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Phenotypic, genetic and genome-wide associations for ketosis and metabolic disease indicators on the basis of cow reference groups

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A matrix	Pedigree relationship matrix
acetyl-CoA	Acetyl-coenzyme A
BCS	Body condition score
BHB	β-hydroxybutyrate
BLUP	Best linear unbiased predictor
bp	Base pairs
BTA	*Bos taurus* autosome
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
CDCB	Council of Dairy Cattle Breeding
CDN	Canadian Dairy Network
d	Day
dGV	Direct genomic value
DHI	Dairy Herd Improvement
DIM	Days in milk
DMI	Dry matter intake
FA	Fatty acids
FPR	Fat-to-protein ratio
FPRbin	Binary distributed FPR
FPRgauss	Gaussian-distributed FPR
FTIR	Fourier-transform infrared spectroscopy
G matrix	Genomic relationship matrix
gEBV	Genomic enhanced breeding value
GEC	Genetic Type I Error Calculator
GLMM	Generalized linear mixed model
GRM	Genomic relationship matrix
GWAS	Genome-wide association studies
H matrix	Combined pedigree and genomic relationship matrix
HMG-CoA	β-hydroxy-β-methylglutaryl-CoA
ICF	Interval from calving to first insemination
kb	Kilobase

KET	Ketosis
LSM	Least squares means
Mbp	Megabase pairs
MDR	Metabolic Disease Resistance
m*M*	Millimolar (concentration, mmol/L)
mo	Month
MUFA	Monounsaturated fatty acids
NAFLD	Nonalcoholic fatty liver disease
NEB	Negative energy balance
NEFA	Non-esterified FA
NTM	Nordic Total Merit
pBF	Genome-wide significance level according to Bonferroni
pCD	Normative significance candidate threshold
PDS	Piecewise direct standardization
PMAS	Poor metabolic adaption
PUFA	Polyunsaturated fatty acids
QQ plot	Quantile-Quantile plot
QTL	Quantitative trait loci
RPS	Retroactive percentile standardization
SCC	Somatic cell count
SCS	Somatic cell score
SFA	Saturated fatty acids
SNP	Single nucleotide polymorphism
ssGBLUP	Single-step genomic BLUP
ssGWAS	Single-step GWAS
UFA	Unsaturated fatty acids
VIT	Vereinigte Informationssysteme Tierhaltung w.V, IT solutions for animal production
WGS	Whole-genome sequence
wk	Week
yr	Year

ZUSAMMENFASSUNG

Ketose zählt zu einer der bedeutendsten Stoffwechselerkrankungen hochleistender Milchkühe in der Frühlaktation. In Abhängigkeit von mit einer Ketose assoziierten schwerwiegenden Sekundärerkrankungen, daraus resultierenden Behandlungskosten, sowie reduzierter Milchleistung, verursacht diese Erkrankung bereits im subklinischen Stadium hohe ökonomische Verluste im Milchviehbetrieb. Hohe wirtschaftliche Defizite verdeutlichen die Wichtigkeit der Einbeziehung von Management- und Zuchtstrategien zur Überwachung und Vorbeugung von Ketose in der Milchviehhaltung. Um Stoffwechselerkrankungen frühzeitig erkennen und vermeiden zu können, kann die Überwachung von Stoffwechselmetaboliten im Blut herangezogen werden. Eine kosteneffiziente, schnelle und zuverlässige Alternative zur Blutuntersuchung stellt die Analyse von Stoffwechselindikatoren der Testtagsmilch dar. Mit Hilfe der Fourier-Transformations-Infrarot Spektroskopie (**FTIR**) lassen sich neben den routinemäßig analysierten Milchleistungsmerkmalen, neue, innovative Indikatormerkmale der Stoffwechsellage in der Frühlaktation determinieren. Insbesondere für solche innovativen Gesundheitsmerkmale stehen historische Daten lediglich in begrenztem Umfang zur Verfügung. Die genomische Selektion hinsichtlich dieser Merkmale ist im Vergleich zu Milchleistungsmerkmalen, aufgrund der bestehenden kleinen Bullenreferenzstichprobe, erschwert. Die Genotypisierung weiblicher Tiere in Betrieben mit zuverlässiger Gesundheitsdatenerfassung wirkt dieser Herausforderung entgegen und ermöglicht eine Verbesserung der Genauigkeit genomischer Zuchtwerte hinsichtlich innovativer Gesundheits- sowie Indikatormerkmale. Die simultane Einbeziehung neuer Phänotypen, wie auch genomischer Informationen weiblicher Tiere im Rahmen einer Kuh-Lernstichprobe, offeriert neue Perspektiven in der Milchrinderzucht, besonders im Hinblick auf eine verbesserte Krankheitsresistenz.

Vor diesem Hintergrund eruiert die vorliegende Arbeit die Beziehung zwischen Ketose, innovativen Biomarkern der Milch, sowie dem Fruchtbarkeitsmerkmal Rastzeit auf verschiedenen Analyseebenen und unter Verwendung einer Kuh-Lernstichprobe: 1) Analyse der Beziehungen zwischen Ketose und Milchindikatoren auf phänotypischer Ebene; 2) Schätzung von (Ko-)Varianzkomponenten für diese Merkmale unter Berücksichtigung verschiedener Verwandtschaftsmatrizen auf quantitativ-genetischer Ebene; 3) Durchführung genomweiter Assoziationsstudien (**GWAS**), Identifikation assoziierter Einzelnukleotid-Polymorphismen (**SNP**) sowie Kandidatengene der Ketosediagnose, Milchindikatoren und der Rastzeit bei Holstein Kühen in der Frühlaktation.

Um in das übergeordnete Thema dieser Dissertation einzuführen, dient **Kapitel 1** als allgemeine Einleitung. Eine Literaturübersicht hinsichtlich Ätiologie und Pathogenese, des ökonomischen Einflusses von Ketose, der Beziehung zu weiteren Erkrankungen, Milchindikatoren und zur Fruchtbarkeit, sowie der Implementierung von Ketose in die Zuchtwertschätzung wird in diesem Kapitel gegeben. Des Weiteren stellt Kapitel 1 die genetische und genomische Architektur der in dieser Arbeit analysierten Merkmale heraus und betrachtet die Perspektiven einer Kuh-Lernstichprobe.

Die mittels Infrarot Spektroskopie detektierten innovativen Milchindikatoren gliedern die Arbeit in drei Studien (**Kapitel 2**, **3**, **4**), welche jeweils, die zuvor benannten Forschungsbereiche bearbeiten. Die vorliegenden Studien umfassen eine eingehende Evaluation der Stoffwechselerkrankung Ketose, innovativer Milchindikatormerkmale und der Rastzeit frühlaktierender Holstein-Friesian Kühe.

Kapitel 2 adressiert Assoziationsanalysen hinsichtlich des Fett-Eiweiß-Quotienten (**FPR**) der Milch und Ketose erstlaktierender Holstein Kühe. Die Assoziationsanalysen basieren auf einem umfassenden Datensatz genotypisierter Kühe großer Testherden Deutschlands. Neben diversen Blutindikatoren dient ein erhöhter FPR als Indikator für eine energiedefizitäre Stoffwechsellage und Ketose. Die Beziehung zwischen Ketose und dem FPR auf phänotypischer Ebene wurde unter Anwendung generalisierter linearer gemischter Modelle eruiert. Die Ergebnisse indizierten einen wechselseitigen signifikanten Einfluss zwischen einer Ketosediagnose und dem FPR in der Frühlaktation. Eine steigende Ketoseinzidenz war signifikant mit einem erhöhten FPR, und umgekehrt, assoziiert. Weiterhin erfolgte die Schätzung von Varianzkomponenten anhand eines univariaten Tiermodells und unter Verwendung verschiedener Verwandtschaftsmatrizen. Die mittels Pedigree-basierter Verwandtschaftsmatrix geschätzte Heritabilität für Ketose lag tendenziell höher als bei Anwendung einer SNP-basierten Verwandtschaftsmatrix (Pedigree-basiert: 0,17; SNP-basiert: 0,11). Im Vergleich zu der Pedigree-Heritabilität des binär verteilten FPR (Grenzwert = 1,5) wurde eine höhere Heritabilität basierend auf der genomischen Verwandtschaftsmatrix geschätzt (Pedigree-basiert: 0,09; SNP-basiert: 0,15). Für den normalverteilten FPR zeigten sich annähernd identische Pedigree- und SNP-Heritabilitäten (Pedigree-basiert: 0,14; SNP-basiert: 0,15). Anhand bivariater linearen Tiermodelle wurden außerdem moderate Pedigree-basierte und genomische Korrelationen zwischen Ketose und dem FPR (0,39 - 0,71) geschätzt. Die Ergebnisse deuten darauf hin, dass sich der FPR ebenfalls auf genetischer Ebene als Indikator

für Ketose in der Frühlaktation eignet. Zur Identifizierung von SNP-Markern und Kandidatengenen, welche mit Ketose und dem FPR assoziiert sind, wurden GWAS durchgeführt. Der mit Ketose assoziierte SNP rs109896020 (*Bos taurus* Autosom (**BTA**) 5, 115.456.438 bp) ist in der Nähe des Kandidatengens *PARVB* lokalisiert, welches beim Menschen im Zusammenhang mit der nicht-alkoholischen Fettlebererkrankung steht. Der am signifikantesten assoziierte SNP für den FPR ist im *DGAT1* Gen auf BTA 14 lokalisiert. Die detektierten SNP Assoziationen hinsichtlich einer Ketose und des FPR weisen auf unterschiedliche, zugrundeliegende genomische Mechanismen beider Merkmale hin.

Aufgrund der leichteren Erfassbarkeit klinischer Ketosen im Vergleich zu subklinischen Ketosen wurden vorwiegend klinische Ketoseaufzeichnungen zur Schätzung quantitativ-genetischer und genomischer Parameter genutzt. Die Einbeziehung subklinischer Ketosefälle könnte aufgrund höherer Inzidenzen und präziserer Phänotypen zu einem Informationsgewinn führen. Zur Detektion subklinischer Ketosen werden jedoch detaillierte Aufzeichnungen von Indikatoren, wie beispielsweise Ketonkörperkonzentrationen in Blut oder Milch, benötigt. Die zweite Studie, **Kapitel 3**, fokussiert sich deshalb auf die Analyse der Beziehung zwischen Ketose und der mittels FTIR detektierten Aceton- und β-Hydroxybutyrat- (**BHB**) Milchkonzentration in der Frühlaktation bei Holstein Kühen der ersten drei Laktationen. Um den phänotypischen Effekt von Ketose auf die Aceton- und BHB-Konzentration des ersten Testtages, und zudem den Einfluss von Aceton sowie BHB auf Milchproduktionsmerkmale zu untersuchen, wurden generalisierte lineare gemischte Modelle verwendet. Eine erhöhte Ketoseinzidenz war dabei mit einer signifikant erhöhten Aceton- und BHB-Konzentration der Milch am ersten Testtag assoziiert. Die deutlichen phänotypischen Assoziationen zwischen Ketose, Aceton und BHB am ersten Testtag induzieren eine routinemäßige Bestimmung und Nutzung von Ketonkörperkonzentrationen der Milch, zur Verbesserung und Erleichterung des Gesundheitsmanagements in der Milchviehhaltung. Zudem wurden positive Korrelationen zwischen Aceton, BHB und dem Fettgehalt, dem FPR und auch dem somatischen Zellscore der Milch detektiert. Des Weiteren dienten bivariate lineare Tiermodelle der Schätzung genetischer Varianzkomponenten innerhalb einzelner Laktationen sowie unter Berücksichtigung aller Laktationen im Rahmen von Wiederholbarkeitsmodellen. Pedigree-basierte Heritabilitäten für Aceton rangierten auf einem niedrigen Niveau zwischen 0,01 in der dritten Laktation und 0,07 in der ersten Laktation sowie zwischen 0,03 und 0,04 für BHB. Basierend auf dem Wiederholbarkeitsmodell betrugen die Heritabilitäten 0,05 für Aceton und 0,03 für BHB. Genetische Korrelationen zwischen Aceton und BHB innerhalb einzelner Laktationen sowie

über die ersten drei Laktationen hinweg lagen in einem moderaten bis hohen Bereich (0,69 – 0,98). Ebenfalls hohe genetische Korrelationen konnten zwischen Aceton, BHB und Ketose einzelner Laktationen geschätzt werden (0,71 – 0,99). Genetische Korrelationen zwischen Aceton einzelner Laktationen sowie BHB einzelner Laktationen lagen zwischen 0,55 und 0,66 und suggerieren eine frühe Selektion hinsichtlich FTIR Ketonkörperkonzentrationen in der ersten Laktation. Zwischen Ketose, Aceton, BHB und dem FPR sowie dem Fettgehalt des ersten Testtages der Milch konnten hohe positive genetische Korrelationen geschätzt werden. Zwischen Ketose, sowie Ketonkörperkonzentrationen und der Milchleistung hingegen, identifizierten wir negative Korrelationen. Basierend auf diesen Ergebnissen ist eine Implementierung der Ketonkörperkonzentrationen des ersten Testtages in Selektionsindizes für Stoffwechselerkrankungen aus züchterischer Sicht empfehlenswert. Im Rahmen der GWAS eruierten wir Kandidaten-SNP für Aceton auf BTA 4, 10, 11 und 29, sowie für BHB auf BTA 1 und 16. Identifizierte Kandidatengene *NRXN3*, *ACOXL*, *BCL2L11*, *HIBADH*, *KCNJ1* und *PRG4* sind in Lipid- und Glucosestoffwechselwege involviert.

