wildtype allele.

To test whether the FN298674:g.90T>C mutation might influence *MLN* expression, samples of abomasal mucosa tissue from 55 previously genotyped cows were taken and analysed using qRT-PCR. The expression levels of *MLN* were decreased by 89% in cows being heterozygous or homozygous for the mutant allele C of the polymorphism FN298674:g.90T>C relative to cows homozygous for the wildtype allele T. Among cows

carrying the mutant allele C homozygously or heterozygously, the expression levels were identical. Therefore, this expression study gave evidence of a significantly decreased *MLN* expression in cows carrying the mutant allele. FN298674:g.90T>C may therefore play a role in bovine LDA via reduction of the motility of the abomasum.

The two most significantly LDA-associated polymorphisms, FN298674:g.90T>C and FN298674:g.1891insG, were tested in 148 German Fleckvieh cattle. German Fleckvieh was used as a reference breed, as this breed is known for its very low incidence of LDA. Genotypes of FN298674:g.90T>C were 0.468 (T/T), 0.371 (C/T), and 0.161 (C/C) in this breed. In comparison to German Holstein cows, German Fleckvieh showed a shift to the wildtype allele T and the homozygous wildtype genotype (T/T). For FN298674:g.1891insG, the genotypic distribution was 0.591 without insertion (WT/WT), 0.295 heterozygous (WT/INS) and 0.114 homozygously inserted (INS/INS).

This study indicates *MLN* to be involved in the etiopathogenesis of LDA in German Holstein cattle. It provides a SNP affecting a predicted NKX2-5 transcription factor binding site, which is associated with LDA and significantly lowers the expression of *MLN*.

3.4 Comprehensive genome-wide association study for left-sided displacement of the abomasum

S. Mömke, M. Sickinger, P. Lichtner, K. Doll, J. Rehage, O. Distl (2013) Genome-wide association analysis identifies loci for left-sided displacement of the abomasum in German Holstein cattle. Journal of Dairy Science: in press.

In this study, we performed a genome-wide association study for 854 German Holstein cows including 225 cases and 629 controls. All cows were genotyped using the Illumina Bovine SNP 50 beadchip. After quality control of genotypes, a total of 36,226 informative SNPs were left for analysis. We employed a mixed linear model (MLM) approach for a genome-wide association study of LDA. At a significance level of $-\log_{10}P>3.0$, we identified a total of 36 SNPs on 17 bovine chromosomes associated with LDA. Two associated SNPs, located on different chromosomes showed genome-wide significant associations with LDA at $-\log_{10}P>4.6$. These were located on BTA11 (46.70 Mb, OR=1.74) and 20 (16.67 Mb, OR=1.06). The SNP on BTA11 was located within the gene *IL1RN* (interleukin 1 receptor antagonist). The SNP on BTA20 was intergenic with *IPO11* (importin 11) being the closest gene.

To detect a possible genetic pattern underlying the 36 SNPs associated with LDA, a pathway analysis was carried out. For each intragenic SNP, the specific gene was chosen (16 SNPs). In the case of an intergenic SNP located between two genes, we chose both of these genes (twelve SNPs) and if only one gene was located within a distance of less than 500 kb, only this gene was chosen (seven SNPs). If no gene was located within a 500 kb distance of the SNP, no gene was included into the analysis (one SNP). Therefore, a total of 46 different genes were used. Functional pathways significant at $-\log_{10}P>2.0$ were regarded. The function annotations most probably related with LDA were deposition of Ca^{2+} ($-\log_{10}P=3.28$, *BMP2K* and *SLC8A1* genes) and insulin-dependent diabetes mellitus ($-\log_{10}P=2.34$, *CUX2*, *IL1RN*, *LRP1B*, and *POLR2M* genes). In a second pathway analysis we used only those genes, which contained the 16 intragenic SNPs. In this analysis, the most probable function annotation was deposition of Ca^{2+} ($-\log_{10}P=4.17$). Insulin-dependent diabetes mellitus was also found in this analysis, but with a lower significance ($-\log_{10}P=1.63$). Therefore, pathway analyses indicate genes involved in calcium metabolism and insulin-dependent diabetes mellitus to be factors in the pathogenesis of LDA in German Holstein cows.

3.5 Genome-wide high density association study for left-sided displacement of the abomasum

S. Lehner, C. Dierks, J. Rehage, O. Distl (2013) A genome-wide association study for left-sided displacement of the abomasum using the Illumina bovine high-density bead chip.

In this study we performed a high density association analysis for LDA using the Illumina Bovine HD beadchip to refine previous genomic locations for LDA and detect new ones. Subsequent to quality control, 588,753 SNPs were left to analyse 126 cases affected by LDA and 280 population-based controls. We employed a mixed linear model approach to perform genome-wide association studies and detected six regions containing SNPs significantly associated with LDA at $-\log_{10}$P-value>5.17, which corresponds to $-\log_{10}$P-value>3.0 after correcting for multiple testing. These regions were detected on BTA2 at 108.7-109.5 Mb (4 SNPs), BTA8 at 5.7-5.8 Mb (10 SNPs), BTA13 at 29.0-29.2 Mb (12 SNPs), BTA20 at 54.1-54.4 Mb (10 SNPs), BTA24 at 61.0-61.2 Mb (7 SNPs), and BTAX at 103.4-103.6 Mb (7 SNPs). Further 19 regions were detected at $-\log_{10}$P-value>4.17, which corresponds to $-\log_{10}$P-value>2.0 after correcting for multiple testing. These are located on BTA1 (3 regions), 2 (4 regions), 3 (2 regions), 4 (1 region), 5 (1 region), 9 (1 region), 14 (1 region), 20 (2 regions), 22 (1 region), 24 (1 region), and 27 (2 regions). In addition, eight single markers associated with LDA at $-\log_{10}$P-value>4.17 were detected on BTA1, 2 (3 markers), 11, 17, 24, and X.

The genomic regions containing SNPs associated with LDA at $-\log_{10}$P-value>5.17 were scanned for positional candidate genes. On BTA2, *SLC4A3* (solute carrier family 4, anion exchanger, member 3) is the closest, protein coding gene and located 408 kb proximal of the LDA associated region. On BTA8, *LOC781874* (heart- and neural crest derivatives-expressed protein 2-like) is located at 5.75 Mb and therefore within the LDA-associated region on this chromosome. On BTA13 the most significant SNP is located 66 kb from the next gene *FRMD4A* (FERM domain containing 4A)*. CDH18* (cadherin 18, type 2) is located within the LDA-associated region on BTA20, and the LDA-associated region on BTA24 contains the genes *PIGN* (phosphatidylinositol glycan anchor biosynthesis, class N) and *KIAA1468*. Five SNPs are located intragenic within *PIGN* and the other two SNPs are located within *KIAA1468*. On BTAX, three markers, including the one most significantly associated with LDA, are located within uncharacterized *LOC100847796*.

The variances explained (r^2) by SNPs of these regions are high and reach maxima of 7.3% on BTA2, 6.4% on BTA8, 6.4% on BTA13, 6.1% on BTA20, 6.2% on BTA24, and 6.9% on the X-chromosome. They might therefore be of use in a genetic test for LDA.

3.6 Next generation sequencing of German Holstein cow genomes to detect polymorphisms for left-sided displacement of the abomasum

S. Lehner, C. Dierks, J. Rehage, O. Distl (2013) Whole genome sequencing of German Holstein cows to detect polymorphisms for left-sided displacement of the abomasum.

The objective of the present study was to perform genome-wide sequencing in individuals and pools of selected German Holstein cows. The genomes of four individual cows and two pools consisting of six cows each were sequenced with a mean coverage rate of 7.3 sequences per base pair. Each two individuals and one pool consisted of cows affected by LDA and of control cows, respectively. The cases and the controls were matched to each other by age and sex. A total of 124.98 Gb sequences were generated from the six samples of two pools and four individuals. Mapping efficiency of the sequences corrected for quality was at 97.9% on the average using the BWA tool. The sequences were trimmed and aligned to the *bos taurus* reference genome.

A total of 7,939,073 different polymorphisms were identified within the six samples compared with the reference sequence. Of these polymorphisms, 7,459,197 were SNPs and 479,876 were indels (insertions or deletions). We compared the putative SNPs in the mapped sequences with the Ensembl variants database (http://www.ensembl.org/Bos_taurus) and discovered 47.9% of the SNPs and 85.7% of the indels were novel. In the next step, indels and SNPs were specified for their location in the bovine genome. Thus, 27.7% of all indels and 26.4% of all SNPs were located intragenic. Of the intragenic SNPs, 96.9% are located within introns, as well as 98.5% of the indels. A total of 681 indels were potentially damaging as they were classified as affecting codons or splice sites or causing frame shift. In addition, 20,536 SNPs caused amino acid exchanges and therefore alterations of the proteins they were coding for, affected start or stop codons, or affected splice sites.

For all SNPs detected in one or more of the six samples used for the NGS, we determined the association with LDA. In the next step, we determined the density of markers associated with LDA. For one genomic location, more than 30 polymorphisms with a significant *P*-value were concentrated on 100 kb. This location was on proximal BTA13. All other locations contained less than 25 LDA-associated polymorphisms per 100 kb. In the next step, polymorphism detection was limited to the genomic regions formerly reported to be linked or associated with LDA. Within these regions, 10,609 intragenic single nucleotide variants (SNVs) and eight intragenic indels significantly associated with LDA among the six samples were detected. 308 of these SNVs and none of the indels were located within coding sequences, three SNVs were located within splice sites, 11 within 5’ UTRs, and 88 within 3’

UTRs. Of the coding SNVs, 179 were synonymous, 127 were non-synonymous, one caused a start codon gain, and one caused a stop codon loss. All non-synonymous SNVs were analysed for their effects on the protein using the PolyPhen2 software (Adzhubei et al. 2010). Ten SNVs within eight genes were classified as probably damaging, four SNVs within four genes as possibly damaging, and nine SNVs within eight genes as unknown. The remaining 104 SNVs were classified as benign (Tables 2 and 3). Potentially damaging SNVs were detected within the genes *SIDT1*, *LOC538060*, and *KALRN* (BTA1), *ARHGAP30*, *SPTA1* (two SNVs)*, IVL* (BTA3), *OR6C2* and *LOC505479* (BTA5), *EGF* and *ADH7* (BTA6), *NWD1* and *LOC509006* (two SNVs, BTA7), *SPG11* (BTA10), *THNSL2* (BTA11), *ZIC2* (two SNVs, BTA12), *DCLRE1C* (BTA13), *GUCY2E* (BTA15), *SDCCAG8* (BTA16), *GPR133* (BTA17), *TRIM65* and *LLGL2* (BTA19), *BDP1* (BTA20), *PHLPP1* and *LOC510913* (BTA24), and *RBP3* (BTA28).