Auf phänotypischer Ebene konnten bereits Beziehungen zwischen dem Fettsäureprofil der Milch sowie dem Energie- und Fruchtbarkeitsstatus der Kuh postpartum detektiert werden. Erstmalig wurden in der dritten durchgeführten Studie, **Kapitel 4**, die Beziehungen zwischen Ketose in der Frühlaktation, dem Fettsäureprofil der Milch am ersten Testtag und dem Fruchtbarkeitsmerkmal Rastzeit erstlaktierender Holstein Kühe auf genetischer und genomischer Ebene evaluiert. In diesem Zusammenhang wurde ein single-step best linear unbiased predictor (**BLUP**) Verfahren der genomischen Zuchtwertschätzung (**ssGBLUP**) angewendet, welches die gleichzeitige Berücksichtigung genotypisierter und nicht genotypisierter Kühe in der Analyse ermöglicht. Varianzkomponenten und Heritabilitäten für die normalverteilten Merkmale wie die Fettsäuren (**FA**) der Milch, die Rastzeit und für die binär verteilte Ketose, wurden unter Verwendung von Linearen- und Schwellenwertmodellen mittels ssGBLUP geschätzt. Die Schätzung genetischer Korrelationen umfasste bivariate Rechenläufe. Die Heritabilitäten für die Milch-FA waren moderat und rangierten zwischen 0,09 und 0,20, die Heritabilität für die Rastzeit (0,08) und Ketose (0,05) hingegen lagen auf einem niedrigen Niveau. Genetische Korrelationen zwischen Ketose, der Konzentration ungesättigter FA (**UFA**), einfach ungesättigter FA (**MUFA**) und der Stearinsäure (**C18:0**) waren hoch (0,74 – 0,85) und leicht positiv zwischen Ketose und der Rastzeit (0,17). Genetische Korrelationen zwischen UFA, MUFA, C18:0 und der Rastzeit rangierten zwischen 0,34 und 0,46. Diese Ergebnisse suggerieren, dass sich die mittels FTIR detektierte FA-Konzentration des ersten

Testtages als nützlicher Prädiktor für Ketose und die Rastzeit eignen. Ferner legen die für die FA determinierten, moderaten Heritabilitäten eine Einbeziehung der FA-Konzentrationen in Selektionsindizes der Stoffwechselgesundheit und der Rastzeit nahe. Die mit Hilfe von ssGBLUP geschätzten genomischen Zuchtwerte bildeten die abhängigen Variablen der single-step GWAS (**ssGWAS**). Ein großer Anteil identischer genomischer Regionen für die verschiedenen FA, insbesondere für UFA, MUFA sowie für gesättigte FA und die Palmitinsäure, konnte unter Verwendung der ssGWAS identifiziert werden. Ein spezifischer SNP auf BTA 15 war dabei signifikant mit C18:0 und Ketose assoziiert.

Es konnte keine Region auf dem Genom detektiert werden, welche gleichzeitig Einfluss auf alle Merkmale besaß. Dennoch sind einige der annotierten Kandidatengene, wie beispielsweise *DGKA*, *IGFBP4* und *CXCL8* in den Lipidstoffwechsel, Fruchtbarkeitsmechanismen und Produktionskrankheiten in der Frühlaktation involviert. Auf genomischer Ebene identifizierten wir signifikant assoziierte SNP und annotierten potenzielle Kandidatengene, die auf gemeinsame physiologische Mechanismen hinsichtlich der FA-Konzentrationen der Milch, Ketose und der Rastzeit hindeuten. Zusammenfassend zeigte diese Studie, dass die Anwendung von ssGBLUP zur Schätzung genetischer Parameter und der ssGWAS im Vergleich zu früheren Ansätzen, basierend auf reinen Pedigree- oder genomischen Verwandtschaftsmatrizen, deutliche genetische Mechanismen für die drei Merkmalskategorien FA, Stoffwechselerkrankung Ketose und das Fruchtbarkeitsmerkmal Rastzeit verifizierten.

Kapitel 5 evaluiert in einer allgemeinen Diskussion die wichtigsten Ergebnisse der drei durchgeführten Studien (**Kapitel 2**, **3**, **4**). Darüber hinaus werden erstmalig detaillierte phänotypische Beziehungen zwischen der spezifischen FA-Konzentration am ersten Testtag und Ketose abgeleitet, welche praktische Auswirkungen auf das Ketosemonitoring im Milchviehbetrieb besitzen. Des Weiteren werden Aspekte epigenetischer Auswirkungen einer Ketose sowie die Nutzbarkeit von FTIR-Messungen in der Milchrinderzucht diskutiert. Auf der Grundlage unserer Studienergebnisse (**Kapitel 2**, **3**, **4**) werden Schlussfolgerungen gezogen und Empfehlungen für künftige Ketose Überwachungs-, Präventions- und Zuchtstrategien unter Berücksichtigung der analysierten innovativen Milchindikatoren abgeleitet.

SUMMARY

Metabolic disorders display a major part of early lactation disease complexes including ketosis as one of the most common metabolic disorder and an access condition for other diseases in high-yielding dairy cattle i.e., Holstein cows. High economic losses for dairy farmers due to ketosis highlighted the importance of incorporating management and breeding strategies to monitor and prevent ketosis. Today, routine milk sampling using Fourier-transform infrared spectroscopy (**FTIR**) allows the determination of novel innovative traits of practical interest for dairy farmers in metabolic disease prevention. In particular, new health traits limited historical data and genomic selection regarding those traits is challenging. A smaller reference population is available to be used in genomic predictions compared with e.g., milk production traits. Genotyping of cows in herds with reliable health records may be one strategy to enhance the accuracy of genomic predictions for health and novel indicator traits. Thus, cow reference groups offer new prospects in dairy cattle breeding for improved disease resistance by combining phenotypes for novel traits with high-density genetic markers. Against this background, we assessed the usability of common and novel milk measurements i.e., the relation between novel functional FTIR measurements, fertility trait interval from calving to first insemination and ketosis diagnosis on the basis of cow reference groups on different scales: 1) Examination of relations between milk indicator traits and ketosis, phenotypically; 2) Estimation of (co)variance components for such traits considering different relationship matrices on quantitative genetic scales; 3) Genome-wide association studies (**GWAS**), identification of associated single nucleotide polymorphisms (**SNP**) and candidate genes for the ketosis diagnosis, innovative milk indicator traits and the fertility interval trait in Holstein cattle.

In order to introduce the overall topic of the present thesis **chapter 1** displays the general introduction. Accordingly, a literature overview on metabolic disease ketosis is provided here. The etiology and pathogenesis of ketosis, economic impacts, ketosis implementation in breeding programs, genomic architecture, and the relation to novel milk indicator traits as well as a fertility interval trait are examined.

The different innovative milk indicator traits measured via routine milk sampling by infrared spectroscopy divides this thesis into three studies (**chapter 2**, **3**, **4**), respectively, addressing the previously mentioned research areas. The present studies are an in-depth evaluation of metabolic disease ketosis and innovative milk indicator traits in Holstein cattle.

Chapter 2 addresses association analysis between first test-day fat-to-protein ratio (**FPR**), a metabolic deficit indicator, and ketosis in first-lactation German Holstein cows based on a large data set of genotyped cows from large scale co-operator herds. In addition to several blood indicators, the FPR is suggested as an indicator for ketosis, because a FPR > 1.5 refers to high lipolysis. Phenotypic trait associations were inferred through the application of generalized linear mixed models and indicate to a strong relationship between ketosis and the FPR. Increasing ketosis incidences were significantly associated with higher FPR and vice versa.
Univariate and bivariate linear animal models were used to estimate genetic (co)variance components, heritabilities and genetic correlations between the traits using pedigree-based or genomic relationship matrix. The ketosis heritability was slightly larger when modeling the pedigree-based relationship matrix (pedigree-based: 0.17; SNP-based: 0.11). For the binary distributed FPR (threshold = 1.5), heritabilities were larger when modeling the genomic relationship matrix (pedigree-based: 0.09; SNP-based: 0.15). For Gaussian-distributed FPR heritabilities were almost identical for both pedigree and genomic relationship matrices (pedigree-based: 0.14; SNP-based: 0.15). Genetic correlations between ketosis with FPR using either pedigree- or genomic-based relationship matrices were in a moderate range from 0.39 to 0.71. Results lead to suggest the utilization of FPR from the first test-day as an indicator for genetic ketosis predictions. Applying GWAS we identified the specific SNP rs109896020 (*Bos taurus* autosomes (**BTA**) 5, 115,456,438 bp) contributing to ketosis. The identified potential candidate gene *PARVB* in close chromosomal distance was associated with nonalcoholic fatty liver disease in humans. The most important SNP contributing to the FPR was located within the *DGAT1* gene. Different SNP significantly contributed to ketosis and FPR indicating different mechanisms for both traits genomically.

As accurate population-wide binary health trait recording is difficult to implement proper Gaussian indicator traits which can be routinely measured in milk are needed. Consequently, the second scientific study, **chapter 3**, focused on the ketone bodies acetone and β-hydroxybutyrate (**BHB**) measured via FTIR in milk in first- to third-parity Holstein cows. Associations between FTIR acetone and BHB with ketosis and with test-day traits were studied phenotypically and quantitative genetically. Generalized linear mixed models were applied to infer the influence of binary ketosis on Gaussian-distributed acetone and BHB (definition of an identity link function) and vice versa i.e., the influence of acetone and BHB on ketosis (definition of a logit link function). Additionally, linear models were applied to study associations between BHB, acetone and test-day traits (milk yield, fat percentage, protein

percentage, FPR, and somatic cell score) from the first test-day after calving. An increasing ketosis incidence was statistically significant associated with increasing FTIR acetone and BHB milk concentrations. The strong phenotypic associations between first test-day FTIR acetone and FTIR BHB with ketosis suggested routine determination and utilization of ketone bodies in milk to improve the dairy cow health management. Acetone and BHB concentrations were positively associated with fat percentage, FPR and somatic cell score. Bivariate linear animal models were applied to estimate genetic (co)variance components for ketosis, acetone, BHB, and test-day traits within parities one to three and considering all parities simultaneously in repeatability models. Pedigree-based heritabilities were quite small i.e., in the range from 0.01 in parity three to 0.07 in parity one for acetone and from 0.03 to 0.04 for BHB. Heritabilites from repeatability models were 0.05 for acetone and 0.03 for BHB. Genetic correlations between acetone and BHB were moderate to large within parities and considering all parities simultaneously (0.69 to 0.98). Genetic correlations between acetone and BHB with ketosis from different parities ranged from 0.71 to 0.99. Genetic correlations between acetone across parities and between BHB across parities ranged from 0.55 to 0.66. Genetic correlations between ketosis, acetone, and BHB with FPR and with fat percentage being large and positive but negative with milk yield. Furthermore, we identified pronounced favorable genetic correlations among ketosis, FTIR acetone, FTIR BHB and FPR within parities one to three, and moderate genetic correlations from the repeatability model. Hence, from a breeding perspective results proposed a consideration of milk ketone bodies in selection indices for metabolic disorders. The strong genetic correlations between FTIR acetone and BHB from first lactation with the respective traits in later lactations indicated the usefulness of early selection in first parity cows. In GWAS, we identified SNP on BTA 4, 10, 11, and 29 significantly influencing acetone and on BTA 1 and 16 significantly influencing BHB. The identified potential candidate genes *NRXN3*, *ACOXL*, *BCL2L11*, *HIBADH*, *KCNJ1*, and *PRG4* were involved in lipid and glucose metabolism pathways.

Furthermore, milk fatty acids (**FA**) have been suggested as novel biomarkers for early lactation metabolic diseases and for the female fertility status. Thus, the evaluation of genetic and genomic associations between ketosis, milk FA and the fertility interval trait, the interval from calving to first insemination (**ICF**) in first-lactation Holstein cows referred to **chapter 4**. In this regard, we focused on a single-step genomic best linear unbiased predictor (**ssGBLUP**) approach allowing a simultaneous consideration of genotyped and ungenotyped cows. Variance components and heritabilities for all Gaussian-distributed FA, for ICF, and for binary ketosis were estimated by applying single-step genomic BLUP single-trait linear and threshold models,

respectively. Genetic correlations were estimated in series of bivariate runs. Heritabilities for FA were moderate in the range from 0.09 to 0.20 but quite small for ICF (0.08) and for ketosis (0.05 on the underlying liability scale). Genetic correlations between ketosis and unsaturated FA (**UFA**), monounsaturated FA (**MUFA**), stearic acid (**C18:0**) were large (0.74 to 0.85) and low positive between ketosis and ICF (0.17). Genetic correlations between UFA, MUFA, and C18:0 with ICF ranged from 0.34 to 0.46. The results indicated that first test-day FTIR FA concentrations in milk were valuable predictors for ketosis and for ICF. Furthermore, the estimated moderate heritabilities for FTIR FA concentrations suggested a consideration of FA in selection indices for female fertility trait ICF and health. Genomic breeding values from the ssGBLUP estimations were dependent traits in single-step GWAS (**ssGWAS**). In ssGWAS, we identified a large proportion of overlapping genomic regions for the different FA, especially for UFA and MUFA, and for saturated FA and palmitic acid. One significantly identical associated SNP was identified for C18:0 and ketosis on BTA 15. However, there was no genomic segment that simultaneously affected significantly all trait categories ICF, FA and ketosis. Nevertheless, some of the annotated potential candidate genes *DGKA*, *IGFBP4* and *CXCL8* played a role in lipid metabolism and fertility mechanisms and influenced production diseases in early lactation. Genomically, we identified significantly associated SNP and annotated potential candidate genes indicating shared physiological mechanisms on FA concentrations, ketosis and ICF. In conclusion, the application of single-step GBLUP genetic parameter estimations and single-step GWAS inferred closer genetic mechanisms of the three trait categories FA, metabolic disorders and female fertility trait ICF compared to previous approaches based on either pure pedigree or pure genomic relationship matrices.

In **chapter 5**, a general discussion evaluated the results presented in this thesis. Additionally, phenotypic relations between first test-day FA concentration and ketosis based on the data set described in chapter 4, were inferred and revealed practical implications for on-farm detection of metabolic disease ketosis. Furthermore, aspects regarding epigenetic impacts of ketosis in dairy cows and the usefulness of FTIR measurements were discussed. Based on our study results conclusions were drawn and recommendations for future ketosis monitoring, prevention and breeding strategies considering analyzed novel milk traits were provided.

CHAPTER 1

General Introduction

Negative energy balance in high-yielding dairy cows

During early lactation of high-yielding dairy cows the increase of nutrient demand for milk production exceeds the slow increase in dry matter intake and causes a state of negative energy balance (**NEB**). The rapidly increasing milk production in the first weeks after calving lead to elevated requirements in glucose, amino- and fatty acids (**FA**) which were two to five times higher postpartum than prepartum (Bell, 1995). This energy deficiency resulted in metabolic stress and increased susceptibility to i.e., mastitis, claw diseases and metabolic disorders (e.g., ketosis (**KET**), Buttchereit et al., 2012). Figure 1.1 displays the relationship between energy intake and energy requirements for a lactation of high producing dairy cows and the increased early lactation disease incidences of first lactating cows.

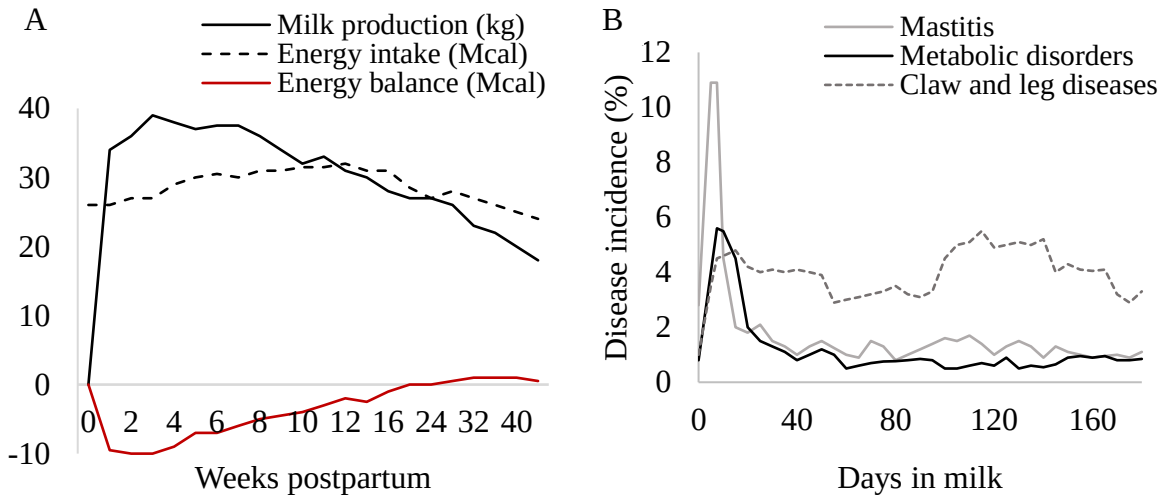

Figure 1.1. (A) Milk yield (kg/day), net energy intake (Mcal/day), net energy balance (Mcal/day) of high-yielding cows, and (B) incidences (%) for mastitis, metabolic disorders and claw and leg diseases of first lactating cows (modified according to Bauman and Currie, 1980; Buttchereit et al., 2012).

Metabolic disorders displayed a major part of early lactation disease complexes including KET as one of the most common metabolic disorders and an access condition for other diseases in dairy cattle (Oetzel, 2004).

Ketogenesis and ketosis

Glucose and FA are usually metabolized to the enzyme acetyl-coenzyme A (**acetyl-CoA**) in hepatocytes which normally enters the citric acid cycle by condensing with oxaloacetate (Figure 1.2, Laffel, 1999). Glycolysis produces pyruvate, functioning as a precursor of oxaloacetate.

Caused by the lack of energy in early lactation glycolysis falls to a very low level and oxaloacetate is preferentially utilized in the process of gluconeogenesis (Figure 1.2, Laffel, 1999). Furthermore, the state of NEB triggers lipolysis and cows adipose tissue mobilization. The mobilized FA are oxidized in the liver via β-oxidation to acetyl-CoA (Zhang and Ametaj, 2020). Throughout the NEB oxaloacetate is not available to condense with acetyl-CoA produced by the FA β-oxidation. Thus, acetyl-CoA becomes diverted from the citric acid cycle to mitochondrial ketone body formation by ketogenesis (Figure 1.2, Laffel, 1999).

During the first step of ketogenesis thiolase condensates two molecules of acetyl-CoA into acetoacetyl-CoA. Acetoacetyl-CoA functions as a substrate for β-hydroxy-β-methylglutaryl-CoA (**HMG-CoA**) synthase conducting the synthesis of HMG-CoA (Harvey et al., 2019). Afterwards, HMG-CoA lyase metabolizes HMG-CoA to the unstable ketone body acetoacetate. The acetoacetate is converted to stable ketone body β-hydroxybutyrate (**BHB**) by D-β-hydroxybutyrate dehydrogenase and a proportion of the acetoacetate is decarboxylized to acetone due to a spontaneous non-enzymatic decarboxylation (Harvey et al., 2019).

The two ketone bodies acetoacetate and BHB function as a short term source of energy for several organs like heart, brain and also skeletal muscle in the physiological state characterized by limited availability of carbohydrates (Robinson and Williamson, 1980). For the usage in extrahepatic tissue BHB is converted to acetoacetate by the enzyme β-hydroxybutyrate dehydrogenase and acetoacetate is converted back to acetyl-CoA by β-ketoacyl-CoA transferase and acetoacetyl-CoA-thiolase (Dhillon and Gupta, 2021). This conversion of the ketone bodies to usable acetyl-CoA occurs exclusively extrahepatic. The resultant acetyl-CoA could then be used in citric acid cycle in previously mentioned tissues. Throughout the oxidation of BHB to acetoacetate and the following usage of acetyl-CoA in the citric acid cycle these processes produce 22 ATP per molecule (Dhillon and Gupta, 2021). The ketone body acetone is not usable for energy provision and, thus, excreted from the body with urine and exhaled by the lungs causing the characteristically sweet, fruity breath (Harvey et al., 2019).

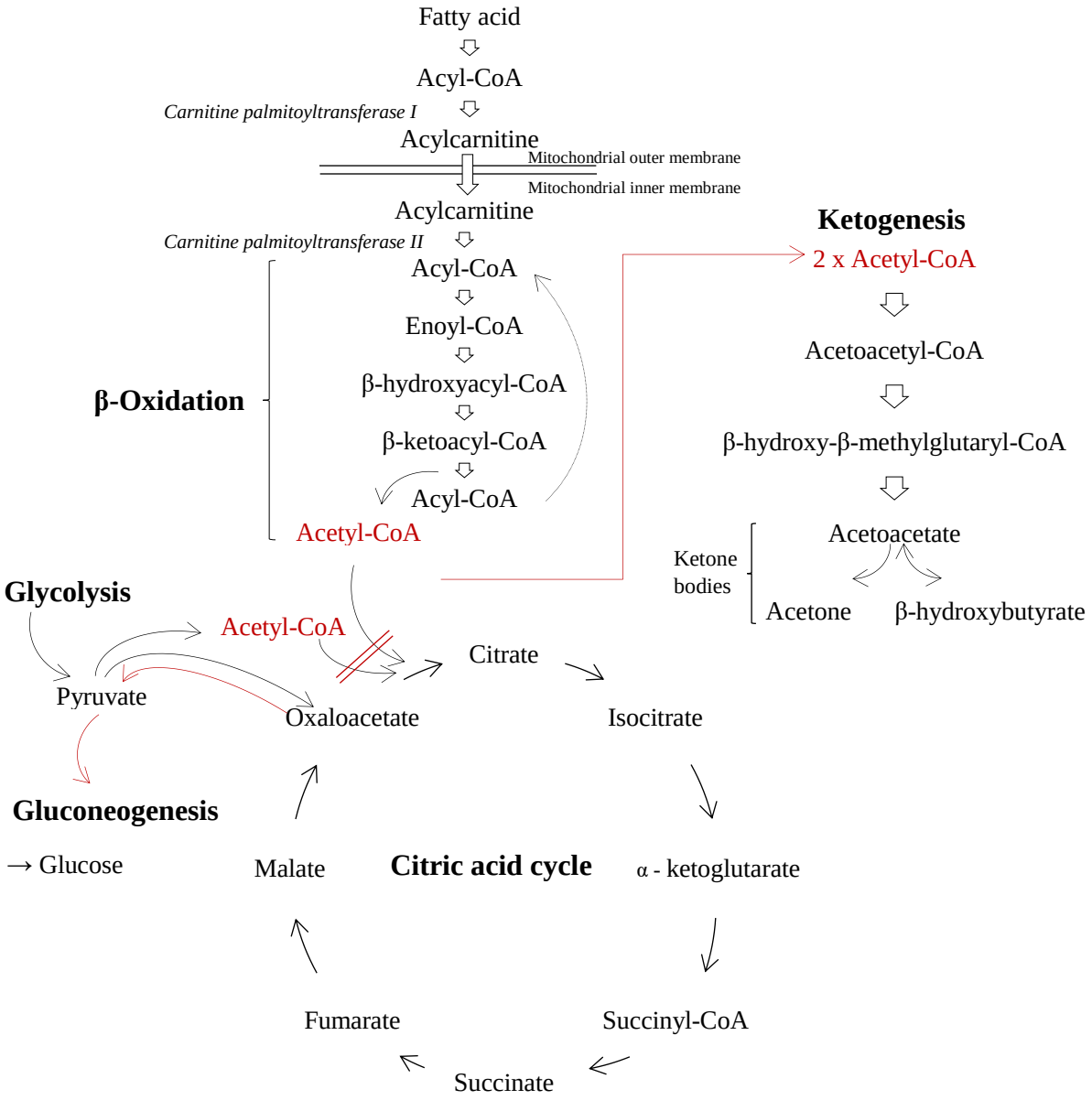

Figure 1.2. Mitochondrial fatty acid β-oxidation, citric acid cycle and ketogenesis in the hepatocyte (modified according to Bergman, 1971; Laffel, 1999).

Also the digestive tract displayed a site of ketone body production in ruminants (Bergman, 1971). In the healthy ruminant ketone bodies were produced by the rumen epithelium from dietary FA, especially from butyrate. But during KET and reduced feed intake the hepatic ketogenesis bases on FA mobilized from body tissue accounts for the majority of ketone body formation in animals (Bergman, 1971).

An excessive concentration of the circulating ketone bodies in extrahepatic tissue caused intoxication and metabolic disease KET in dairy cows. Typical clinical signs of KET were depressed milk yield and feed intake, weight loss, lethargy. Also nervous disturbances e.g., obsessive licking and excess salivation could be observed in some cases (Benedet et al., 2019a).

The metabolic disease KET is reflected by high concentrations of ketone bodies in blood, milk and urine (Enjalbert et al., 2001; Benedet et al., 2019a). As summarized by Benedet et al. (2019a) a blood BHB level greater than 1.2 mmol/l was generally defined as the KET threshold value. The additional classification of BHB blood levels between 1.2 mmol/l and 2.9 mmol/l was considered to define subclinical KET without any clinical disease signs while a BHB value higher than 3.0 mmol/l reflected clinical KET (Benedet et al., 2019a). The measurement of BHB concentration in blood displayed the gold standard in KET diagnostics (Benedet et al., 2019a). Furthermore, different types of KET were distinguished in literature (Zhang and Ametaj, 2020). Type I KET, primary KET, occured within six weeks postpartum according to the increasing milk production in early lactation. Type II KET, secondary KET, appeared early after calving and was coincident with further diseases. Obesity and overfeeding during the dry period could lead to this type of KET. Another type of KET not related to early lactation energy balance arised due to the intake of ketogenic precursors i.e., the consumption of silage with high amounts of butyrate (Zhang and Ametaj, 2020).