Of the genes containing potentially damaging SNVs, *EGF* (epidermal growth factor) on BTA6, *SDCCAG8* (serologically defined colon cancer antigen 8) on BTA16 and *PHLPP1* (PH domain and leucine rich repeat protein phosphatase 1) on BTA24 may be regarded as functional candidate genes for LDA, as they play roles in gastric motility, obesity, or type 2 diabetes mellitus. Of these genes, *PHLPP1* is of certain interest, because its position was indicated by the linkage analysis and both previous association studies for LDA.

IV General Discussion

Discussion of the individual studies

Functional genomics research to successfully unravel genetic variants with large influence on trait expression is strongly connected with the status of the reference genome for the target species and with the number of markers available. Linkage analyses with medium dense marker sets have been the method of choice in the pre-genomic era of domestic animals. This type of analysis indicates genomic regions, which most likely contain mutations segregating for the families under investigation. The power depends on the number of families and the number of individuals per family. In the case of LDA in German Holstein cows, samples including half-sib families were collected by veterinary clinics. As LDA is prevalent in progeny of sires used for artificial insemination, many half-sib daughter groups, each containing large numbers of individuals, were available for linkage analysis. This daughter design was used instead of a grand-daughter design based on progeny-tested sons. The later one was not feasible as a state-wide record of diseases in dairy cattle is not yet operating in Germany. Furthermore, a granddaughter design may be questionable as the most powerful design for linkage analysis when many daughter groups do not exceed 100 to 150 animals and the number of grandsires with a higher number of sons segregating for LDA is limited. Therefore, in the case of LDA, a daughter design appeared much more powerful. Using a daughter design for linkage analysis, we detected two genome-wide and three chromosome-wide significant QTL for LDA on five bovine chromosomes. This was the first report of genetic loci identified for LDA in dairy cattle. Compared to analyses for functional or production traits, studies on disease traits are rare. This might be explained by the fact, that performance records for cows are easily available, while health records are rarely gathered centrally by a breeding organization. We were able to employ an affected half-sib design of 14 large half-sib families including five even larger grandsire families segregating for LDA. The affection status of each cow was confirmed by veterinary experts of university clinics for cattle during LDA-surgery. LDA is a multifactorial trait with a heritability estimated at up to 50% (Hamann et al. 2004, Ricken et al. 2004) and presumably a multitude of genes influencing the affection risk of a cow for this disease. Not all of these LDA-related genes might even segregate in all sire families, which might lower the power to detect these loci. In addition, linkage analysis with half-sib families depends on the heterozygous carrier status for the disease allele in question in the common sires. Loci genetically fixed in most of the common sires are not detectable using linkage analysis. In order to control genetic fixation, we included as many sires as possible in our set of samples and in addition a number of

daughters unaffected by LDA. Therefore, the five QTL significantly linked with LDA across all families might be the most important ones in this study, but grandsire-family- or family-specific QTL must not be disregarded. All in all, eleven family-specific QTL were detected for LDA. This linkage study was the first step towards a molecular genetic characterization of LDA.

In the past, the prevalence of LDA was reported to be genetically correlated with milk yield (MY), milk-fat yield and percentage (FY, FP), and milk-protein yield and percentage (PY, PP) (Ricken et al., 2004). Therefore, it could be assumed, that some QTL for milk performance traits previously detected in Holstein cows might overlap with QTL for LDA. These QTL for milk performance traits could be retrieved using by the Cattle Quantitative Trait Locus database (Cattle QTLdb, Hu et al. 2013). The QTL for LDA on BTA1 showed joint QTL with two milk performance traits. Two QTL for MY (Nadesalingam et al. 2001, Daetwyler et al. 2008) as well as three QTL for PY (Zhang et al. 1998, Nadesalingam et al. 2001, Daetwyler et al. 2008) were mapped to the genomic region on BTA1 where the LDA-QTL was located. On BTA3, QTL for all five milk performance traits were detected in the region of the LDA-QTL. There were one QTL for MY, one for FP, three for FY, four for PP, and two for PY (Heyen et al. 1999, Rodriguez-Zas et al. 2002, Boichard et al. 2003, Ashwell et al. 2004, Daetwyler et al. 2008, Martínez-Royo et al. 2010). On BTA21, each one QTL for MY, FP, and PP overlapped with the QTL for LDA on this chromosome (Pimentel et al. 2011), and each one QTL for MY, PY, PP, and FY where found in the region of the LDA-QTL on BTA23 (Bennewitz et al. 2003, Bennewitz et al. 2004, Daetwyler et al. 2008). Only on BTA24, no QTL for milk performance traits were found. Therefore, the joint distribution of QTL for milk performance traits and LDA suggests that a co-segregation of loci influencing both trait complexes may be possible.

Though all QTL for milk performance traits overlapping with QTL for LDA mentioned above were detected in Holstein cattle, it was not clear if they were also segregating in our material. Therefore, we made up a new design to test for this co-segregation directly. In order to identify QTL influencing LDA as well as milk performance traits, somatic cell score (SCS) and length of productive life (LPL), we employed the same paternal German Holstein half-sib families as used in the linkage analysis for LDA. As we wished to detect only those traits segregating with LDA, we used only the daughters affected by LDA. The fundamental idea of this study was that the QTL for milk production traits and LPL, which are identified in this specific material, should generally be dependent on a disposition for LDA. This study design also permits a direct comparison of QTL. If the QTL detected for LDA and those for milk production traits or LPL share a common position in the genome, the allelic distribution

among the daughters of the half-sib families automatically shows a significant deviation from the expected normal distribution. However, if the differences for milk performance traits or LPL among the daughters of the half-sib families are low, QTL detection is complicated. Common QTL among the traits may be caused by pleiotropic effects or linkage disequilibrium with QTL for LDA. In this study, we detected four QTL for LPL, all of which were congruent with QTL for LDA. This is in accordance with previous studies, where cows affected by LDA showed significantly increased culling rates (Hamann et al. 2004, Ricken et al. 2005) and therefore a reduced LPL. In addition to the negative effect of LDA itself onto LPL, two of the QTL for LPL are in agreement with former studies for this trait. On BTA1, a SNP within the third exon of the *POU1F1* (POU class 1 homeobox 1) gene was reported to be associated with LPL in US Holsteins (Huang et al., 2008). Other studies in US Holsteins reported a QTL for LPL on BTA21 (Heyen et al. 1999) containing SNPs within the uterine milk protein (*UTMP*) gene, which were associated with LPL (Khatib et al. 2005, 2007). Both genetic variants associated with LPL might also affect the disposition of a cow for LDA or influence the convalescence. These results indicated that a general selection for a long productive life seems to have also effects on LDA reduction and the identification of common QTL for both traits can even help to intensify this dual selection process.

Aside from LPL, also QTL for FY and PP showed genetic co-segregation with QTL for LDA. This is consistent with statistical analyses by Ricken et al. (2004), who reported positive genetic correlations between LDA and FY and negative genetic correlations between LDA and PP. The QTL for the traits MY, FP, PY, and SCS in our study showed common positions only with family-specific QTL for LDA, but not with the QTL detected across all families. The genetic associations of these traits might therefore be considerably smaller and merely family-dependent. In summary, LDA seems to be genetically correlated mainly with LPL, FY, and PP. This is of importance, as markers for milk production traits are used on a large scale for genomic selection of cattle. Unravelling co-localized QTL of LDA and desired traits can help to improve the selection methods and reduce genetic antagonisms.

In addition to the shared QTL, we examined the impact of two SNPs within the genes *DGAT1* (Diacylglycerol O-acyltransferase 1) and *ABCG2* (ATP-binding cassette, sub-family G (WHITE), member 2) for LDA. Both mutations are associated with milk performance in dairy cattle to a huge extent (Cohen-Zinder et al. 2005, Grisart et al. 2002) and they are intensely used for genetic selection. The SNP within *DGAT1* was not linked nor associated with LDA and the SNP within *ABCG2* was monomorphic for all German Holstein cows. Therefore, selecting cows for these genetic variants does not seem to have an effect on the LDA affection risk.

Subsequently to the linkage analyses, we scanned the five main genomic regions for LDA detected by genome-wide linkage analysis for functional candidate genes. On proximal BTA23, we discovered the *MLN* (motilin) gene. This gene is expressed mainly in the gastrointestinal tract and encodes for a hormone which controls gastrointestinal motility and actuates phase III of the migrating motor complex (Wierup et al. 2007, Poitras et al 2008). Within interdigestive phases, plasma levels of motilin are raised every 1.5 to 2 hours for some minutes, causing strong peristaltic contractions from the stomach towards and along the intestine (Poitras et al. 2008), which promotes gastric emptying in fasting periods. Other studies showed that higher doses of motilin caused stronger contractile responses in house musk shrews (Tsutsui et al. 2009) and even reported therapeutical approaches using motilin against human diabetic gastroparesis (McCallum et al. 2007a,b). Our own analyses showed associations between *MLN* and the LDA affection risk of German Holstein cows. LDA is usually preceded by a reduced gastrointestinal peristalsis up to gastroparesis (Geishauser et al. 1998b), which seems to be partly caused by a lowered level of *MLN* expression. As LDA is a genetically complex disease, the influence of *MLN* is fractional. For our analyses, we sequenced the complete genomic DNA of *MLN* and examined all polymorphisms detected for associations with LDA. After correcting the data for stratification, the most strongly LDA-associated SNP was FN298674:g.90T>C, located within the first, non-coding exon of *MLN*. This SNP affected a predicted NKX2-5 transcription factor binding site. NKX2-5 is known to influence gene expression (Riazi et al. 2009) and mutations within the binding sites of this transcription factor were reported to decrease expression of the respective genes and to be involved in the development of complex diseases (Oishi et al. 2008). We therefore carried out an expression analysis for *MLN*. Biopsies of abomasal tissue were taken of 55 German Holsteins, whereof 26 were homozygous for T/T, 20 were heterozygous (C/T), and nine were homozygous for C/C at FN298674:g.90T>C. Cows homozygous and heterozygous for the mutant allele C showed a significantly lowered expression of *MLN* compared with the cows homozygous for the wildtype allele T. Therefore, the mutant allele of FN298674:g.90T>C is supposed to lower expression of *MLN*, leading to a reduced gastrointestinal peristalsis and in succession to a higher affection risk of cows for LDA. While for the homozygous genotype there was complete accordance between association analysis and expression study, the heterozygous individuals were mainly unaffected by LDA, but their *MLN* expression was lowered to a high extent. Therefore, heterozygous cows might show a low basis expression of *MLN*, but they might be able to raise the *MLN* expression more often or to a higher level compared with cows homozygous for the mutant allele. They might therefore reach a threshold of the expression, which is sufficient to prevent LDA. The variance explained for LDA by the SNP FN298674:g.90T>C was at 3.1% in all German Holstein cows genotyped. When the SNP FN298674:g.90T>C was combined with the FN298674:g.1891insG mutation

within the first intron of *MLN*, the variance explained increased to 3.9%. This might partly be caused by the strong linkage disequilibrium of r^2=0.8 among FN298674:g.90T>C and FN298674:g.1891insG. To further verify the effect of *MLN*, we examined German Fleckvieh individuals for the two mutations most significantly associated with LDA, as this breed is known to rarely contract a LDA (Doll et al. 2009). In German Fleckvieh, the frequencies of the homozygously mutant genotype as well as of the mutant allele were exceptionally low for both markers analysed, which corroborated the assumption of MLN being one of the causal genes for LDA. In conclusion, we detected a polymorphism affecting a NKX2-5 transcription factor binding site, causing a lowered expression of MLN and conferring a higher risk for LDA in German Holstein cows. This is the first report on a polymorphism showing a functional association with LDA. It is the first step in the development of a genetic test for LDA-susceptibility in this breed. As gastric dilatation and gastric rotation disorders are known not only in cattle but also in humans (Tsai and Tseng 2009), dogs (Monnet 2003), cats (Bredal et al. 1996), pigs (Bethlehem and Hilvering 1987), guinea pigs (Willemse 1975), and horses (Hudson and Merritt 2008), our study might advance research of gastric motility disorders in these species.

Genome-wide association studies (GWAS) with dense SNP maps are effective approaches for detection of genomic regions in high linkage disequilibrium with the trait of interest, independent of the family structure (Grapes et al. 2004). While linkage analyses are expected to yield QTL extending over tens of centimorgans (Weller and Ron 2011), genomic regions associated with the same traits are usually much smaller in size. On the other hand, the number of significantly trait-associated genomic locations is higher than in linkage analyses, mainly due to the dense coverage of the genome with SNPs and the employment of unrelated individuals leading to a higher sample size (Weller and Ron 2011). For our GWAS, individuals unrelated at the grandsire level were preferably chosen. This led to genomic regions, associated with LDA in a wide range of individuals within the population. However, in this approach it was not assessed if an individual was of a family, which accounts for a big number of individuals or of a very small one. Therefore, the genetic locations for traits influenced by many genes usually vary between association and linkage analyses. We first used the Illumina Bovine SNP 50K beadchip for a large number of individuals due to its high cost-benefit ratio. A significance threshold of a $-\log_{10}P$-value>3.0 was chosen to be the lower limit for a SNP to significantly influence the affection risk of a cow for LDA. That way, a total of 36 SNPs on 17 different bovine chromosomes were identified for LDA. One of these SNPs, located at 57.91 ($-\log_{10}P$-value=3.2) on BTA24, was detected within a QTL for LDA of the previous linkage study. Two further SNPs at BTA17 and BTA19 are located within or closely adjacent to family-specific QTL of that study. The differences

among both studies arise from the differences in the study design. Regions detected by linkage analysis depend on the segregation pattern of LDA-alleles within the paternal half-sib families. While the sire has to be heterozygous for an allele to detect the QTL in question, this plays no role in association analysis. Linkage analyses also show QTL that may be specific for the families analysed. Due to the diverse ancestry used in the present GWAS, most of the detected regions deviate from those of the linkage analysis. However, the ones with matching positions might also be the ones with the highest influence on LDA.

Using a method suggested by Lander and Kruglyak (1995), correction for multiple testing was performed to determine the threshold of genome-wide significance. This method had been used before in the GWAS of Schulman et al. (2011). The threshold for significance was at $-\log_{10}P = 4.6$ in the latter study and the same threshold was applied for the present study. Therefore, SNPs on BTA11 at 46.7 Mb and on BTA20 at 16.7 Mb show genome-wide significance. Another SNP on BTA11 at 102.9 Mb is just below this threshold. Both SNPs genome-wide significantly associated with LDA showed explained variances at 2.5% and 2.8%. The SNP on BTA11 at 46.7 Mb was located intragenic within *IL1RN* (interleukin 1 receptor antagonist) and the SNP on BTA11 at 102.9 Mb was located intragenic within *AK8* (adenylate kinase 8).

In the pathogenesis of LDA, factors like insulin concentration, calcium metabolism, potassium homeostasis, metabolic alkalosis as well as the parasympathetic nervous system were reported to play main roles (van Winden and Kuiper 2003, Doll et al. 2009, Türck and Leonhard-Marek 2010, Zurr and Leonhard-Marek 2012). Therefore, *IL1RN* was an obvious functional candidate gene for LDA as it plays a role in insulin secretion. Decreased expression of this gene was observed in type 2 diabetes (Maedler et al. 2004). *IL1RN* also protects pancreatic beta cells from glucose induced production of interleukin-1-beta and therefore affects insulin secretion, pancreatic cell proliferation, and apoptosis.

Pathway analyses also detected a pathway for insulin-dependent diabetes mellitus for the genes *IL1RN*, *CUX2*, *LRP1B*, and *POLR2M.* Of these genes, *IL1RN* and *CUX2* contain intragenic SNPs associated with LDA. Diabetes mellitus is caused by insulin insufficiency leading to hyperglycemia, which is an often finding in LDA affected cows (van Winden and Kuiper, 2003). Elevated plasma glucose levels were shown to cause reduced outflow of fluid off the abomasum in dairy cows and therefore supposed to be a risk factor in the pathogenesis of LDA (Holtenius et al. 2000). Another pathway for LDA, which might be of functional importance was detected for calcium metabolism and comprised the genes *BMP2K* and *SLC8A1*. Both of these genes contained intragenic SNPs associated with LDA.

In the past, correlations between metabolic factors and LDA have been reported and much research has been done in this direction. Those studies, however, were carried out in vitro or on cows acutely affected by LDA. In this study, a pathway analysis for positional candidate

genes, which were chosen based on a GWAS, was carried out. The analysis of pathways using genes from LDA-associated genomic regions gave new insights into the genetic portion of LDA and suggested calcium metabolism and insulin-dependent diabetes mellitus to be the main players in the genetic pathogenesis of LDA.

Following the GWAS on the Illumina Bovine SNP 50 beadchip, an Illumina bovine high density bead chip with more than 700,000 SNPs became available for association analysis. This analysis was performed to detect genomic regions, which might not have been detected by the former analysis with a lower density of SNPs, and also aimed at getting a higher resolution of genomic loci associated with LDA that were already identified. The regions associated with LDA using this high density beadchip showed higher significance levels than those of the 50K association analysis. Six main significant regions were detected for LDA and further 27 significant locations showed associations with LDA to a lesser extent. Though regions containing more SNPs associated with LDA are commonly preferred to single markers, as they are not that prone to being merely an artefact, the eight single markers identified should not be excluded hastily. German Holstein cattle show a median length of linkage disequilibrium (LD) blocks at 144 kb (Qanbari et al. 2010). Therefore, in this study the Illumina Bovine HD beadchip contains an average of about 30 genotyped SNPs per LD block. As this is the average, there are much smaller blocks of LD containing far less SNPs and some of the SNPs on the beadchip might be also monomorphic. Single marker associations therefore might be based on such small blocks of LD containing only one polymorphic SNP. The maximal variance explained by SNPs of the six main regions for LDA ranged from 6.1% to 7.3%. These values are even higher than the ones found for the SNPs within *MLN*. Therefore, the locations identified in this association analysis might have a big impact on the affection risk of a cow for LDA. They might be of use in a genetic test for LDA, combined with the *MLN* SNPs.

Locations detected in this analysis were compared with those of the linkage analysis and the 50K beadchip analysis. Of the six main regions detected for LDA, the location on BTA8 is in complete concordance with the one of the 50K beadchip. The region associated with LDA on BTA24 is located within a QTL for LDA of the linkage analysis and in the 50K beadchip analysis an adjacent region was detected. Therefore, these regions might contain the genes with the biggest impact on the affection risk of a cow for LDA. Of the markers associated with LDA at a lower extent in this high density study, a single marker located on BTA1 is located within a QTL for LDA and another single marker on BTA17 is located within a family-specific QTL for LDA. Furthermore, locations on BTA14 and BTA24 are in complete agreement with and locations BTA2 and BTA3 are adjacent to those of the 50K beadchip analysis.

The positional genes *SLC4A3, EPHA4, HAND2, LOC781304, LOC100847275, FRMD4A, CDH18, PIGN, KIAA1468*, and *LOC100847796* are located within or close to the regions containing significantly LDA-associated SNPs. Though *SLC4A3, FRMD4A, PIGN,* and *KIAA1468* are expressed in bovine intestine, and *PIGN* and *KIAA1468* are even expressed in the abomasum (http://www.ncbi.nlm.nih.gov/unigene), most of the genes are no obvious functional candidate genes for LDA. However, because of the complex genetic of LDA, these genes could influence the affection risk of a cow for LDA by other, currently unknown mechanisms. The only strong functional candidate gene is *HAND2*, which codes for a transcription factor required for enteric neuron differentiation. In haploinsufficient mice, a reduced expression of *HAND2* caused decreased numbers of neurons, numbers of neuronal nitric oxide synthase, and calretinin (D'Autréaux et al. 2011). Due to the abnormal enteric nervous system of these mice, gastrointestinal motility was also reduced. The region on BTA24 associated and linked with LDA was searched for functional candidate genes. In studies on human, gastrin-releasing peptide (*GRP*) was shown to increase plasma gastrin, pancreatic polypeptide, glucagon, gastric inhibitory peptide, and insulin (Naylor et al. 1987). Among others it has effects on the gastrointestinal and central nervous systems as the release of gastrointestinal hormones or smooth muscle cell contraction. A further functional candidate gene is *PHLPP1* (PH domain and leucine rich repeat protein phosphatase 1). Dysfunctions of this gene are involved in obesity and type 2 diabetes (Andreozzi et al. 2011). This study identified six regions showing high significant associations with LDA. The explained phenotypic variance of SNPs within these regions was at up to 7.3%. This is of value for a genetic test against LDA. Functional candidate genes for LDA were detected for two of the regions.