Incidences for clinical KET ranged between 0.7% and 3.5% in European dairy herds (Berge and Vertenten, 2014). However, regarding the high incidences for subclinical KET (up to 49%) in early lactation and the relationship to further cost-intensive diseases, KET is one of the most important metabolic disorders in dairy farms (Suthar et al., 2013; Berge and Vertenten, 2014; Vanholder et al., 2015).

Relationship of ketosis with health and fertility

Besides other metabolic disorders, KET is considered as an access condition for further cost-intensive metabolic diseases such as retained placenta, metritis, laminitis, and displaced abomasum. Suthar et al. (2013) assessed the relationship of subclinical KET with postpartum diseases in European dairy farms. Multivariate binary logistic regression models revealed that cows with subclinical KET had 1.5, 9.5, and 5.0 times greater odds of developing metritis, clinical KET, and displaced abomasum in early lactation, respectively (Suthar et al., 2013). According to that, Duffield et al. (2009) estimated the influence of serum BHB concentrations on subsequent diseases. Elevated BHB concentration (≥ 1,200 µmol/l) in the first week after calving was associated with an increased risk of displaced abomasum and metritis. Duffield et al. (2009) presumed that a similar etiology of KET and displaced abomasum could be the reason for the disease association. Due to the reduced feed intake and the anorexia during KET, the deficit in the rumen fill probably caused displaced abomasum (Shaver, 1997). The association between elevated BHB concentration and metritis might be based on the influence of increased

BHB concentrations on the immune function (Hammon et al., 2006). Recent study results by Lei and Simões (2021) associated average milk BHB concentration with left displaced abomasum in Holstein cows. Cows suffering from displaced abomasum showed significantly higher BHB milk concentrations (0.18 ± 0.02 mmol/l) in the first month postpartum than healthy cows (0.07 ± 0.02 mmol/l). Results of Lei and Simões (2021) confirmed the thesis by Duffield et al. (2009) that KET contributed to the etio-pathophysiology of displaced abomasum. A significant correlation between high blood BHB concentrations, oxidative stress and liver apoptosis in bovine hepatocytes was described by Song et al. (2016). Excessive ketone bodies accumulated in blood and liver cells and induced KET. Song et al. (2016) showed that BHB levels were positively related to oxidative indicators (e.g., malondialdehyde, glutathione disulfide) while a negative relationship was detected between BHB levels and total antioxidant capacity indicating that high BHB levels induced hepatocyte oxidative stress. Furthermore, the hepatocyte apoptotic rate was significantly elevated in cells exposed to subclinical and clinical KET BHB concentrations confirming that high BHB concentrations caused hepatocyte apoptosis and hence liver damage due to oxidative stress (Song et al., 2016).

Furtheron, negative impact of KET on cows fertility complex was detected. Negative energy balance generated an endocrine environment that depressed the growth of the follicle and reduced its functionality (Lucy, 2019). The increase of circulating ketone bodies, especially BHB, and FA caused minor fertility of the oocyte and immune dysfunction with detrimental impact on uterine recovery (Wathes et al., 2009). Holstein cows with increased blood BHB had a significantly delayed interval from calving to first observed oestrus and interval from calving to first insemination (**ICF**) (Rutherford et al., 2016). Thus, KET reduced intensity and duration of oestrus activity. The fertility trait ICF measures the days from calving to a cow's ability to resume cyclicity after calving and to show oestrus behaviour. Hence, the ICF displays an integral part of cows fertility complex (Mehtiö et al., 2020). Recent results of Li et al. (2019) confirmed that BHB induced oxidative stress which caused upregulated release of pro-inflammatory factors in bovine endometrial cells. The enhanced reliance on FA as an energy source postpartum induced oxidative damage to mitochondria in metabolically active tissues including the liver and also the reproductive system. The excess lipid accumulation in oocytes and the regenerating endometrium reduced fertility due to increased inflammatory alterations (Wathes et al., 2013).

Genetic correlation of ketosis to dairy cattle breeding goal and health traits

Genetic correlations between KET and milk production traits were not consistent among different studies. While Belay et al. (2017) estimated positive genetic correlations of 0.17 between clinical KET (veterinarian-recorded) and milk yield in first- to fourth-lactation Norwegian Red cows, Koeck et al. (2013) found a negative genetic correlation (-0.07) between the traits in early first-lactation Canadian Holsteins. Low positive genetic correlations of 0.15, 0.002 and 0.16 were detected among KET, fat, protein and lactose yield, respectively (Belay et al., 2017). Fat, protein and lactose percentage were negative genetically correlated to KET, with -0.02, -0.33 and -0.04, respectively (Belay et al., 2017). Again, early first lactation correlations between KET, fat percentage (0.33) and protein percentage (-0.06) (Koeck et al., 2013) differed to correlations detected by Belay et al. (2017). Vosman et al. (2015) exposed correlations between breeding values for KET (based on milk BHB and acetone concentrations), production, health traits, and fertility. Thereby, a higher breeding value for KET resulted in less KET. Breeding values for KET were negative correlated to breeding values for milk (-0.28), fat (-0.15), and protein yield (-0.08) and positive correlated to fat (0.16) and protein (0.39) percentage. Correlations with fertility (0.29), udder health (0.19), mastitis (0.19-0.21), and somatic cell score (0.16) were positive, which means less KET results in better fertility and udder health (Vosman et al., 2015).

Generally, studies analyzing genetic correlations between KET and other diseases suggested positive genetic correlations. Strong positive genetic correlations were determined between first lactation KET and displaced abomasum (0.79) and low to moderate correlations were found for retained placenta (0.07 - 0.21), metritis (0.62), clinical mastitis (0.26) and milk fever (0.19) in Canadian Holsteins and Norwegian Red cattle (Heringstad et al., 2005; Jamrozik et al., 2016). Also for second- and third-lactation dairy cows genetic correlations of KET to described diseases stayed positive (Heringstad et al., 2005).

Economic impact of ketosis

Clinical and also subclinical KET were associated with lower milk production, increased probability of production diseases, lower reproductive performance, and thus increased culling of dairy cows (Steeneveld et al., 2020). Mostert et al. (2018) estimated the economic impact of subclinical KET in dairy cattle using a dynamic stochastic simulation model in consideration of the reduced milk production, treatment, culling, related diseases, and different parities during the first 30 days after calving. Estimations revealed total costs of €130 per case per year, ranging between €39 and €348 (5 to 95 percentiles) while those costs increased from €83 per year in

parity one up to €175 in parity three (Mostert et al., 2018). Previous studies of McArt et al. (2015) and Gohary et al. (2016) verified the high economic impacts or rather losses induced by KET for dairy farmers. A recent study by Steeneveld et al. (2020) quantified the losses due to KET in order to support decision making regarding prevention and treatment of the disease by veterinarians and farmers. Taking into consideration the different treatment strategies for clinical and subclinical KET, the occurrence of clinical, subclinical KET, displaced abomasum, mastitis, and the effect of KET on reproduction, culling and milk yield a cow simulation model was applied (Steeneveld et al., 2020). The biological output results of the simulation study showed average annual milk production losses due to six clinical KET cases of 1,199 kg and due to 36 subclinical KET cases of 6,126 kg in high risk scenarios. Additionally, an increase in the number of displaced abomasum cases, mastitis cases, of inseminations and culled cows was observed with an increased KET risk. The economic output results indicated overall costs for clinical and subclinical KET in a 130 cow herd of €7,371 per year in the high risk scenario due to decreased milk revenues, higher culling costs, higher insemination costs, and costs for related diseases (Steeneveld et al., 2020). These high economic losses highlighted the importance of incorporating all possibilities to prevent KET which means considering breeding aspects besides management aspects to generate more disease resistant animals.

Ketosis related milk indicators

Aside from the gold standard (blood BHB measurement) several milk indicators were suggested for KET monitoring and prevention in early lactation. The increased body fat mobilization in state of NEB and KET lead to an increased milk fat synthesis and thus an elevated fat content of milk (Duffield et al., 2009). According to Zhang et al. (2015) ketone body BHB facilitated the FA synthesis in the mammary epithelial cells. The treatment of mammary epithelial cells with different concentrations of BHB induced a significant increase in the expression of genes involved in FA synthesis resulting in an enhanced triglyceride secretion. In contrast to this the inadequate feed intake during the first third of lactation caused insufficient protein provision from ruminal bacteria leading to decreased milk protein content (Gürtler and Schweigert, 2005). Hence, the milk fat-to-protein ratio (**FPR**) higher than 1.5 referred to high lipolysis and could serve as a valuable, easily available indicator for the energy status postpartum and KET (Heuer et al., 1999).

As a non-invasive, rapid and inexpensive method Fourier-transform infrared spectroscopy (**FTIR**) is globally used to analyze milk samples in dairy herds. Besides milk fat, protein and lactose concentrations infrared spectroscopy enabled the prediction of additional valuable novel

phenotypes of importance to breeding programs (Tiplady et al., 2020). Milk recording organizations around the world already provide novel milk phenotypes such as milk ketone body concentration for monitoring metabolic health status in early lactations (Schwarz, 2017). With regard to the economic consequences of KET in dairy herds (Steeneveld et al., 2020) early detection of metabolic milk indicators via FTIR might be effective in disease and, therefore, treatment costs prevention (Benedet et al., 2019a). Although the determination of blood BHB concentration was considered as the gold standard to predict subclinical and clinical KET, the detection of milk ketone body concentrations is a promising approach to assess the metabolic status of a cow (Schwarz, 2017; Gross and Bruckmaier, 2019). High correlations between blood and milk ketone body concentrations were identified. Enjalbert et al. (2001) analyzed the relationship between ketone body concentration in milk and blood and detected strong phenotypic correlations for acetone of 0.96 and BHB of 0.66. The primary use of BHB by mammary gland for FA synthesis in ruminants is a possible reason for a lower correlation coefficient between blood and milk BHB (Smith et al., 1974). In general, due to the usage of ketone bodies for FA synthesis in the mammary gland the concentrations of milk ketones were approximately ten times smaller than blood concentrations (Enjalbert et al., 2001). Furthermore, a high correlation between milk acetone and milk BHB of 0.68 was found (Enjalbert et al., 2001) and the detected prediction accuracy of FTIR milk BHB and acetone concentration was high with 71% and 73%, respectively (Grelet et al., 2016). Santschi et al. (2016) exposed the usefulness of the routine infrared measurement of ketones in milk via FTIR and confirmed the usability of milk ketone body concentration evaluated in monthly milk samples as monitoring tool in early lactation Holstein cows. The FTIR technic reflects a cost-efficient, fast and reliable alternative to the blood ketone analysis due to the implementation in routine monthly milk recording.

Different thresholds for KET monitoring by milk ketone body concentrations were recommended. A threshold of 0.15 mmol/l for milk acetone and of 0.10 mmol/l for milk BHB was defined as threshold for subclinical KET by de Roos et al. (2007). In contrast to that, Santschi et al. (2016) suggested higher milk BHB thresholds of 0.15 mmol/l to 0.19 mmol/l to detect suspect cows and milk BHB $\geq$ 0.20 mmol/l as KET positive. Recent study results by Churakov et al. (2021) proposed a milk BHB threshold of 0.19 mmol/l as a predictor for severe NEB in dairy cows. According to Schwarz (2017) FTIR KET screening on herd level is already implemented in Belgium (MCC Flanders), Canada (Ketoscreen, Ketolab, CanWest DHI), Denmark (Danish Cattle Federation), Germany (KetoMIR), Netherlands (Qlip, CRV), France (CetoDetect, CLASEL), Japan (Tokachi DHI), Poland (Polish Breeders Association), Spain

(LIGAL), Sweden (Eurofins), and the United States (KetoMonitor, AgSource), where the Dairy Herd Improvement (**DHI**) reports inform farmers about the herd KET risk.

Furthermore, changes in the FA profile of milk allowed conclusions about cows metabolic health in early lactation (Gross and Bruckmaier, 2019). Since the analysis of milk FA by gas chromatography is expensive recent studies investigated the potential of FTIR milk FA as novel biomarkers for dairy cows health (Jorjong et al., 2015; Dettmann et al., 2020). With regard to high prediction accuracies for milk FA composition analyzed by mid-infrared spectroscopy (Fleming et al., 2017) and recommended by Gross and Bruckmaier (2019) an incorporation of FA besides milk ketone body concentrations would possibly lead to higher accuracies in detecting KET in early lactation dairy cows. Strong phenotypic associations between NEB and increased ketone bodies with specific milk FA concentration in early lactation Holstein cows were detected in previous studies (Gross et al., 2011; Nogalski et al., 2015). Due to high body fat mobilization during NEB, especially, unsaturated (**UFA**) and monounsaturated FA (**MUFA**) milk concentration significantly increased (Nogalski et al., 2015). In contrast to that, proportion of most de novo synthesized milk FA ≤ palmitic acid (**C16:0**) was decreased during the NEB (Gross et al., 2011). Accordingly, Dettmann et al. (2020) explained that the proportions of short chain saturated FA (**SFA**, C10:0 to C14:0) were significantly lower in early lactation than in mid lactation suggesting an inhibition of the de novo synthesis of short chain FA by the long chain FA from mobilized body fat. Proportions of stearic acid (**C18:0**), MUFA and oleic acid (**C18:1**) were increased and C16:0 was decreased in the early-lactation period (Dettmann et al., 2020). The contrasting decrease of C16:0 in milk in early lactation might be due to the double origin of the FA derived from mobilization of body fat reserves and partly originated from de novo synthesis in the mammary gland (Grummer, 1991).