In the last study, next generation sequencing (NGS) was performed to bring the previous studies to a favorable issue. Fast sequencing methods of complete genomes have been enhanced not only in human but also in cattle in recent time. This approach is much more time and cost efficient today than previous Sanger chain termination sequencing methods. The complete genomes of individuals can be scanned for SNPs within all positional and functional candidate genes located in genomic regions, which show linkage or association with LDA. Six samples were used for NGS. Of these samples, two individuals and one pool of six cows were affected by LDA, two further individuals and another pool of six cows were unaffected by LDA. In these samples, 7,939,073 different polymorphisms were detected and the proportion of novel SNPs was at 47.9%. In previous NGS studies in cattle, higher numbers of novel SNPs were found (Eck et al. 2009, Kawahara-Miki et al. 2011, Stothard et al. 2011), but the number of already known SNPs is rising in the databases with each study performed. Therefore, each new study is expected to reveal less novel SNPs, especially, as

the breed Holstein cattle has been sequenced before (Zhan et al. 2011, Stothard et al. 2011). When starting to evaluate the data for LDA, a first approach located one region showing a high density of LDA associated polymorphisms in the sequenced samples on BTA13. This region is in consistency with one of the main regions for LDA detected on the high density beadchip. In this region, the two genes *DCLRE1C* and *FRMD4A* contained a total of four single nucleotide variants (SNVs) within their coding sequence. One of these SNVs within *DCLRE1C* was predicted to cause a potentially damaging, unknown effect. Though both genes are no obvious functional candidate genes for LDA, they might influence the disease by still unknown mechanisms. Furthermore, causal SNVs might not only be found within the coding sequences, but also affect transcription factors, branch sites, promotors or other functional sites, which have to be regarded in future. This is supported by the study on the causal polymorphism for LDA within *motilin*, which affects a transcription factor binding site and is located in the 5'-untranslated region of this gene. However, coding SNVs with a predicted damaging effect, which are also associated with LDA and within or closely neighboring to the regions detected for LDA in previous analyses, can be expected to play a role for LDA. This is corroborated as the causal polymorphisms identified for milk performance are missense mutations (Cohen-Zinder et al. 2005, Grisart et al. 2002). In the present study, a total of 28 potentially damaging, coding SNVs were located within or closely neighboring to regions described in the previous studies for LDA. Within the regions of the linkage analysis for LDA, 20 potentially damaging polymorphisms were detected within 18 genes. Accordingly, nine potentially damaging polymorphisms within eight genes were located within or adjacent to the regions of the Illumina Bovine SNP 50 beadchip analysis, and seven potentially damaging polymorphisms within eight genes were located within or adjacent to the regions of the Illumina Bovine HD beadchip analysis. Of the genes containing the potentially damaging SNVs, *EGF*, *SDCCAG8*, and *PHLPP1* can be regarded functional candidate genes for LDA. The protein of *EGF* (epidermal growth factor) acts as a potent inhibitor of gastric acid secretion and motility (Smith et al. 1982). *SDCCAG8* (serologically defined colon cancer antigen 8) was robustly associated with early-onset obesity (Scherag et al. 2010) and an increased expression of *PHLPP1* can cause obesity and type 2 diabetes (Andreozzi et al. 2011). The region of this later gene was indicated by all three previous whole genome analyses for LDA. Therefore, some of the discovered SNVs might already be the causal mutations which generated the QTL for LDA. However, for the development of a genetic test for a polygenic trait, the detection of causal mutations is not even required (Weller and Ron 2011) as it can be replaced by marker analyses. This study therefore provides polymorphisms within the coding sequences of functional and positional candidate genes for LDA, which may facilitate a better understanding of the pathogenesis of LDA and also lead to an improved genetic test for this disease.

Table 2. Genomic regions detected in the association analyses for LDA with their position according to the UMD3.1 map (Pos.) and the bovine chromosome (BTA) combined with the results of the next generation sequencing. The association studies of the Illumina bovine 50K (50K) and the Illumina bovine HD beadchip (HD) are given with their P-value corrected for multiple testing of $-\log_{10}P_{corr}>1.0$ (1), $-\log_{10}P_{corr}>2.0$ (2), $-\log_{10}P_{corr}>3.0$ (3), and $-\log_{10}P_{corr}>4.0$ (4). Candidate genes at a maximum distance of 5 Mb for each location are given, if they contain LDA-associated single nucleotide variants (SNVs) affecting the protein. Their effect was predicted by the PolyPhen2 software (Adzhubei et al. 2010).

Study	BTA	Pos. (Mb)	LDA associated SNVs with effect on protein		
			Gene	SNV Position (Mb)	Predicted effect
50K (1)	1	14.7	0	0	0
HD (2)	1	26.1-27.3	0	0	0
HD (2)	1	39.4	*PROS1*	37.8	Benign
			PROS1	37.8	Benign
			ENSBTAG00000022908	42.1	Benign
HD (2)	1	81.4-81.6	0	0	0
HD (2)	1	153.5	0	0	0
HD (2)	1	157.2	0	0	0
HD (2)	2	3.9	0	0	0
HD (2)	2	51.4	0	0	0
50K (1)		54.5			
HD (2)	2	59.1-59.9	0	0	0
HD (2)	2	94.3-94.5	0	0	0
HD (2)	2	104.5	*CCDC108*	107.7	Benign
HD (4)		108.7-109.5	*NHEJ1*	107.8	Benign
			OBSL1	108.2	Benign
			OBSL1	108.2	Benign
HD (2)	2	114.3-114.6	0	0	0
HD (2)	2	120.2-120.4	0	0	0
HD (2)	3	14.5	*SPTA1*	11.1	Probably damaging
			SPTA1	11.1	Probably damaging
			SPTA1	11.1	Benign
			CD1E	11.7	Benign
			ENSBTAG00000038502	11.8	Benign
			PAQR6	14.6	Benign
			ASH1L	15.2	Benign
			UBAP2L	16.3	Benign
			IVL	17.9	Unknown
50K (1)	3	58.4	*ENSBTAG00000021501*	57.5	Benign
			CLCA1	57.7	Benign
50K (1)	3	65.5	*AK5*	67.6	Benign
			ASB17	69.2	Benign
50K (1)	3	78.1	0	0	0
HD (2)	3	107.2	*EPHA10*	108.7	Benign
50K (1)		110.9	*GJB5*	111.5	Benign
			ENSBTAG00000005784	112.9	Benign
HD (2)	4	66.2-66.3	0	0	0
50K (1)	4	75.3	*C7orf31*	71.3	Benign
			TNS3	75.5	Benign

Study	BTA	Pos. (Mb)	LDA associated SNVs with effect on protein		
			Gene	SNV Position (Mb)	Predicted effect
50K (1)	4	115.9	*RNF32*	118.9	Benign
50K (1)	5	22.8	*PLXNC1*	24.2	Benign
HD (2)	5	33.1	*RHEBL1*	30.9	Benign
50K (1)	6	95.4	0	0	0
50K (2)	6	107.4	0	0	0
50K (1)	7	5.6	*ENSBTAG00000008021*	5.7	Benign
			CPAMD8	6.0	Benign
			NWD1	6.2	Probably damaging
50K (1)	7	29.0	*C5orf63*	28.2	Benign
50K (1)	7	43.0	*LOC509006*	41.8	Benign
			LOC509006	41.8	Probably damaging
			LOC509006	41.8	Probably damaging
			ENSBTAG00000038623	42.6	Benign
			ENSBTAG00000019925	43.3	Benign
			ENSBTAG00000047519	43.7	Benign
HD (3)	8	5.7-5.8	0	0	0
50K (2)		5.8			
HD (2)	9	62.0-62.8	0	0	0
50K (1)	10	52.8	*ENSBTAG00000031396*	54.5	Benign
50K (1)	11	7.2	*IL18RAP*	7.2	Benign
50K (2)	11	22.7-23.9	0	0	0
HD (2)	11	30.6	0	0	0
50K (3)	11	43.9-46.7	*IL36B*	46.6	Benign
50K (1)		54.2	*IL36B*	46.6	Benign
			THNSL2	47.7	Benign
			THNSL2	47.7	Possibly damaging
			RETSAT	49.5	Benign
50K (2)	11	102.9	*COQ4*	98.9	Benign
			SPTAN1	99.1	Benign
			PPAPDC3	101.5	Benign
50K (1)	12	59.1-59.2	0	0	0
HD (3)	13	29.0-29.2	*FRMD4A*	28.7	Benign
			DCLRE1C	29.8	Unknown
50K (1)	14	67.0	*SNX31*	65.9	Benign
HD (2)		67.0-68.7	*RGS22*	66.5	Benign
HD (2)	17	53.9	*DHX37*	53.1	Benign
50K (1)		57.2	*DNAH10*	54.1	Benign
			TCTN2	54.3	Benign
			DDX55	54.3	Benign
			DDX55	54.3	Benign
50K (1)	19	4.2	0	0	0
50K (1)	19	59.0	*TRIM65*	56.4	Probably damaging
			LLGL2	56.6	Splice site
			ABCA10	62.0	Benign
HD (2)	20	5.2	*FAM169A*	6.6	Benign
			BDP1	9.9	Possibly damaging
			BDP1	9.9	Benign
50K (3)	20	16.7	*NLN*	13.7	Benign
50K (1)	20	35.3	0	0	0
HD (3)	20	54.1-54.4	0	0	0

Study	BTA	Pos. (Mb)	LDA associated SNVs with effect on protein		
			Gene	SNV Position (Mb)	Predicted effect
HD (2)	20	60.1	0	0	0
HD (2)	22	17.5-17.6	*CPNE9*	17.0	Benign
HD (2)	24	16.9	0	0	0
50K (1)	24	46.1	0	0	0
HD (2)		46.1-46.2			
50K (1)	24	57.9	*LOC510913*	58.6	Unknown
HD (3)		61.0-61.2	*PHLPP1*	61.5	Unknown
			SERPINB10	62.6	Benign
HD (2)	27	26.2	*0*	0	0
HD (2)	27	31.8-32.2	*ENSBTAG00000018713*	34.9	Benign
HD (2)	27	43.5-44.4	*0*	0	0
50K (1)	28	8.8	0	0	0
50K (2)	28	43.6	*ANTXRL*	42.3	Benign
			RBP3	42.6	Unknown
			MAPK8	43.2	Benign
HD (3)	X	103.3-103.6	0	0	0
HD (2)	X	128.9	0	0	0

Table 3. Quantitative trait loci (QTL) detected in the linkage analysis for LDA with their position (QTL Pos.) on the UMD3.1 map and the bovine chromosome (BTA) combined with the results of the next generation sequencing. The QTL type differentiates the main QTL of the linkage analysis (L) and the family specific QTL (L fs). The total number of intragenic single nucleotide variants (SNVs) associated with LDA (SNVs total) is given for each location as well as the number of SNVs located within coding sequences or splice sites (SNVs func.). SNVs causing an amino acid exchange are given with their respective gene, position (SNV Pos.) and their effect predicted by the PolyPhen2 software (Adzhubei et al. 2010).

QTL type	BTA	QTL Pos. (Mb)	SNPs total	SNPs func.	LDA associated SNVs with effect on protein		
					Gene	SNV Pos. (Mb)	Predicted effect
L	1	32.6-81.0	931	23	*PROS1*	37.8	Benign
					PROS1	37.8	Benign
					LOC783843	42.1	Benign
					EFHB	44.7	Benign
					CD96	56.8	Benign
					TMPRSS7	57.2	Benign
					TMPRSS7	57.3	Benign
					C3ORF52	57.3	Benign
					SIDT1	58.7	Possibly Damaging
					LOC538060	62.2	Start gain
					POLQ	66.6	Benign
					GOLGB1	66.8	Benign
					GOLGB1	66.8	Benign
					PARP14	67.6	Benign
					KALRN	69.7	Splice site
					MUC4	71.1	Benign
					MUC4	71.1	Benign
L	3	1.7-17.2	743	28	*ILGR2*	2.0	Benign
					LOC787714	2.6	Benign
					LOC787714	2.6	Benign
					USP21	8.4	Benign
					ARHGAP30	8.4	Unknown
					SLAMF9	8.9	Benign
					SPTA1	11.1	Probably Damaging
					SPTA1	11.1	Probably Damaging
					SPTA1	11.1	Benign
					CD1E	11.7	Benign
					LOC521663	11.8	Benign
					PAQR6	14.6	Benign
					ASH1L	15.2	Benign
					UBAP2L	16.3	Benign
L fs	5	48.1-84.6	759	21	*RDH16*	56.9	Benign
					MMP19	57.8	Benign
					METTL7B	57.9	Benign
					OR6C2	58.6	Probably Damaging
					LOC788438	59.7	Benign
					AMDHD1	60.6	Benign
					LOC505479	67.9	Benign
					LOC505479	67.9	Probably Damaging
					CSF2RB	75.7	Benign

QTL type	BTA	QTL Pos. (Mb)	SNPs total	SNPs func.	LDA associated SNVs with effect on protein		
					Gene	SNV Pos. (Mb)	Predicted effect
L fs	5	90.1-97.5	145	3	*PIK3C2G*	92.0	Benign
					DDX47	97.5	Benign
L fs	6	13.8-31.7	333	9	*EGF*	16.6	Stop loss
					MGC157237	26.0	Benign
					ADH7	26.6	Probably Damaging
L fs	10	92.5-121.2	210	8	*C14ORF102*	103.0	Benign
					SPG11	104.0	Benign
					SPG11	104.0	Splice site
L fs	12	72.4-90.6	521	10	*DZIP1*	76.9	Benign
					ZIC2	80.7	Unknown
					ZIC2	80.7	Unknown
					CCDC168	83.0	Benign
					ARHGEF7	89.5	Benign
L fs	15	51.5-59.2	327	10	*OR52K2*	51.3	Benign
					LOC617692	55.9	Benign
					GUCY2E	56.9	Probably Damaging
L fs	16	25.8-54.9	790	13	*KIF26B*	32.4	Benign
					SDCCAG8	34.4	Unknown
					C1ORF167	42.8	Benign
					C1ORF167	42.8	Benign
					LOC513399	46.1	Benign
L fs	17	40.4-54.3	310	13	*ZNF10*	45.2	Benign
					GPR133	47.0	Possibly Damaging
					DHX37	53.1	Benign
					DNAH10	54.1	Benign
					TCTN2	54.3	Benign
					DDX55	54.3	Benign
					DDX55	54.3	Benign
L fs	19	56.1-61.7	244	6	*TRIM65*	56.4	Probably Damaging
					LLGL2	56.6	Splice site
					ABCA10	62.0	Benign
L	21	62.4-66.7	72	1	0	0	0
L	23	0.0-8.1	248	3	0	0	0
L fs	23	29.8-45.6	518	12	*LOC528343*	29.5	Benign
					LOC511103	29.5	Benign
					LOC528373	30.6	Benign
					OR2B6	30.6	Benign
					SCGN	32.0	Benign
L	24	57.2-62.7	134	3	*LOC510913*	58.6	Unknown
					PHLPP1	61.5	Unknown
					SERPINB10	62.6	Benign
L fs	26	25.6-51.9	102	11	*SORCS3*	25.7	Benign
					PAOX	25.8	Benign
					KNDC1	50.8	Benign

Comprehensive discussion

Many genomic regions each exhibiting a small to medium effect seem to be involved in LDA in German Holstein cows. This result was not unexpected because the genetic variance of polygenic traits as LDA is commonly assumed to be aroused by a large number of genetic variants contributing a small effect each (Manolio et al. 2009, Weller and Ron 2011). It is even a known fact that many of these variants contributing to the phenotypic variation often do not reach genome-wide significance (Lango Allen et al. 2010). Even traits showing a higher heritability than LDA, like for example human body height (h^2=80-90%) (Maher 2008), depend on variants within a high number of genes. At least 180 loci were shown to be responsible for the adult human height, but nevertheless only about 20% of the heritable phenotypic variance is explained by them (Lango Allen et al. 2010). Similarly, 144 SNPs have been reported for milk protein yield in Holstein cows (Daetwyler et al. 2008). Hundreds of variants have been identified for many other traits, but in the most cases, they explained just a small proportion of the estimated heritability (Maher 2008). Different studies tried to answer the question of this so-called missing heritability, which is unexplained by GWAS (genome-wide association studies) findings. This question is of importance, as compelling genetic tests for polygenic diseases are demanded especially in livestock selection. A complete identification of the genes influencing LDA would lead to a better prevention and even treatment of this disease. Missing heritability was attributed to even higher numbers of presently undiscovered variants with small effects, to structural variants, which are not dependably detected by GWAS or to very rare variants present in less than 5% of the population, which are hardly detected by a standard GWAS (Manolio et al. 2009). For some diseases, rare variants with a very high penetrance were detected. These mutations are able to cause the disease even if there are no other defect alleles. However, those variants are an extremely rare finding in polygenic diseases as they usually fall easy prey to selection (Maher 2008). On the other hand, cattle are selected artificially and therefore some variants might not be eliminated, if to humans they seem worth preserving (Goddard and Hayes 2009) due to pleiotropic effects. German Holstein cows are subject of intensive selection for longevity, which is affected by LDA to a big extent (Geishauser et al. 1998a, Wolf et al. 2001, Hamann et al. 2004). Therefore, variants affecting LDA are unlikely to be protected by such measure. On the other hand, it is discussed controversially if (Ricken et al. 2004) or if not (Wolf et al. 2001, Hamann et al. 2004) LDA is more common in cows intensely selected for milk production. If this was the case, it might provoke a selection of these antagonistic traits.
Phenotypic variation might be also caused by allelic heterogeneity (Lango Allen et al. 2010), with many different variants within the same gene each of different origin. This is supported by a study of Zhang et al. (2012), who reported allelic heterogeneity to be present in a

substantial number of loci for the trait human height. Those variants are as difficult to detect by GWAS as any other rare variant. However, if a genetic variant with a small effect on a trait was identified within a specific gene, chances are good for the identification of even more variants in this gene for the same trait, maybe with even larger effects (Manolio et al. 2009). This implicates, that the potentially damaging single nucleotide variants (SNVs) identified by NGS within regions previously shown to be linked or associated with LDA might be causal ones even if their frequency within the German Holstein population is small. SNVs classified as possibly or probably damaging by the PolyPhen software (Adzhubei et al. 2010) are predominantly rare variants (Gorlov et al. 2008). Therefore, it might be promising to first genotype a larger number of LDA-affected German Holstein cows for these mutations. In the next step, the genes, in which the mutations were identified, should be sequenced in a larger number of cows to detect possible allelic heterogeneity.

In our GWAS for LDA, SNPs showing a minor allele frequency (MAF) < 5% were excluded from further analyses during quality control. Therefore, we were able to detect a good portion of the regions containing common variants for LDA, but hardly those containing rare variants. Rare variants have been tried to address by focusing on SNPs showing a MAF < 5%. It has even been suggested that the detection of variants with 0.5% < MAF < 5% in large studies might be more promising for causal SNV identification than focusing on common SNPs, while detection of variants with a MAF < 0.5% was only recommended to be addressed by sequencing (Gorlov et al. 2008). Other authors like Siu et al. (2011) even regarded GWAS inappropriate for the variants at 0.5% < MAF < 5%. The locations detected by our linkage analysis for LDA can be expected to contain rare variants for LDA neither, as the effect sizes of those variants are commonly insufficient for linkage analyses (Manolio et al. 2009). For detection of rare variants for LDA, a study design based on massive parallel next generation sequencing or exome sequencing might be necessary. However, a large number of regions containing common variants for LDA have been detected by our studies until now. The polymorphisms we identified for LDA show high individual phenotypic variances explained and taken together should explain already a large part of the heritability of LDA.

The small effects contributed by each variation for LDA may also explain the differences between our linkage analysis and both GWAS as well as between both GWAS. In each study, another design with another composition of animals had to be used. While in the linkage analysis only large half-sib families were employed, the GWAS on the Illumina bovine 50K beadchip was conducted on a large number and the association study on the Illumina bovine HD beadchip was conducted on a smaller number of mainly unrelated German Holstein cows. Therefore, the detection of causal variants showing a small frequency within the population depends on the percentage of cows within the chosen study design carrying these variants. In the linkage study, only causal variants heterozygous in the common sire of

a half-sib family can be detected. An association study using a large number of cows can be expected to locate other regions than a study using fewer cows. In addition, the number of simultaneously genotyped SNPs differs among both beadchips. The Illumina bovine 50K beadchip contains about 50,000 markers, while the Illumina bovine HD beadchip contains more than 700,000. All SNPs of the 50K beadchip are also contained on the HD beadchip. As German Holstein cattle show a median length of linkage disequilibrium (LD) blocks at 144 kb (Qanbari et al. 2010), variants located on small blocks of LD are not detected by the 50K beadchip. This can be easily observed in our results of both beadchips. Of the six main regions associated with LDA on the HD beadchip, only the one on BTA8 was also detected on the 50K beadchip. This region includes ten SNPs, one of them also present on the 50K beadchip and also associated with LDA in the GWAS using that beadchip. This suggests that these SNPs are located on a small block of LD. Using the 50K beadchip, only a single marker was associated with LDA at this position. Of the remaining five regions detected by the HD beadchip, four on BTA2, 13, 24, and X only contained SNPs not present on the 50K beadchip, indicating, that on the latter beadchip no SNPs existed at this position or that existing SNPs were uninformative. Only one location of the HD beadchip (BTA20) contained a SNP also present on the 50K beadchip, which was not associated with LDA in the study of the 50K beadchip. This is supposed to be caused by the differences in the number of individuals used between both beadchips and due to a rare genotype of the SNPs within this region causing a large effect in the cows chosen for the HD beadchip. Furthermore, confirmation of SNPs detected by GWAS for complex traits in different independent samples is generally poor due to the usually small effect sizes and the absence of the variation in another family even within the same breed (Goddard and Hayes 2009).

A challenge in the identification of the effect of variations on the phenotype is the epistasis effect among genes. Many genes work collectively with other ones and it is important to know about those pathways. A GWAS alone is not able to indicate those networks of genes well, as it only indicates the effect of one specific location on a phenotype. For example Maher (2008) stated "*Two genes may each add a centimetre to height on their own, for example, but together they could add five*" and a setting published by Hoh and Ott (2003) constitutes the combined presence of certain genotypes of three different loci only cause the disease if present in this constellation. For LDA, a first step was taken in this direction by doing a pathway analysis to identify genetic networks among genes within genomic locations for LDA. Further analyses are necessary to define the effects of the resulting genes onto each other.