In general, results of Bastin et al. (2011) revealed that UFA and especially MUFA were more variable than SFA throughout the lactation. The changes in milk FA contents could be related to the underlying physiology and energy balance of the cows in early lactation (Bastin et al., 2011). At the beginning of the lactation the mobilization of adipose FA and the incorporation of FA in milk occured due to the NEB (Palmquist et al., 1993; Bastin et al., 2011). The NEB and KET caused catabolism of adipose tissue and thus, the release of particular long-chain FA from mobilized tissue (Kay et al., 2005). Major mobilized FA from adipose tissue are MUFA, C18:1, C18:0, and C16:0. This explains the increase in MUFA and C18:0 milk FA at the beginning of lactation. Park et al. (2020) observed low levels of SFA content during the phase of NEB in early lactation and increasing SFA afterwards. Those findings suggested that a synthesis of SFA requires sufficient energy provision. Milk FA profiles did not only differ with

lactation stages but also with parity (Dettmann et al., 2020). Dettmann et al. (2020) confirmed that the concentration of FA increased with increasing parity. The rise might be due to the greater milk fat production induced by a stronger body fat mobilization and a stronger NEB in later parity cows (Dettmann et al., 2020).

First cut-off values for specific FTIR FA regarding NEB detection were identified by Churakov et al. (2021). According to their study results a C18:0 concentration ≥ 0.47 g/100 g of milk or a C18:1 concentration ≥ 1.16 g/100 g of milk displayed a state of severe NEB in early lactation. Furthermore, an influence of FA on cows fertility was discussed. Stádník et al. (2015) described an antagonistic relationship between milk MUFA and female fertility whereas increased SFA levels were associated with improved fertility traits. Significantly increased number of services per conception and days open were observed with rising MUFA and decreasing SFA milk contents in Fleckvieh cows (Stádník et al., 2015).

How successful incorporation of new innovative FTIR predicted traits as indicator traits in breeding programs will be depends on the heritability on the one hand and on genetic correlation with the real trait values on the other hand (Miglior et al., 2017; Tiplady et al., 2020). Thus, the next sections deal with the implementation of KET in dairy cattle breeding goals as well as the genetic and genomic background of KET, indicator traits and fertility trait ICF.

Ketosis in dairy cattle breeding goals

In the past, breeding goals in dairy cattle mainly focused on increasing milk production. Due to ascending health and fertility problems the selection shifted away from pure production oriented selection towards more balanced breeding goals by considering health, fertility and longevity in selection indices (Miglior et al., 2017). The Nordic countries have recorded health data since 1974. And also Finland, Schweden and Denmark established a health recording system in 1980. A routine genetic health trait evaluation has taken place, for example, in Austria and Germany since 2010, in France since 2012, and in Canada since 2013 (Egger-Danner et al., 2015). The following section describes the implementation of KET in dairy cattle breeding goals in Germany as well as different breeding organizations and countries of great importance for dairy cattle breeding.

In Germany, most health data are collected in herds participating in dairy cattle health related projects e.g., KUH-L and KuhVision. In these herds standardized direct health trait recording has been performed and cows have been genotyped. That led to an unselected cow reference population for direct health traits comprising 100,000 cows and 6,500 bulls in April 2019 (VIT, 2021). In the same year, genomic breeding values for direct metabolic health traits,

e.g., displaced abomasum, milk fever, and KET were implemented in German Holsteins. The three traits were combined to direct health composite RZmetabol being part of the RZhealth. In the total breeding value RZhealth the four sub-values, RZudderfit (clinical and subclinical mastitis), RZhoof (hoof diseases), RZmetabol (metabolic diseases), and RZrepro (reproduction disorders) were weighted according to their economic importance. Correspondingly, the RZmetabol was weighted with 25%, thereby KET was weighted with 30% into the index (Figure 1.3, VIT, 2021). The reliability of RZmetabol was 55% and will increase with rising data basis in the future (Rensing, 2019). Until now, no additional milk records e.g., the milk ketone body concentrations have been used in breeding value estimation regarding KET in Germany but an integration is planned.

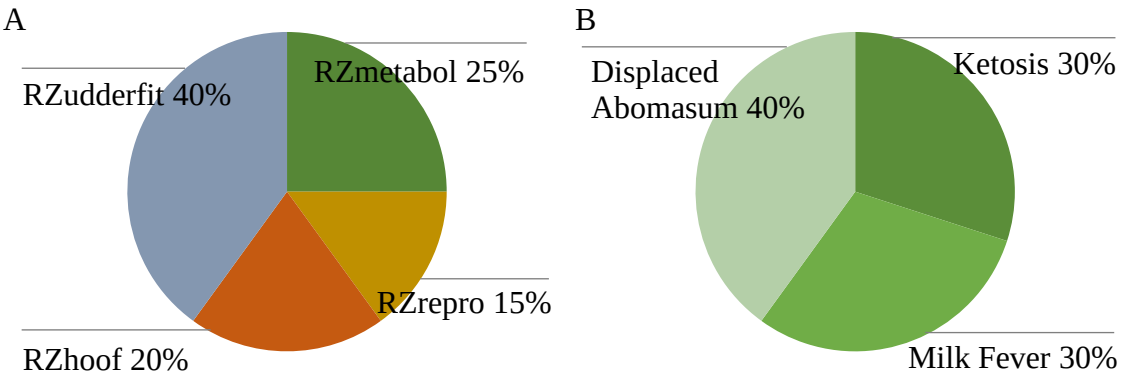

Figure 1.3. Complexes in the genetic evaluation for (A) RZhealth and (B) individual traits in the RZmetabol with corresponding index weights (modified according to VIT, 2021).

The Council of Dairy Cattle Breeding (**CDCB**) in the United States introduced new genetic evaluations for the six most common health traits, KET, mastitis, metritis, displaced abomasum, milk fever, and retained placenta in Holsteins in April 2018 (CDCB, 2018). As described by CDCB (2018) genetic and genomic KET evaluations were based on producer-recorded health data collected by the DHI organization across the United States. Moreover, evaluations were provided for males and females and expressed as percentage points of resistance above or below the breed average. Estimated KET predicted transmitting ability represented the resistance of the offspring to KET, whereby, larger positive values are more favorable. So far, average genomic reliabilities of the health traits ranged from 40 to 49 in young animals and from 44 to 56 in progeny-tested animals. The average genomic reliability for KET ranged between 41 (young animals) and 46 (progeny-tested animals) (CDCB, 2018). Since August 2018, disease resistance traits were included in the Net Merit $, a measure of lifetime profit, through the new

Health $ trait sub-index with a weighting value of 2.3% (Figure 1.4). KET was considered in Health $ with a weighting of 4.7% (Figure 1.4, VanRaden et al., 2021).

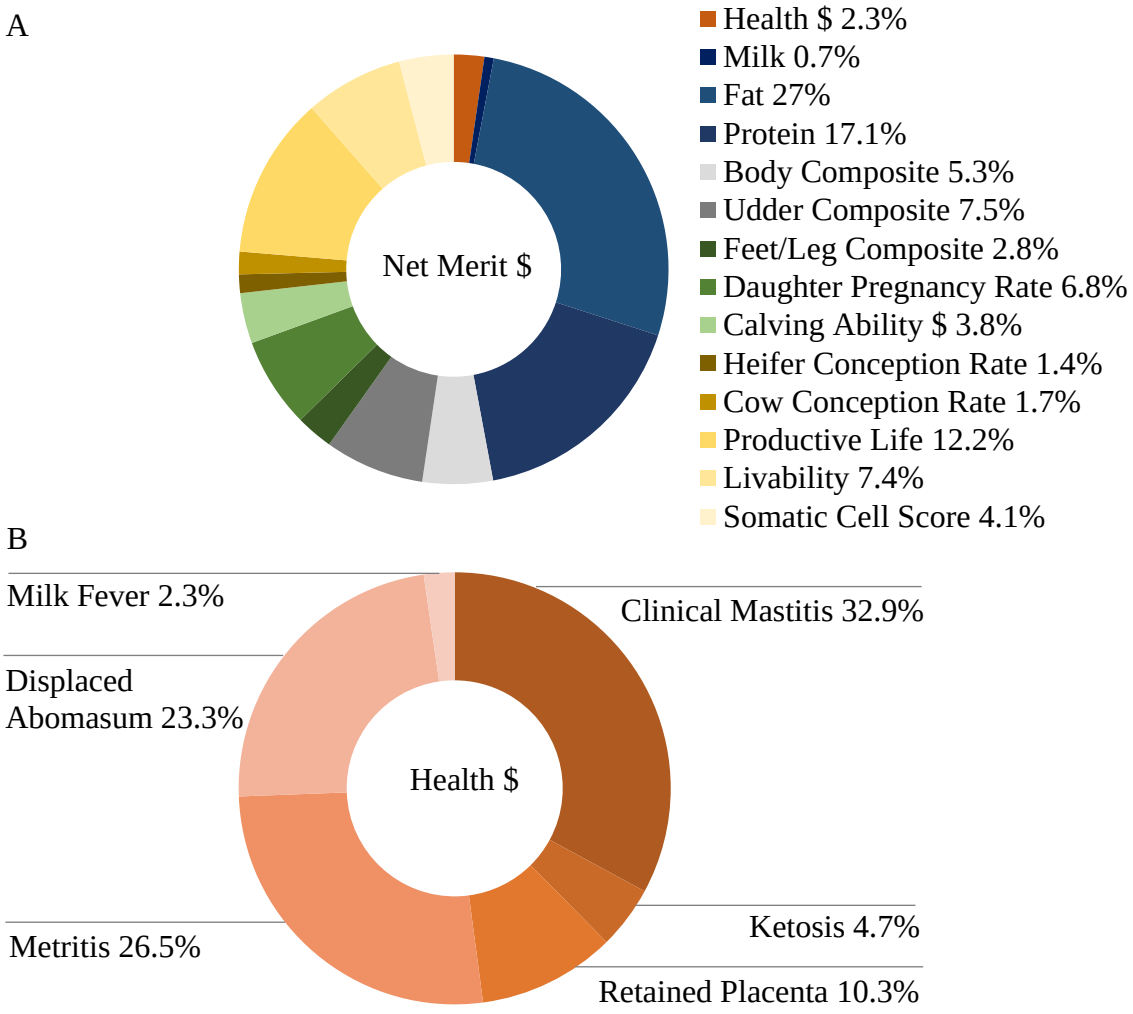

Figure 1.4. Weighting of individual traits in (A) the Net Merit $ and (B) the Health $ index (modified according to CDCB, 2020; VanRaden et al., 2021).

Furthermore, several breeding organizations and countries already considered KET milk indicators in breeding value estimation. In December 2016, the Canadian Dairy Network (**CDN**) introduced genetic evaluations for the Metabolic Disease Resistance (**MDR**) index including the traits subclinical KET, clinical KET, and displaced abomasum (Beavers and Van Doormaal, 2016). Already since 2007, voluntarily health event recording have taken place in Canadian herds which are used for genetic disease evaluations. Additionally, DHI laboratories provide milk BHB levels which are incorporated in genetic evaluations for subclinical KET. The overall MDR index combined the traits into a single value for genetic selection with a

weighting of 50% for subclinical KET, 25% for clinical KET and displaced abomasum, respectively (Figure 1.5., Beavers and Van Doormaal, 2016).

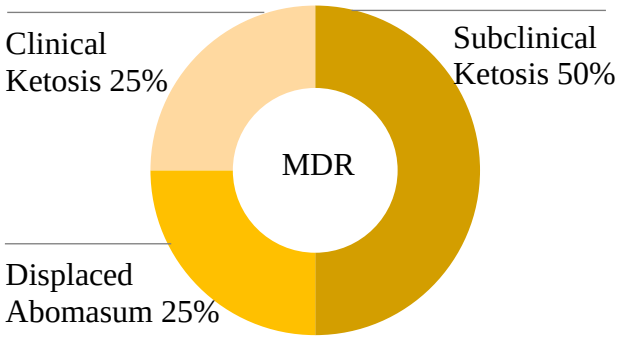

Figure 1.5. Weight on each trait in the Metabolic Disease Resistance (MDR) index.

Moreover, since 2016, Genex in Canada has included the sub-index subclinical KET with a weighting of 6% in the health trait HLTH$ which is part (with 24%) of the ICC$, the ideal commercial cow index in Holsteins (Figure 1.6).