The traditional method for dairy cattle selection based upon the sires pedigree information, phenotype ascertainment, and progeny testing based on the phenotype and performance of his daughters. This was necessary, as data on production traits in dairy cattle can be

collected in females only. Although this method results in accurate breeding values, the generation interval is five years at minimum (Hayes et al. 2012). A further disadvantage was that only few traits could be considered. In dairy cattle breeding, selection for milk-performance traits has been performed at a large scale, while other traits as for example fertility, disease affection risks, and methane production were neglected in the past. This was mainly due to difficulties in measuring or recording the latter traits or because the genetic influence upon these traits was disregarded or because there was no awareness of the importance to select for a specific trait in the past, as for example the bovine production of methane (Hayes et al. 2012). In the case of LDA, it was not possible to obtain sufficient records on the affection state of German Holstein cows to apply a granddaughter design. Therefore, selection did not include this and the most other diseases. Selection for a restricted number of production traits has indeed lead to a deterioration of many functional traits in Holstein cows, as fertility, longevity, and metabolic stability (Boichard and Brochard 2012). This is also the case for the LDA affection risk and especially health traits are seen as a major challenge in future. There are efforts in organizing a standardized collection of disease data on farms all over the world (Boichard and Brochard 2012). New opportunities in cattle selection have been achieved by the development of genomic selection programs. Genomic selection does not require causal mutations for specific traits, but utilizes genome-wide panels of dense SNPs (Goddard and Hayes 2009). This marker assisted selection bases on linkage disequilibrium between the SNPs and the still undiscovered causal variant (Hayes et al. 2012). For the establishment of genomic selection of a specific trait, only a few thousand cows are needed and phenotype ascertainment may be decoupled from selection (Boichard and Brochard 2012). Genomic selection has become an important method in livestock breeding. It may raise the rate of genetic improvement per year by 100% (Goddard and Hayes 2009) and lower the generation interval to breeding sires down to two years (de Haas et al. 2011, Hayes et al. 2012). In USA, almost all top bulls were evaluated by genomic estimated breeding values but not by progeny (Weller and Ron 2011). Therefore, genomic selection could be a new opportunity to select against LDA using SNPs detected within the present studies. Weller and Ron (2011) propose the application of an *a posteriori* granddaughter design based on genomic regions detected by GWAS. For all sires, haplotypes including the SNPs with largest impact on the trait in question are determined and the sires can be grouped accordingly. However, even genomic selection is limited in its possibilities. The breeding value has to be estimated from a reference population genotyped for all SNPs, in which all individuals have been phenotypically categorized. This is one of the main challenges in genomic selection approaches, as the reference population has to be sufficiently large containing thousands to tens of thousands cows with secured phenotypes. If a routinely report of a trait is not possible or too expensive in a population, it is also difficult to

obtain large reference samples (Hayes et al. 2012) as it is the case in LDA. Furthermore, as genomic breeding values depend on linkage disequilibrium, which changes over generations, the accuracy of prediction will decay rapidly (Weller and Ron 2011, Hayes et al. 2012). Therefore, this approach is not feasible for LDA, as the reference population had to be adjusted continually. A suitable solution of this problem is the utilization of causal variants. The identification of the causal mutation of a QTL is time-consuming and expensive, but reasonable if some criteria are fulfilled. These are among others a sufficiently large effect of the QTL as well as a low frequency of the favorable allele in the present population (Weller and Ron, 2011). Next to the bias reduction in selection, further advantages are the possibilities to select for the causal variants among different cattle breeds and the high economic profit which is obtained by an even small genetic gain (Weller and Ron 2011, Hayes et al. 2012). For LDA, an approach into this direction was done by determining part of the causal variants. These variants can now, combined with the results of the GWAS, be utilized for selection against LDA and to provide a genetic test. The causal and potentially causal mutations discovered may provide deeper insights into the pathogenesis of LDA and into genetic interactions. They might also lead on a track to new medical treatments and prevention of LDA by drug discovery.

V Summary

Left-sided displacement of the abomasum (LDA) is a common and economically important disease in German Holstein cows. Pathogenesis of LDA is multifactorial, but nevertheless genetic components are important as demonstrated by the high heritability at up to 50%. Therefore, molecular genetic analyses should identify LDA-associated genomic regions and unravel single nucleotide variants (SNVs) responsible for LDA. For this purpose, we performed linkage analysis and genome-wide association analyses (GWAS) using beadchips with medium and high density SNP microarrays to detect regions for LDA. Subsequently, next generation sequencing (NGS) was used to target SNVs of those regions.

The linkage analysis revealed two regions on BTA1 and 3 showing genome-wide and three regions on BTA21, 23, and 24 showing chromosome-wide significant linkage with LDA. Eleven further regions showed linkage with LDA on a family-specific basis. To provide a better understanding of genetic traits co-segregating with LDA, we analysed the same data for milk performance traits and length of productive life. As the results show, LDA seems to be especially correlated with the length of productive live, milk-fat yield, and milk-protein percentage. Unravelling those co-localized genomic regions was an important task to improve genomic selection methods, as the incautious use of production trait markers might lead to an increase of the LDA prevalence and in consequence to a decrease in productivity and longevity of dairy cows. Furthermore, polymorphisms located within the *DGAT1* and *ABCG2* genes, currently used for selection of milk performance traits in cattle were analysed in our data. As no co-segregation of polymorphisms within these genes had been found with LDA, a selection on certain genotypes of these genes will not raise the incidence of LDA.

For association-mapping and to identify new regions for LDA, we employed the Illumina bovine 50K and the Illumina bovine high density beadchips. Using the Illumina bovine 50K beadchip, a total of 36 significantly associated SNPs on 17 bovine chromosomes were identified for LDA at $-\log_{10}P>3.0$. One of these SNPs was located within the QTL for LDA on BTA24 and two further SNPs were located within or closely adjacent to family-specific QTL of the previous linkage study. Two SNPs on BTA11 and 20 even showed genome-wide significant associations with LDA. The SNP on BTA11 is located within a functional candidate gene for LDA. Pathway analyses using the data of this association study detected genetic pathways for insulin-dependent diabetes mellitus and calcium metabolism associated with LDA. This provides a new insight into the genetic pathogenesis of LDA.

The SNPs associated with LDA using the Illumina bovine HD beadchip showed higher $-\log_{10}P$-values and explained larger proportions of the phenotypic variance than the LDA-associated SNPs using the 50K beadchip. Six regions showing highly significant associations with LDA were detected and further 27 locations also showed significant associations with

LDA. The highest variance explained by single LDA-associated SNPs of each of the six main regions for LDA was at 6.1% - 7.3%. This confirms the predictive value of these SNPs for a genome-wide LDA-test. Of these six regions detected for LDA, the location on BTA8 is in complete concordance with the one of the 50K beadchip. The region associated with LDA on BTA24 is located within the QTL for LDA of the linkage analysis, and in the 50K beadchip analysis an adjacent region was detected. Therefore, these regions might contain genes with high impact on the affection risk of a cow for LDA. Functional candidate genes for LDA were detected on both chromosomes.

Differences between our linkage analysis and both GWAS as well as between both GWAS are explained by the small effects contributed by each region for LDA as well as the different groups of animals employed and the different marker density used in the studies. Therefore, implementing different study designs for LDA, we were able to detect a good portion of the regions containing common variants for LDA. Combining the polymorphisms of these regions for LDA, it should be possible to explain a large part of LDA heritability.

Based on the linkage analysis for LDA, a search for candidate genes for LDA was performed. The bovine motilin (*MLN*) gene was a functional candidate for LDA due to its known influence on gastrointestinal peristalsis in humans. Sequencing of this gene revealed a SNP (FN298674:g.90T>C) within the first, non-coding exon of *MLN*, which affected a NKX2-5 transcription factor binding site and showed significant associations with LDA. Furthermore, heterozygous and homozygous mutant genotypes of FN298674:g.90T>C were shown to significantly decrease expression of *MLN*. The SNP explains a proportion of 3.1% of the phenotypic variance and can be used for selection to reduce the LDA-affection risk in the German Holstein population.

Next generation sequencing (NGS) subsequently was performed to identify SNPs for LDA based on the locations of the previous studies. In total, six samples were sequenced and yielded 7,939,073 different SNPs and indels (insertions and deletions). The proportion of novel SNPs was at 47.9% and the proportion of novel indels was even higher. Analysing the SNPs associated with LDA among the sequenced samples, a high density of LDA associated polymorphisms was found for a region on BTA13, which was also detected on the Illumina bovine HD beadchip. The *DCLRE1C* gene in this region contains a potentially damaging SNP with unknown effect. In addition to this SNP, 27 further potentially damaging, coding SNPs were located within or closely neighboring to regions described in the previous studies for LDA. Of all genes containing these SNPs, *EGF*, *SDCCAG8*, and *PHLPP1* can be regarded as functional candidates for LDA. This study therefore provides SNVs within the coding sequences of functional and positional candidate genes for LDA.

All causal and potentially causal mutations, which have been discovered for LDA, may provide deeper insights into the pathogenesis of LDA and illuminate an important part of the

genetic background of this disease. It may also lead on a track to LDA prevention by drug discovery or selection based on a genome-wide LDA-test.

VI Zusammenfassung

Die linksseitige Labmagenverlagerung (LMV) ist eine häufige und wirtschaftlich bedeutsame Erkrankung der Milchrinderrasse Deutsche Holstein. Die Pathogenese der LMV ist ein multifaktorielles Geschehen, jedoch ist die genetische Komponente sehr groß. Die Heritabilität wurde in diversen Studien auf bis zu 50% (Wolf et al. 2001, Hamann et al. 2004) geschätzt. Dies ist verglichen mit anderen, häufingen Rinderkrankheiten, ein sehr hoher Wert. Daher war es von großer Bedeutung, die LMV molekulargenetisch zu analysieren und Genomregionen sowie möglichst kausale Mutationen zu identifizieren. Mehrere verschiedene Verfahren wurden dazu herangezogen. Zu diesen gehörten eine Kopplungsanalyse und genomweite Assoziationsanalysen (GWAS) auf dem Illumina Bovine 50K beadchip sowie dem hochauflösenderen Illumina Bovine HD beadchip. Im Anschluss daran wurde eine genomweite Sequenzierung (next generation sequencing, NGS) an sechs Proben durchgeführt, um kausale Varianten (SNVs, single nucleotide variants) in den ermittelten Regionen zu identifizieren.

In der Kopplungsanalyse wurden fünf Genomregionen auf den Chromosomen BTA1, 3, 21, 23 und 24 für LMV identifiziert, davon zwei mit genomweiter und drei mit chromosomenweiter Signifikanz. Außerdem zeigten elf weitere Regionen eine Kopplung mit LMV, die sich auf einzelne Familien beschränkte. Um darüber hinaus ein besseres Verständnis genetischer Zusammenhänge von LMV und anderen Merkmalen zu erhalten wurde dasselbe Familienmaterial LMV-erkrankter Kühe aus der Kopplungsanalyse erneut für die Merkmale Milchmenge, Milch-Fettmenge, Milch-Proteinmenge, Milch-Fettgehalt, Milch-Proteingehalt, somatischen Zellgehalt und die Länge des produktiven Lebens analysiert. Die Ergebnisse belegen, dass LMV insbesondere mit der Länge des produktiven Lebens und darüber hinaus mit Milch-Fettmenge und Milch-Proteingehalt korreliert. Eine Erforschung dieser genetischen Zusammenhänge ist von großer Bedeutung für die heutzutage in großem Stil angewendeten Verfahren der genomischen Selektion, da eine unbedachte Selektion auf Marker für Milchleistungsmerkmale auch zu einem Anstieg unerwünschter Nebeneffekte – wie etwa zu einem Anstieg der LMV Inzidenz – führen könnte. In der Folge könnten sich dadurch Produktivität und Lebensleistung sogar verschlechtern. Neben diesen Analysen wurden Polymorphismen der Gene *DGAT1* und *ABCG2,* die derzeit für die Selektion auf Milchleistungsmerkmale genutzt werden, in unserem Material LMV-erkrankter Kühe untersucht. Zwischen diesen Polymorphismen und LMV wurde keine Kosegregation gefunden. Daher ist anzunehmen, dass eine Selektion auf bestimmte Genotypen dieser Gene die Inzidenz von LMV in der Deutschen Holstein Population nicht erhöht.

Um die bekannten Genomregionen für LMV einzugrenzen und um zusätzliche Regionen aufzufinden wurden Assoziationsanalysen auf den Illumina Bovine 50K und Illumina Bovine HD beadchips durchgeführt. Die Analyse auf dem Illumina Bovine 50K beadchip ergab 36 SNP auf 17 Rinderchromosomen, die signifikante Assoziationen mit LMV von $-\log_{10}P>3.0$ zeigten. Einer dieser SNPs wurde innerhalb eines in der Kopplungsanalyse auf LMV beschriebenen Genombereichs auf BTA24 gefunden und zwei weitere SNPs lagen innerhalb von oder benachbart zu familienspezifischen QTL. Diese Positionen dürften einen besonderen Einfluss auf LMV ausüben. Zwei SNPs auf BTA11 und BTA20 erreichten genomweit signifikante Assoziationen mit LMV, wobei der SNP auf BTA11 innerhalb eines funktionellen Kandidatengens für LMV lokalisiert war. Im Anschluss an die Assoziationsanalyse wurde auf Basis der Ergebnisse eine Analyse auf genetische Stoffwechselpfade durchgeführt. Dabei ergaben sich funktionelle Stoffwechselpfade für den Kalziummetabolismus und insulinabhängigen Diabetes mellitus. Diese Ergebnisse sind von Interesse, da sie neue Erkenntnisse hinsichtlich der genetischen Pathogenese für LMV liefern.

In der im Anschluss durchgeführten Analyse auf dem hochauflösenden Illumina Bovine HD beadchip wurden sechs hochsignifikant mit LMV assoziierte Genomregionen identifiziert. Diese zeigten eine höhere Signifikanzen als solche aus der vorherigen Assoziationsanalyse auf dem Illumina 50K beadchip. Außerdem wurden 27 weitere Regionen mit geringeren LMV-Assoziationen gefunden. Die maximale phänotypische Varianz, die durch die gefundenen SNPs erklärt wurde, liegt für die SNPs der sechs hochsignifikant mit LMV assoziierten Regionen zwischen 6,1% und 7,3%. Sie ist damit sogar höher als die durch den SNP im *MLN* Gen erklärte Varianz. Diese SNPs sind dementsprechend von besonderer Bedeutung für einen kombinierten genetischen Test auf LMV-Anfälligkeit bei Deutschen Holsteins. Von den sechs hauptsächlichen, auf dem hochauflösenden beadchip gefundenen Regionen für LMV stimmt eine Region auf BTA8 komplett mit einer Region der 50K beadchip Analyse überein. Eine auf BTA24 identifizierte Region liegt innerhalb eines QTL aus der Kopplungsanalyse und ist direkt zu der durch den 50K beadchip identifizierten Region benachbart. Daher sind in diesen Bereichen Gene mit hoher Auswirkung auf die Anfälligkeit einer Kuh für LMV zu erwarten. Eine nähere Untersuchung dieser Lokalisationen ergab funktionelle Kandidatengene auf beiden Chromosomen.

Die Unterschiede zwischen der Kopplungsanalyse und den GWAS sowie zwischen beiden GWAS sind durch die jeweils kleinen Effekte der einzelnen Regionen für LMV sowie durch die unterschiedliche Tierauswahl und die unterschiedliche Markerdichte zwischen den Studien zu erklären. Durch diese Anwendung unterschiedlicher Analysemethoden waren wir in der Lage, einen großen Teil der Genomregionen zu ermitteln, die bei Deutschen Holsteins

verbreitete SNVs enthalten. Damit sollte es möglich sein einen großen Teil der Heritabilität für LMV zu erklären.

Basierend auf den Ergebnissen der Kopplungsanalyse für LMV wurde eine Suche nach funktionellen Kandidatengenen unternommen. Das bovine *MLN* (motilin) Gen war in einer Genomregion auf BTA23 aus der vorangegangenen Kopplungsanalyse lokalisiert und wurde aufgrund seiner Wirkung auf die Labmagenmotilität als Kandidatengen für LMV ausgewählt. Die Sequenzierung dieses Gens ergab einen SNP im ersten, nicht kodierenden Exon dieses Gens, der eine NKX2-5 Transkriptionsfaktorbindungsstelle zerstört und eine signifikante Assoziation mit LMV aufweist. Des Weiteren wurde gezeigt, dass der heterozygote als auch der homozygot mutierte Genotyp die Expression von *MLN* signifikant herabsetzt. Dieser SNP erklärt eine phänotypische Varianz von 3,1% und kann bereits genutzt werden, um Kühe und Besamungsbullen zu selektieren und die Anfälligkeit für LMV in der Deutschen Holstein Population zu reduzieren.

Um die in den vorangegangenen Studien identifizierten genomischen Positionen effizient auf für LMV kausale Polymorphismen untersuchen zu können, wurde im nächsten Schritt jeweils das komplette Genom für sechs Proben sequenziert. Dies geschah durch das seit kurzem kostengünstig anwendbare „Next-Generation Sequencing“ (NGS) Verfahren. Insgesamt wurden in den sequenzierten Proben 7.939.073 verschiedene Polymorphismen gefunden, von denen 47,9% noch nicht zuvor veröffentlicht wurden. Alle SNPs wurden innerhalb der sechs Proben auf Assoziation mit LMV getestet. Eine hohe Dichte mit LMV assoziierter Polymorphismen wurde auf BTA13 gefunden und entspricht der durch den Illumina Bovine HD beadchip identifizierten Region. Das in dieser Region lokalisierte Gen *DCLRE1C* enthält einen potenziell schädigenden SNV (single nucleotide variant) mit unbekanntem Effekt in seiner kodierenden Sequenz. Neben *DCLRE1C* wurden weitere 27 mit LMV assoziierte, intragenische SNVs mit potenziell schädigendem Effekt in oder direkt neben den bisher beschriebenen Regionen für LMV gefunden. Von den Genen, die diese SNVs enthalten, sind *EGF* (BTA6), SDCCAG8 (BTA16), and PHLPP1 (BTA24) als funktionelle Kandidatengene für LMV anzusehen.

Die vorliegenden Untersuchungen der genetischen Einflüsse auf LMV ermöglichen einen neuen Blick auf die Pathogenese der LMV und decken einen wichtigen Anteil des genetischen Hintergrundes dieser Erkrankung auf. Die vorliegenden Ergebnisse könnten zu einer verbesserten Prävention von LMV durch Entwicklung neuer Medikationen führen und ermöglichen einen genetischen Test auf LMV.

VII Literature

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Zwald NR, Weigel KA, Chang YM, Welper RD, Clay JS (2004b) Genetic Selection for Health Traits Using Producer-Recorded Data. II. Genetic Correlations, Disease Probabilities, and Relationships with Existing Traits. J Dairy Sci 87: 4295–4302.

VIII Characterization of own contributions to the scientific work

Author contributions to the scientific publications which are part of this postdoctoral thesis are characterized using the following criteria:

1. Idea and study design
2. Realization of the study and molecular genetic analyses
3. Statistical Analysis
4. Interpretation of Results
5. Preparation of the manuscript

In addition, institutions involved in the studies and external support is specified.

Publication 1

S. Mömke, H. Scholz, K. Doll, J. Rehage, O. Distl (2008)
Mapping Quantitative Trait Loci for Left-sided Displaced Abomasum (LDA) in German Holstein Dairy Cows.
Journal of Dairy Science 91: 4383-4392.

Involved institutions:

- Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover (Foundation), Germany
- Clinic for Cattle, University for Veterinary Medicine Hannover, Germany
- Clinic for Ruminants, Faculty for Veterinary Medicine, Justus-Liebig-University Giessen, Germany

Support:

- DFG (German Research Foundation), MO1691/1-1: Lehner (geb. Mömke)

Contributions:

1. Idea and study design: Lehner (geb. Mömke), Distl
2. Realization of the study and molecular genetic analyses: Lehner (geb. Mömke)
3. Statistical Analysis: Lehner (geb. Mömke), Distl
4. Interpretation of Results: Lehner (geb. Mömke)
5. Preparation of the manuscript: Lehner (geb. Mömke), Distl

Publication 2

S. Mömke, W. Brade, O. Distl (2011)

Co-segregation of quantitative trait loci (QTL) for milk production traits and length of productive life with QTL for left-sided displacement of the abomasum in German Holstein dairy cows.

Livestock science 140: 149-154.

Involved institutions:

- Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover (Foundation), Germany
- Chamber of Agriculture (LWK) Lower Saxony, 30159 Hannover, Germany Clinic for

Support:

- DFG (German Research Foundation), MO1691/1-1 and 1-2: Lehner (geb. Mömke)

Contributions:

1. Idea and study design: Lehner (geb. Mömke), Distl
2. Realization of the study and molecular genetic analyses: Lehner (geb. Mömke)
3. Statistical Analysis: Distl
4. Interpretation of Results: Lehner (geb. Mömke)
5. Preparation of the manuscript: Lehner (geb. Mömke), Distl

Publication 3

S. Mömke, M. Sickinger, J. Rehage, K. Doll, O. Distl (2012)
Transcription factor binding site polymorphism in the *motilin* gene associated with left-sided displacement of the abomasum in German Holstein cattle.
PLoS ONE 4: e35562.