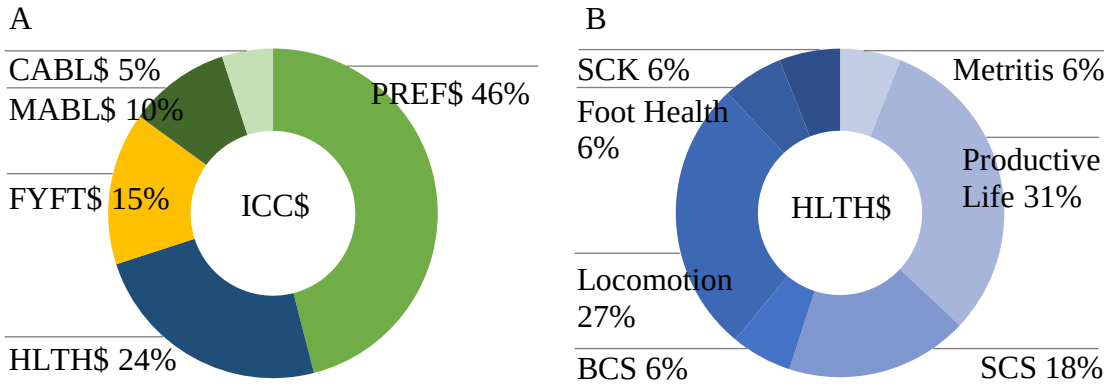

Figure 1.6. (A) Sub-indexes included in the ideal commercial cow index (ICC$) and (B) health traits included in the health (HLTH$) sub-index with respective weights (modified according to Genex, 2016).
CABL$ = calving ability, FYFT$ = fertility and fitness, HLTH$ = health, MABL$ = milking ability, PREF$ = production efficiency, SCK = subclinical ketosis, BCS = body condition score, SCS = somatic cell score.

Since 2014, the breeding organization CRV has presented breeding values for KET for each Holstein bull. A value higher than 100 means that the progeny of the bull will have a lower risk for KET. Thus, using bulls with a higher breeding value for KET will cause less KET

susceptible cows. Besides breeding values for udder health, claw health, calving ease, birth ease, viability, and daughter fertility the KET breeding value is part of the Better Life Health index (CRV, 2017). Weighting is not applied to the breeding values in the index. The expression of the breeding value in percentages less mastitis, lameness, difficult birth etc. determines the weight in Better Life Health. For example, a bull with all breeding values that were included in the Better Life Health index equal to 104, udder health, claw health, fertility and livability has a weight of about 20%, respectively, the birth traits have a weight of 13% and KET is weighted with 9% (personal correspondence with CRV, July, 22, 2021). Three milk indicators, milk acetone, BHB levels and the FPR from milk recording (day five to day 60), are included in KET breeding value estimations. Thereby, traits were corrected for herd, season, days in lactation, age of calves, parity, heterosis, recombination, cow and permanent environmental effect in the animal model based on best linear unbiased predictor (**BLUP**) technic (CRV, 2017).

In 2008, the joint Nordic genetic evaluation for health traits was implemented for Denmark, Finland and Sweden (Rius-Vilarrasa et al., 2018). Until 2017, these breeding value estimations were based on veterinary treatment records for reproduction disorders, metabolic diseases, feet and leg problems in sire models. However, with the availability of metabolic biomarkers (e.g., BHB, acetone) and genomic prediction models for inclusion of the cow reference population the application of an animal model for health evaluation is preferred. The health evaluation included early and late reproductive disorders, feet and leg problems and metabolic disorders. Due to the inclusion of BHB and acetone concentrations (10 to 60 days in milk (**DIM**)) as indicator traits for KET the trait metabolic disorders was divided into KET and other metabolic disorders. The additional indicator trait information improved the reliability of breeding values for KET and other metabolic disorders, from 0.29 to 0.34 and 0.36 in Holstein cows, respectively (Rius-Vilarrasa et al., 2018). The Nordic Total Merit (**NTM**) index includes the trait complex health and reproduction (45%), production efficiency (40%), conformation and workability (15%). The health and reproduction complex considers the general health index, which describes a bull's daughters genetic potential to resist reproduction, metabolic and feet diseases and also includes the breeding value for KET (Vikinggenetics, 2021). The traits are weighted in NTM according to their economic value which quantify the value of a marginal change in the trait i.e., the value of one unit improvement of the trait while the remaining traits are constant. The overview on the incorporation of KET in breeding programs displays differences among its implementation (usage of direct health traits, usage of indicator traits e.g., BHB concentrations) and reflects the increasing importance of cows metabolic health in dairy cattle breeding.

Genetic and genomic architecture of ketosis, milk indicators and fertility interval trait

Heritabilities

Ketosis

Heritabilities for KET ranged between 0.02 and 0.16 depending on the used genetic-statistical modeling approach (Table 1.1). Heritabilities for KET based on linear models were situated between 0.01 and 0.08 while heritability estimates using threshold models ranged from 0.02 up to 0.16 (Table 1.1, Pryce et al., 2016). Thereby, estimates based on farmer-recorded data were similar to those based on veterinarian records (Pryce et al., 2016). The majority of heritability estimates for KET was based on pedigree relationship (**A**) matrices (Table 1.1). Only a few studies additionally considered genomic information and genomic relationship matrices (**G**) in a combined relationship (**H**) matrix. The single-step genomic BLUP (**ssGBLUP**) approach combining genomic and pedigree relationships has been shown to increase reliability and reduce bias of breeding values of young animals for test-day traits compared to traditional BLUP in dairy cattle (Oliveira et al., 2019). Furthermore, single-step methodology has been proven to enhance prediction accuracy, especially, for complex traits with lower heritability (Guarini et al., 2018) and to be valuable for complex data sets where only a proportion of pedigreed animals were genotyped (Aguilar et al., 2019). Hence, ssGBLUP is routinely used for genomic evaluation in many livestock species and currently under implementation for dairy cattle (Misztal et al., 2020). In general, the small heritabilities for KET (Table 1.1) might base on the complex nature of disease traits (Kemper and Goddard, 2012). The pathogenesis of complex diseases is often regulated by intermediate phenotypes with quantitative inheritance. Blanco-Gómez et al. (2016) argued that not-detectable intermediate phenotypes explain a major part of the missing heritability. Consideration of continuous KET indicators such as FTIR milk BHB concentration might contribute to a clear detection of subclinical cases, higher disease incidences and increasing heritability estimates (Belay et al., 2017).

Interval from calving to first insemination

Heritabilities for the ICF ranged on low levels between 0.03 and 0.11 (Table 1.2). Also for ICF, heritability estimates considering G or combined H matrix were scarce (Table 1.2). Although the heritabilities for KET and ICF were low a genetic improvement of metabolic health and fertility traits was possible due to sufficient genetic variability, high accuracy and intensity of selection (Berry et al., 2019). Hence, breeding programs for improved animal health and disease resistance should be an integral part of the disease control approach (Berry et al., 2011).

Table 1.1. Heritabilities for producer/veterinarian-recorded ketosis in dairy cattle.

Model[1]	RM[2]	Breed[3]	Parity	DIM	Heritability	Reference
T	A	HOL	All	1 - 305	0.08	Uribe et al., 1995
L, T	A	HOL	1 - 5	1 - 305	0.01, 0.02	Kadarmideen et al., 2000
T	A	HOL	1, All	1 - 50	0.11, 0.06	Zwald et al., 2004
T	A	RDC	1, 2, 3	-15 - 120	0.14, 0.16, 0.15	Heringstad et al., 2005
T	A	HOL	1 - 4+	1 - 60	0.09	Neuenschwander et al., 2012
L	A	HOL	1	1 - 100	0.02	Koeck et al., 2013
L	A	HOL	1	1 - 100	0.02	Koeck et al., 2014
T	A	HOL	1, 2 - 5	1 - 400	0.09, 0.04	Parker Gaddis et al., 2014
	H				0.14, 0.08	
L	A	RDC	1 - 4	-15 - 120	0.08	Belay et al., 2017
T	H	JER	1 - 5	1 - 60	0.08	Parker Gaddis et al., 2018
T	H	JER	1 - 5	All	0.10	Gonzalez-Peña et al., 2020

[1] L = linear model, T = threshold model.

[2] RM = relationship matrix, A = pedigree relationship matrix, H = combined pedigree and genomic relationship matrix.

[3] HOL = Holstein-Friesian, JER = Jersey, RDC = Red dairy cattle (Norwegian Red).

Table 1.2. Heritabilities for the interval from calving to first insemination in dairy cattle.

Model[1]	RM[2]	Breed[3]	Parity	DIM	Heritability	Reference
L	A	HOL	1 - 5	20 - 200	0.03	Kadarmideen et al., 2000
L	A	HOL	1	30 - 190	0.06	Weigel and Rekaya, 2000
L	A	HOL	1	10 - 200	0.11	Berry et al., 2012
L	A	RDC	1	20 - 230	0.04	Negussie et al., 2013
L	A	HOL	1 - 4+	30 - 250	0.10	Tenghe et al., 2016
L	A	HOL	1, 2, 3	20 - 230	0.10, 0.08, 0.07	Liu et al., 2017a
L	H	RDC	1, 2, 3	20 - 230	0.04	Matilainen et al., 2018
L	A	HOL	1 - 3	≤ 230	0.07	Häggman et al., 2019
L	A	HOL	1, 2, 3	20 - 230	0.06, 0.05, 0.07	Muuttoranta et al., 2019
		RDC			0.05, 0.02, 0.03	
L	A	HOL	1 - 3	20 - 230	0.06	Zhang et al., 2019
	H				0.05 - 0.09	
L	A	RDC	1	20 - 230	0.03	Mehtiö et al., 2020

[1] L = linear model.

[2] RM = relationship matrix, A = pedigree relationship matrix, H = combined pedigree and genomic relationship matrix.

[3] HOL = Holstein-Friesian, RDC = Red dairy cattle (Norwegian Red).

Milk fat-to-protein ratio and ketone bodies

In most studies clinical KET cases were the trait basis for genetic parameter estimations because clinical cases are quite easy to detect (Pryce et al., 2016). Nevertheless, consideration of more precise phenotypes in genetic analyses i.e., of subclinical KET cases contributed to increasing incidence rates and more accurate breeding value estimations. The detection of subclinical KET implied detailed recording of biomarkers, e.g., ketone body concentrations in blood or in milk (König and May, 2018). Alternatively, test-day FPR is suggested for indirect selection strategies on subclinical KET (van Knegsel et al., 2010). Estimated heritabilities for the FPR were larger than for the complex disease trait KET and ranged from 0.07 to 0.31 (Table 1.3). Thereby, differences in heritabilities were observed depending on the distribution of the trait. While Gaussian-distributed FPR heritability was 0.15, a lower heritability of 0.07 for binary FPR was identified (Koeck et al., 2013). Additionally, several studies determined an influence of the lactation period on FPR heritability. Higher heritabilities for the FPR were assigned later in lactation than in earlier periods (Negussie et al., 2013; Mehtiö et al., 2020). Estimated genetic correlations between FPR in different lactation stages ranged from 0.61 to 0.97 suggesting that the FPR in early lactation and in mid to late lactation were not exactly the same traits (Negussie et al., 2013).

Correspondingly, estimated heritabilities for milk BHB and acetone varied in dependence of lactation number and period between low and moderate levels (Table 1.3). Heritabilities for milk ketone bodies ranged from 0.03 to 0.36 and 0.002 to 0.36 for milk BHB and acetone, respectively. According to Koeck et al. (2014) and Lee et al. (2016) early lactation heritability estimates for milk BHB and acetone were lower than estimates based on later lactation periods probably caused by large phenotypic variances during the early lactation (Table 1.3). Furthermore, decreasing early lactation heritabilities were observed with increasing parity due to lower additive genetic and higher permanent environmental variances in later lactations (Lee et al., 2016). Häggman et al. (2019) also estimated low heritabilities of 0.07 for milk BHB traits with linear models and of 0.12 with threshold models on the underlying liability scale.

Table 1.3. Heritabilities for potential ketosis milk indicator traits fat-to-protein ratio and ketone bodies in dairy cattle.

Trait[1]	Model[2]	RM[3]	Breed[4]	Parity	DIM	Heritability	Reference
FPR	L, T	A	HOL	1	5 - 30	0.07 - 0.15	Koeck et al., 2013
					31 - 60	0.03 - 0.14	
	L	A	RDC	1	30, 60, 110	0.16, 0.19, 0.23	Negussie et al., 2013
					160, 260, 310	0.25, 0.25, 0.24	
	L	A	HOL	1	11 - 180	0.30	Buttchereit et al., 2012
	L	A	HOL	1	5	0.17	Bastin et al., 2014
	L	A	FL	1 - 2	8 - 49, 40 - 90	0.16, 0.14	Ederer et al., 2014
	L	A	HOL	1	5 - 40	0.12	Koeck et al., 2014
	L	A	HOL	1 - 3	5 - 305	0.31, 0.27, 0.24	Satoła and Ptak, 2019
	L	A	RDC	1	8 - 35, 36 - 63, 64 - 91	0.08, 0.07, 0.09	Mehtiö et al., 2020
ACE	L, T	A	HOL	1 - 4+	5 - 305	0.002 - 0.009	Wood et al., 2004
	L	A	HOL	1 - 4+	5 - 60	0.10	Van der Drift et al., 2012
	L	A	HOL	1, 2, 3	30	0.18, 0.16, 0.05	Lee et al., 2016
					150	0.29, 0.30, 0.30	
					250	0.36, 0.35, 0.27	
	L	A	HOL	1, 2, 3	5 - 305	0.23, 0.29, 0.31	Ranaraja et al., 2018
				4, All		0.29, 0.29	
	L	G	HOL	1 - 2	3 - 517	0.10	Gebreyesus et al., 2020
	L	A	RDC	1	8 - 35, 36 - 63, 64 - 91	0.18, 0.15, 0.15	Mehtiö et al., 2020
BHB	L	A	HOL	1 - 4+	5 - 60	0.16	Van der Drift et al., 2012
	L	A	HOL	1	5 - 20, 21 - 40	0.14, 0.14	Koeck et al., 2014
					41 - 60	0.17	
					61 - 80	0.22	
					81 - 100	0.29	
	L	A	HOL	1, 2, 3	30	0.10, 0.10, 0.04	Lee et al., 2016
					150	0.07, 0.09, 0.12	
					250	0.09, 0.14, 0.11	
	L	A	RDC	1 - 4	11 - 30, 1 - 60	0.25, 0.28	Belay et al., 2017
					61 - 90, 91 - 120	0.32, 0.36	
					All	0.27	
	L	A	HOL	1, 2, 3	5 - 305	0.14, 0.11, 0.09	Ranaraja et al., 2018
				4, All		0.09, 0.19	
	L	A	RDC	1	5 - 70	0.04 - 0.09	Häggman et al., 2019
	L	G	HOL	1 - 2	3 - 517	0.03	Gebreyesus et al., 2020
	L	A	RDC	1	8 - 35, 36 - 63, 64 - 91	0.16, 0.15, 0.15	Mehtiö et al., 2020

[1] ACE = acetone, BHB = β-hydroxybutyrate, FPR = fat-to-protein ratio.

[2] L = linear model, T = threshold model.

[3] RM = relationship matrix, A = pedigree relationship matrix, G = genomic relationship matrix.

[4] FL = Fleckvieh, HOL = Holstein-Friesian, RDC = Red dairy cattle (Norwegian Red).

Milk fatty acid profile

Heritability estimates for milk FA indicated higher heritabilities for SFA (0.09 – 0.47) than for UFA (0.08 – 0.33) (Table 1.4, Bastin et al., 2011). This might be due to the fact that most of the SFA in milk originates from de novo synthesis by acetyl-CoA carboxylase and FA synthase in the mammary gland while the long chain UFA derived from preformed circulating blood lipids from intestinal absorption and body fat mobilization (Grummer, 1991; Bastin et al., 2012). The metabolic enzymes involved in de novo synthesis seemed to be under stronger genomic control (Bastin et al., 2011; Knutsen et al., 2018). Nevertheless, heritabilities for UFA, MUFA, and C18:0 were smaller than for the SFA group and for C16:0 but still moderate (Table 1.4) indicating that processes involved in the intake of these FA in milk fat i.e., mobilization of FA from adipose tissue may be partly under genetic control. Narayana et al. (2017) and Freitas et al. (2020) identified FA heritability alterations by reason of the lactation stage. Smaller early lactation heritabilities of SFA and UFA were originated by the increased residual variance (Narayana et al., 2017). Residual variances for FA concentrations decreased in mid and late lactation (Narayana et al., 2017). Heritability estimates of milk FA concentration by Soyeurt et al. (2008) also varied through the dairy cows lactation. Soyeurt et al. (2008) described large changes in heritabilities for SFA and MUFA across the lactation. In this study heritability values ranged from 0.09 to 0.42 for SFA and from 0.14 to 0.43 for MUFA. In contrast to estimates by Narayana et al. (2017) the greatest heritability estimates were observed at the early stage and at the end of lactation.

Soyeurt et al. (2008) assumed that the lipid mobilization from adipose tissue at the beginning of the lactation is based on genetically regulated mechanisms and the greater heritability at the end of lactation might be related to lactation persistency. Moderate heritabilities (0.09 – 0.34) for FA based on G matrix using a small number of genotyped Holsteins were estimated by Krag et al. (2013). In this study, higher heritabilities were observed for groups of UFA (0.33), MUFA (0.34) and PUFA (0.28) than for groups of SFA (0.09) although individual SFA had higher heritability estimates compared to individual UFA reflecting the general pattern (Krag et al., 2013). So far, studies considering combined H matrix in variance estimations for milk FA are scarce (Table 1.4). According to results of Petrini et al. (2016) the whole lactation heritabilities for different milk FA were quite similar when A or H matrix was used (Table 1.4). If the difference between A and G matrix is low, an addition of genomic information in genetic predictions will only cause small gains in accuracy (Petrini et al., 2016). However, as outlined above, the combination of pedigree and genomic information in genetic evaluation was recommended to enhance genomic predictions (Guarini et al., 2018).

Table 1.4. Heritabilities for potential ketosis milk indicator fatty acid profile in dairy cattle.

Trait[1]	Model[2]	RM[3]	Breed[4]	Parity	DIM	Heritabilities	Reference
SFA	L	A	HOL	1	5 - 365	0.24 - 0.42	Soyeurt et al., 2008
	L	A	HOL	1	5, 5 - 305	0.25, 0.42	Bastin et al., 2011
	L	G	HOL	1 - 3	129 - 227	0.09	Krag et al., 2013
	L	A, H	HOL	1 - 6	5 - 305	0.25, 0.25	Petrini et al., 2016
	L	A	HOL, JER	1	8 - 305	0.15, 0.10	Hein et al., 2018
	L	A	HOL	1	5 - 305	0.50	Fleming et al., 2018
	L	A	HOL	1 - 4+	5 - 480	0.20	Bobbo et al., 2020
	L	A	HOL	1	5 - 305	0.47	Freitas et al., 2020
UFA	L	A	HOL	1	5, 5 - 305	0.13, 0.22	Bastin et al., 2011
	L	G	HOL	1 - 3	129 - 227	0.33	Krag et al., 2013
	L	A, H	HOL	1 - 6	5 - 305	0.08, 0.08	Petrini et al., 2016
	L	A	HOL	1	5 - 305	0.26	Fleming et al., 2018
	L	A	HOL	1	5 - 305	0.24	Freitas et al., 2020
MUFA	L	A	HOL	1	5 - 365	0.14 - 0.27	Soyeurt et al., 2008
	L	A	HOL	1	5, 5 - 305	0.13, 0.21	Bastin et al., 2011
	L	G	HOL	1 - 3	129 - 227	0.34	Krag et al., 2013
	L	A, H	HOL	1 - 6	5 - 305	0.07, 0.07	Petrini et al., 2016
	L	A	HOL, JER	1	8 - 305	0.15, 0.10	Hein et al., 2018
	L	A	HOL	1 - 4+	5 - 480	0.07	Bobbo et al., 2020
PUFA	L	A	HOL	1	5, 5 - 305	0.20, 0.29	Bastin et al., 2011
	L	G	HOL	1 - 3	129 - 227	0.28	Krag et al., 2013
	L	A, H	HOL	1 - 6	5 - 305	0.11, 0.11	Petrini et al., 2016
	L	A	HOL, JER	1	8 - 305	0.08, 0.11	Hein et al., 2018
	L	A	HOL	1 - 4+	5 - 480	0.07	Bobbo et al., 2020
C16:0	L	A	HOL	1	5, 5 - 305	0.24, 0.40	Bastin et al., 2011
	L	G	HOL	1 - 3	129 - 227	0.14	Krag et al., 2013
	L	A, H	HOL	1 - 6	5 - 305	0.26, 0.26	Petrini et al., 2016
	L	A	HOL, JER	1	8 - 305	0.14, 0.16	Hein et al., 2018
	L	G	HOL	1 - 6	60+	0.34	Gebreyesus et al., 2019
	L	A	HOL	1 - 4+	5 - 480	0.21	Bobbo et al., 2020
C18:0	L	A	HOL	1	5, 5 - 305	0.14, 0.23	Bastin et al., 2011
	L	G	HOL	1 - 3	129 - 227	0.19	Krag et al., 2013
	L	A, H	HOL	1 - 6	5 - 305	0.13, 0.14	Petrini et al., 2016
	L	A	HOL, JER	1	8 - 305	0.11, 0.09	Hein et al., 2018
	L	G	HOL	1 - 6	60+	0.25	Gebreyesus et al., 2019
	L	A	HOL	1 - 4+	5 - 480	0.08	Bobbo et al., 2020

[1] SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, C16:0 = palmitic acid, C18:0 = stearic acid.
[2] L = linear model.
[3] RM = relationship matrix, A = pedigree relationship matrix, G = genomic relationship matrix, H = combined relationship matrix.
[4] HOL = Holstein-Friesian, JER = Jersey.

Genetic correlations between ketosis, milk indicator traits and the interval from calving to first insemination

According to phenotypic correlations genetic correlations between KET and potential milk indicator traits the FPR and ketone body concentrations were positively moderate to high. Favorable genetic correlations based on A matrix for KET diagnosis with FPR and with milk BHB concentration of 0.56 and 0.47 to 0.70, respectively, were observed in first-lactation Canadian Holsteins (Koeck et al., 2014; Koeck et al., 2016). In consideration of the strong genetic correlation between KET diagnosis and milk BHB concentration Koeck et al. (2016) proposed the usage of BHB as an indicator trait for indirect selection for KET. Also genetic correlation among KET and milk acetone concentration was described as highly positive with 0.74 to 0.76 in Holstein and Red Dairy Cattle (Rius-Vilarrasa et al., 2018).

So far and in contrast to phenotypic analyses, studies regarding genetic correlation estimations between producer-recorded KET and the specific milk FA profile in early lactation are scarce. Previous studies focused on blood non-esterified FA (**NEFA**) concentration and did not base on specific milk FA profile. Preceding estimated genetic correlation between KET indicator blood BHB and NEFA concentration in Holstein cows was high at the early beginning of lactation (0.78). Results suggested that both traits should be taken into account in selection strategies against metabolic diseases (Benedet et al., 2020).

Furthermore, moderate genetic correlations between cows energy balance and fertility interval traits have been detected by Mehtiö et al. (2020). Genetic correlation between early lactation milk ketone body and NEFA concentration with ICF were positive with 0.38 and 0.39, respectively (Mehtiö et al., 2020). Genetic correlation estimations of the KET breeding value with breeding values for fertility traits also revealed positive correlations between 0.26 and 0.33, meaning less KET results in better fertility (Vosman et al., 2015). Additionally, Bastin et al. (2012) estimated positive genetic correlations between early lactation milk UFA, MUFA, C18:0 and interval fertility trait days open and negative genetic correlations for those traits after 100 DIM in first lactating Holstein cows. Among SFA, C16:0 and days open the genetic correlations were negatively across the whole lactation (Bastin et al., 2012). These results suggest an association between NEB, KET, the milk FA profile, and female fertility interval traits on genetic levels. Thus, profound investigations on genetic relationships between these traits are needed.

In general, just a few studies focused on quantitative genetic parameter estimation for KET milk indicators in early-lactation periods. There is a need to focus on this early-lactation period in genetic (co)variance estimations to reflect its association with KET during the NEB.

Additionally, most studies put their focus on analyses based on A matrix and genomic investigations considering single nucleotide polymorphism (**SNP**) information, G matrix or combined H matrix were rare.

Genome-wide associations for ketosis, milk indicator traits and the interval from calving to first insemination

Genome-wide association studies (**GWAS**) identified associated genomic variants and thus revealed insights into the genomic architecture of complex traits e.g., diseases (Freebern et al., 2020). The following section provides an overview on genomic regions and candidate genes related to KET, indicator traits, and the ICF.

Ketosis

Only a limited number of studies focused on GWAS and potential candidate gene identification for producer-recorded KET (Table 1.5). Using GWAS, genomic regions mainly located on *Bos taurus* autosomes (**BTA**) 2, 3, 6, 10, 11, 13, 14, 16, 23, 25, 27, and 29 were identified for binary distributed KET. Parker Gaddis et al. (2018) identified SNP significantly associated with producer-recorded KET on BTA 10, 11, 14, and 23 in US Jersey cattle. Genes i.e., *ATP6V1B1*, *RASGRP3*, *DDHD1*, *CYP11B1*, *NLRC4* involved in insulin regulation, lipid metabolism, and immune response were located near to the associated regions (Parker Gaddis et al., 2018). Six KET candidate genes *FN1*, *ACSL1*, *CPT1A*, *IDH3B*, *PC*, *HMGCS2* on BTA 2, 3, 13, 27, and 29 involved in FA metabolism, gluconeogenesis, citric acid cycle, and ketogenesis were identified in a study of Kroezen et al. (2018). Also six genomic regions on BTA 10, 13, 14, and 25 showed association with KET in Chinese Holstein cattle in a single-step GWAS (**ssGWAS**) by Huang et al. (2019). Detected candidate genes e.g., *BMP4*, *HNF4A*, *APOBR*, *SOCS4*, *GCH1*, *ATG14*, *RGS6*, *CYP7A1*, and *MAPK3* involved in insulin or lipid metabolism implicated the contribution of energy metabolism-associated genes to the genetic background of KET (Huang et al., 2019). Furthermore, Freebern et al. (2020) assigned potential candidate regions on BTA 14 and BTA 16 associated with KET using GWAS, fine mapping, and analyses of multi-tissue transcriptome data in Holstein cattle. Candidate genes *LY6K* and *KCNT2* were participating in milk and fat metabolism (Freebern et al., 2020). The large number of identified significant SNP indicated that KET is a polygenic trait, influenced by numerous genomic regions, with comparatively small but additive effect.