Involved institutions:

- Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover (Foundation), Germany
- Clinic for Cattle, University for Veterinary Medicine Hannover, Germany
- Clinic for Ruminants, Faculty for Veterinary Medicine, Justus-Liebig-University Giessen, Germany

Support:

- DFG (German Research Foundation), MO1691/1-2 and 2-1: Lehner (geb. Mömke)

Contributions:

1. Idea and study design: Lehner (geb. Mömke)
2. Realization of the study and molecular genetic analyses: Lehner (geb. Mömke)
3. Statistical Analysis: Lehner (geb. Mömke), Distl
4. Interpretation of Results: Lehner (geb. Mömke)
5. Preparation of the manuscript: Lehner (geb. Mömke), Distl

Publication 4

S. Mömke, M. Sickinger, P. Lichtner, K. Doll, J. Rehage, O. Distl (2013)
Genome-wide association analysis identifies loci for left-sided displacement of the abomasum in German Holstein cattle.
In Review (Journal of Dairy Science).

Involved institutions:

- Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover (Foundation), Germany
- Clinic for Cattle, University for Veterinary Medicine Hannover, Germany
- Clinic for Ruminants, Faculty for Veterinary Medicine, Justus-Liebig-University Giessen, Germany
- Institute of Human Genetics, Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany

Support:

- DFG (German Research Foundation), MO1691/2-1: Lehner (geb. Mömke)

Contributions:

1. Idea and study design: Lehner (geb. Mömke), Distl
2. Realization of the study and molecular genetic analyses: Lehner (geb. Mömke), Lichtner
3. Statistical Analysis: Distl
4. Interpretation of Results: Lehner (geb. Mömke)
5. Preparation of the manuscript: Lehner (geb. Mömke), Distl

Publication 5

S. Lehner, C. Dierks, J. Rehage, O. Distl (2013)

A genome-wide association study for left-sided displacement of the abomasum using the Illumina bovine high-density bead chip.

Involved institutions:

- Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover (Foundation), Germany
- Clinic for Cattle, University for Veterinary Medicine Hannover, Germany

Support:

- DFG (German Research Foundation), MO1691/2-1: Lehner (geb. Mömke)

Contributions:

1. Idea and study design: Lehner (geb. Mömke), Distl
2. Realization of the study and molecular genetic analyses: Lehner (geb. Mömke)
3. Statistical Analysis: Distl
4. Interpretation of Results: Lehner (geb. Mömke)
5. Preparation of the manuscript: Lehner (geb. Mömke), Distl

Publication 6

S. Lehner, C. Dierks, J. Rehage, O. Distl (2013)

Whole genome sequencing of German Holstein cows to detect polymorphisms for left-sided displacement of the abomasum.

Involved institutions:

- Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover (Foundation), Germany
- Clinic for Cattle, University for Veterinary Medicine Hannover, Germany

Support:

- DFG (German Research Foundation), MO1691/2-1: Lehner (geb. Mömke)

Contributions:

1. Idea and study design: Lehner (geb. Mömke), Distl
2. Realization of the study and molecular genetic analyses: Lehner (geb. Mömke)
3. Statistical Analysis: Lehner (geb. Mömke), Distl
4. Interpretation of Results: Lehner (geb. Mömke)
5. Preparation of the manuscript: Lehner (geb. Mömke), Distl

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Appendix

Publication 1

Mapping Quantitative Trait Loci for Left-sided Displaced Abomasum (LDA) in German Holstein Dairy Cows.

Mömke S, Scholz H, Doll K, Rehage J, Distl O.

Journal of Dairy Science 2008; 91: 4383-4392.

doi: 10.3168/jds.2008-1260
http://www.ncbi.nlm.nih.gov/pubmed/18946144

Abstract

A whole-genome scan using an affected paternal half-sib design was utilized to detect quantitative trait loci (QTL) for left-sided displaced abomasum (LDA) in German Holsteins. A total of 360 animals from 14 paternal half-sib families were genotyped, for a total of 306 polymorphic microsatellites. For a whole-genome scan, 221 markers were equally distributed over all 29 bovine autosomes, with an average distance of 13.7 cM. For fine-mapping, a total of 85 additional microsatellites were used. We identified genome-wide significant QTL on Bos taurus autosome (BTA) 1 (54.6 to 58.3 cM) and on BTA3 (5.9 cM). Furthermore, 3 chromosome-wide significant QTL were located on bovine chromosomes 21, 23, and 24. In addition, we found 11 QTL that cosegregated in grandsire families but that were not significant in the across-family analysis. These QTL were located on BTA5, 6, 10, 12, 15, 16, 17, 19, 23, and 26. This study is the first report on QTL for LDA and is a first step toward identifying single nucleotide polymorphisms for LDA-QTL.

Publication 2

Co-segregation of quantitative trait loci (QTL) for milk production traits and length of productive life with QTL for left-sided displacement of the abomasum in German Holstein dairy cows.

Mömke S, Brade W, Distl O.

Livestock science 2011; 140: 149-154.

doi: 10.1016/j.livsci.2011.03.001
http://www.livestockscience.com/article/S1871-1413%2811%2900084-9/abstract

Abstract

Left-sided displacement of the abomasum (LDA) is a commonly observed disease in Holstein dairy cows, previously shown to be genetically correlated with milk performance traits and length of productive life (LPL). The objective of this study was to detect joint quantitative trait loci (QTL) among milk production traits and LDA using a paternal half-sib design including only LDA affected daughters. Joint QTL for LDA and milk production traits in LDA-affected daughters may indicate genetic correlations among these traits due to linkage disequilibrium or even pleiotropic genes. We genotyped 14 paternal half-sib groups including 328 LDA affected daughters and eight sires for 302 microsatellites. Linkage analyses were performed for milk performance traits, somatic cell score (SCS), and LPL. In total, we identified seven genome-wide and further 30 chromosome-wide significant QTL for milk performance traits, SCS, and LPL. All four QTL for LPL, two of the QTL for fat yield, and one QTL for protein percentage overlapped with QTL identified for LDA and twelve further ones overlapped with or were adjacent to family-dependent QTL for LDA. Furthermore, polymorphisms located within the DGAT1 and ABCG2 genes, which were previously reported to be associated with milk performance traits were analysed for all cows. The DGAT1 SNP was associated with milk yield, fat yield, fat percentage, and protein percentage, but showed no association with LDA, while the ABCG2 SNP was monomorphic. We can conclude that genetic correlations among LDA and LPL may arise from these joint QTL. Therefore, selection for longevity should even lower LDA incidence in German Holsteins. A lower co-incidence for LDA-QTL locations was found for fat yield and protein percentage. This study is a step towards better understanding of genetic correlations of LDA with milk performance traits and identification of possible side-effects due to selection for milk production in dairy cows.

Publication 3

Transcription factor binding site polymorphism in the *motilin* gene associated with left-sided displacement of the abomasum in German Holstein cattle.

Mömke S, Sickinger M, Rehage J, Doll K, Distl O.

PLoS ONE 2012; 4: e35562.

doi: 10.1371/journal.pone.0035562
www.ncbi.nlm.nih.gov/pubmed/22536407

Abstract

Left-sided displacement of the abomasum (LDA) is a common disease in many dairy cattle breeds. A genome-wide screen for QTL for LDA in German Holstein (GH) cows indicated *motilin* (*MLN*) as a candidate gene on bovine chromosome 23. Genomic DNA sequence analysis of *MLN* revealed a total of 32 polymorphisms. All informative polymorphisms used for association analyses in a random sample of 1,136 GH cows confirmed *MLN* as a candidate for LDA. A single nucleotide polymorphism (FN298674:g.90T>C) located within the first non-coding exon of bovine *MLN* affects a NKX2-5 transcription factor binding site and showed significant associations (ORallele = 0.64; −log10Pallele = 6.8, −log10Pgenotype = 7.0) with LDA. An expression study gave evidence of a significantly decreased *MLN* expression in cows carrying the mutant allele (C). In individuals heterozygous or homozygous for the mutation, *MLN* expression was decreased by 89% relative to the wildtype. FN298674:g.90T>C may therefore play a role in bovine LDA via the motility of the abomasum. This *MLN* SNP appears useful to reduce the incidence of LDA in German Holstein cattle and provides a first step towards a deeper understanding of the genetics of LDA.

Publication 4

Genome-wide association analysis identifies loci for left-sided displacement of the abomasum in German Holstein cattle.

Mömke S, Sickinger M, Lichtner P, Doll K, Rehage J, Distl O.

Journal of Dairy Science 2013; 96: 3959-3964.

doi: 10.3168/jds.2012-5679
http://www.ncbi.nlm.nih.gov/pubmed/23548285

Abstract

Left-sided displacement of the abomasum (LDA) is one of the most common disorders of the digestive system in many dairy breeds and particularly in Holstein dairy cows. We performed a genome-wide association study for 854 German Holstein cows, including 225 cases and 629 controls. All cows were genotyped using the Illumina Bovine SNP50 BeadChip (Illumina Inc., San Diego, CA). After quality control of genotypes, a total of 36,226 informative single nucleotide polymorphisms (SNP) were left for analysis. We used a mixed linear model approach for a genome-wide association study of LDA. In total, 36 SNP located on 17 bovine (Bos taurus) chromosomes (BTA) showed associations with LDA at nominal -log10P-values >3.0. Two of these SNP, located on BTA11 at 46.70 Mb and BTA20 at 16.67 Mb, showed genome-wide significant associations with LDA at -log10P-values >4.6. Pathway analyses indicated genes involved in calcium metabolism and insulin-dependent diabetes mellitus to be factors in the pathogenesis of LDA in German Holstein cows.

Publication 5

A genome-wide association study for left-sided displacement of the abomasum using the Illumina bovine high-density bead chip.

Lehner S, Dierks C, Rehage J, Distl O.

Abstract

Left-sided displacement of the abomasum (LDA) is a frequent disease in dairy cattle causing significant financial losses for dairy farmers. Heritability of this complex disease was estimated at up to h2=0.5 in German Holstein cows. Using the Illumina bovine high density bead chip comprising 588,753 SNPs after quality control for 126 LDA-cases and 280 population-based controls, we employed a mixed linear model analysis for a genome-wide association study. We identified six genomic regions for LDA on bovine chromosomes 2, 8, 13, 20, 24, and X significantly associated with LDA and covered by 4-12 LDA-associated SNPs. Single SNPs within these regions explained up to 7.3% of the phenotypic variance and therefore might be useful for a genome-wide LDA test.

Publication 6

Whole genome sequencing of German Holstein cows to detect polymorphisms for left-sided displacement of the abomasum.

Lehner S, Dierks C, Rehage J, Distl O.

Abstract

Left-sided displacement of the abomasum (LDA) is a dairy cattle disease with complex genetics and heritabilities estimated at up to 0.5. Great efforts have been made to analyse the genetic factors contributing to this disease in the past. In this study, we determined complete genomic sequences of a total of 16 cows, with each two individual cases and controls and each one pool consisting of six cows, by massively parallel paired-end sequencing. Polymorphisms were detected on basis of the UMD3.1 bovine reference sequence using the CASAVA and GATK software packages. Following association analysis and selection of regions previously reported to be associated with LDA, a total of 312 SNPs were detected within coding sequences of genes or splice sites. Of these SNPs, 28 were potentially damaging, three of which were located within functional candidate genes for LDA.