Table 1.5. Potential candidate genes for producer-recorded ketosis.

Breed[1]	Parity	BTA[2]	Candidate gene	Reference
HOL	1	13	*IDH3B*	Kroezen et al., 2018
		27	*ACSL1*	
		29	*PC*, *CPT1A*	
	2 - 5	2	*FN1*	
		3	*HMGCS2*	
JER	1 - 5	3	*TTLL7*	Parker Gaddis et al., 2018
		6	*ARAP2*	
		10	*FERMT2*, *DDHD1*, *BMP4*,	
		11	*CYP26B1*, *EXOC6B*, *NAGK*, *ATP6V1B1*, *CD207*, *CLEC4F*, *ZNF638*, *XDH*, *SRD5A2*, *SPAST*, *SLC30A6*, *NLRC4*, *RASGRP3*, *FAM98A*	
		14	*SULF1*, *SLCO5A1*	
		23	*PRP1*, *PRP4*, *PRP6*, *PRP8*	
		25	*CLCN7*, *PTX4*, *TELO2*, *MAPK8IP3*, *UBE21*	
HOL	1 - 5	10	*BMP4*, *SOCS4*, *GCH1*, *ATG14*, *RGS6*	Huang et al., 2019
		13	*HNF4A*	
		14	*CYP7A1*	
		25	*APOBR*, *MAPK3*	
HOL		14	*LY6K*, *PARP10*, *DGAT1*	Freebern et al., 2020
sires		16	*KCNT2*, *LOC783947*	

[1] HOL = Holstein-Friesian, JER = Jersey.

[2] BTA = *Bos taurus* autosome.

Milk fat-to-protein ratio and ketone bodies

Also for the milk indicator traits i.e., the milk FPR and BHB concentrations, SNP located in genomic regions related to lipid metabolism, energy balance, immune system, and milk production were identified. As displayed in Table 1.6, GWAS for the FPR mainly identified associated SNP on BTA 1, 11, 13, 14, 16, and 27. The strongest effect on FPR showed SNP rs109421300 located in the well-known candidate gene *DGAT1* on BTA 14 (Tetens et al., 2012). Gene *DGAT1* was associated with milk yield, fat, protein percentage, FA composition, and also energy balance in Holstein cows (Oikonomou et al., 2009; Bovenhuis et al., 2016). Further candidate genes for the FPR contributed to steroid hormone synthesis (*CYP11B1*), alanine, aspartate, glutamate metabolism (*GLUL*), and FA biosynthesis (*OXSM*) (Table 1.6, Tetens et al., 2012).

Genome-wide associations for milk ketone body BHB were located on BTA 3, 6, 11, 14, 17, 19, 20, 22, and 25 (Table 1.6). A first GWAS for FTIR BHB in milk was conducted by Nayeri et al. (2019). Nayeri et al. (2019) identified significantly associated SNP marker on BTA 6, 14, and a novel region on BTA 20. According to results of Tetens et al. (2012) regarding the FPR the determined SNP on BTA 14 was located within the *DGAT1* gene. Candidate genes e.g.,

CXCL8 and *CSN1S1* close to SNP on BTA 6 were reported to influence the innate immune system and milk production traits.

Table 1.6. Potential candidate genes for ketosis milk indicators the fat-to-protein ratio and ketone body β-hydroxybutyrate.

Trait[1]	Breed[2]	Parity	BTA[3]	Candidate gene	Reference
FPR	HOL	-	13	*SRC*	Liu et al., 2010
	HOL	1	14	*DGAT1*, *CYP11B1*	Tetens et al.,
			16	*GLUL*	2012
			27	*OXSM*	
BHB	HOL	1	6	*CXCL8*, *SLC4A4*, *FAM47E*, *GC*, *NPFFR2*, *ADAMTS3*, *FRAS1*, *RASSF6*, *MTHFD2L*, *SLC4A4*, *FAM47E*	Nayeri et al., 2019
		2+	3	*KIAA1324*	
			6	*GNRHR*, *SLC4A4*, *UGT2A1*, *BTC*, *FAM47E*, *SDAD1*, *CSN1S1*, *NPFFR2*, *EPHA5*, *RASSF6*, *SULT1E1*, *DCK*, *GC*, *LOC781988*, *YTHDC1*, *CXCL8*, *UGT2B10*, *MTHFD2L*	
			11	*BIRC6*, *TTC27*	
			14	*LY6K*, *GRINA*, *WDR97*, *C14H8orf33*, *RHPN1*, *GRHL2*, *GML*, *FOXH1*, *ARHGAP39*, *TONSL*, *NRBP2*, *SCRIB*, *LY6H*, *SHARPIN*, *MAF1*, *ZNF34*, *MROH1*, *SMPD5*, *SLURP1*, *LYPD2*, *OPLAH*, *RNF19A*, *ZNF7*, *RGS22*, *HSF1*, *ZC3H3*, *DGAT1*, *CYHR1*, *PUF60*, *CCDC166*, *MIR1839*, *PTP4A3*, *PSCA*	
			17	*ORAI1*	
			20	*OTULINL*, *FBXL7*, *TRIO*, *ANKH*, *MYO10*, *DNAH5*	
			25	*IL4R*, *XPO6*, *PSPH*, *CLN3*, *APOBR*, *IL2*	
	HOL	1	6	*ACOX3*	Soares et al.,
			22	*SLC26A6*, *SLC25A20*	2021
		2 - 5	14	*RASSF6*, *CXCL8*	
			19	*HADHA*, *HADHB*	

[1] BHB = β-hydroxybutyrate, FPR = fat-to-protein ratio.
[2] HOL = Holstein-Friesian.
[3] BTA = *Bos taurus* autosome.

Genomic studies regarding milk ketone body concentrations are scarce, thus, a GWAS study of Pralle et al. (2020) comprising blood BHB concentrations is outlined in the following. Analysis of blood BHB concentrations in early lactation Holstein cows annotated candidate genes that had important roles in hyperketonaemia etiology. Several candidate genes on BTA 6 and BTA 14 contributed to adipogenesis, obesity or fatty liver disease i.e., *NPFFR2*, *ENPP2*, *DEPTOR*, *COL14A1*, *MRPL13*, and *SLC4A4*. Additionally, SNP on BTA 6, 10, and 14 close to genes i.e., *GC*, *TRIM36*, and *ENPP2* associated with type-2 diabetes mellitus and insulin resistance were

detected (Pralle et al., 2020). To the best of our knowledge not any study focused on GWAS regarding the milk acetone concentration.

Milk fatty acid profile

For the FA profile in milk candidate genes involved in oxidative stress (i.e., *MGST1*), glucose homeostasis (i.e., *MFSD4A*) and FA synthesis (i.e., *PAEP*, *CEL*) were detected (Iung et al., 2019). Multi-population GWAS results by Gebreyesus et al. (2019) based on a sample population of Chinese, Danish, and Dutch Holsteins detected 56 genomic regions significantly associated with at least one milk FA. Candidate genes in novel regions e.g., *OSBPL6*, *AGPS* on BTA 2, *PRLH* on BTA 3, *SLC51B* on BTA 10, *ABCG5/8* on BTA 11, and *ALG5* on BTA 12 were involved in lipid binding, lipid biosynthesis, or lipid transport processes. Also in this study *DGAT1* gene was found to be significantly associated with several de novo synthesized FA (C8:0, C10:0, C14:0), medium to long chain FA (C15:0, C16:0), and also UFA (C14:1, C16:1, C18:1c9, C18:2n6, C18_3n3, CLA). Cruz et al. (2019) conducted a GWAS for milk FA in North American Holstein cattle accounting for the *DGAT1* gene effect. Results suggested that *DGAT1* accounted for the most of the variability in milk FA. When fitting the *DGAT1* gene effect as a covariate in the GWAS model additional important identified candidate genes for the majority of FA groups were *PLBD1* and *MGST1* on BTA 5. The gene *PLBD1* was related to milk fat percentage and *MGST1* metabolized lipid and FA hydroperoxides, lipid peroxidation products and oxidized phospholipids (Cruz et al., 2019).

Genomic regions ascertained by Freitas et al. (2020) for milk long, medium, short chain FA, SFA, and UFA were located on BTA 5, 13, and 14 in North American Holstein cows. The main biological pathways related to the candidate genes are carbohydrate, lipid metabolism, cellular lipid metabolic, and catabolic processes. Despite its important function in the synthesis of triacylglycerol *DGAT1* was not found in this study, probably, due to the absence of marker located in its proximity (Freitas et al., 2020). An overview on identified candidate genes for the FA profile, including SFA, UFA, MUFA, PUFA, C16:0, and C18:0 is given in Table 1.7.

Table 1.7. Potential candidate genes for the fatty acid profile in milk.

Trait[1]	Breed[2]	Parity	BTA[3]	Candidate gene	Reference
SFA, MUFA, PUFA	HOL	1 - 2	2	*INSIG2*	Rincon et al., 2012
SFA, MUFA			4	*SCD5*, *INSIG1*	
PUFA			14	*DGAT1*	
SFA, MUFA			22	*SCAP*	
MUFA			26	*SCD*	
C18:0	JER	1 - 3	10	*TDP*, *KCNK13*, *TTC7B*, *CASC4*, *CTTDSPL2*	Buitenhuis et al., 2014
C16:0			14	*DGAT1*	
C16:0, C18:0			27	*SUPT3H*, *RUNX2*	
C18:0	HOL	All	1	*SI*	Li et al., 2014
C18:0			2	*RUNX3*, *FABP3*, *IFFO2*	
C18:0			5	*USP44*, *CHST11*	
SFA, UFA			10	*SLC38A6*	
SFA, UFA			14	*DGAT1*	
C18:0			11	*REG3A*, *LRRTM4*	
C18:0			12	*DCT*, *ABCC4*	
C18:0			16	*LOC101902340*	
C18:0			17	*MYO18B*	
C18:0			24	*CDH7*	
C16:0	HOL	1	5	*MGST1*	Maurice-Van Eijndhoven et al., 2015
C16:0, C18:0			14	*DGAT1*	
SFA, MUFA	HOL	1 - 5	6	*TACR3*	Ibeagha-Awemu et al., 2016
SFA			14	*TONSL*	
MUFA			19	*ITGB4*	
SFA, MUFA			25	*ACHE*	
PUFA	RDC	1 - 4	1	*SLC37A1*, *ABCG1*, *AGPAT3*	Olsen et al., 2017
C18:0			15	*APOA1*, *APOA3*, *APOA4*, *APOA5*	
C16:0, C18:0	HOL,	1 - 6	5	*MGST1*	Benedet et al.,
C16:0, C18:0	JER		14	*DGAT1*	2019b
C16:0			20	*GHR*	
SFA	HOL	1	5	*MGST1*	Cruz et al., 2019
SFA, UFA			14	*DGAT1*, *FOXH1*, *CYHR1*	
C16:0, C18:0	HOL	1 - 6	2	*OSBPL6*, *AGPS*	Gebreyesus et al.,
C16:0			2	*MOGAT1*, *FABP3*, *MECR*	2019
C16:0			5	*MGST1*, *PLBD1*, *LRP6*	
C16:0			14	*DGAT1*, *GPAA1*	
C18:0			15	*APOA1*, *APOA4*, *APOA5*, *DPAGT1*	
C16:0			19	*ACLY*, *BRCA1*, *FASN*, *STAT5A*	
C18:0			20	*PRKAA1*	
C16:0, C18:0			26	*SCD*, *ELOVL3*, *ACSL5*, *GPAM*	
C16:0			29	*TKFC*	

[1] SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, C16:0 = palmitic acid, C18:0 = stearic acid.
[2] HOL = Holstein-Friesian, JER = Jersey, RDC = Red dairy cattle (Norwegian Red).
[3] BTA = *Bos taurus* autosome.

Table 1.7. Potential candidate genes for the fatty acid profile in milk (continued).

Trait[1]	Breed[2]	Parity	BTA[3]	Candidate gene	Reference
SFA	HOL	1 - 6	5	*MGST1*	Iung et al., 2019
MUFA, PUFA			11	*LTBP1*, *PAEP*, *CEL*, *GBGT1*, *ABCA2*, *PTGDS*	
SFA, UFA, MUFA, C16:0, C18:0			14	*DGAT1*, *CYHR1*, *VPS28*, *MROH1*, *OPLAH*, *GPR20*	
C18:0			16	*MFSD4A*, *SLC41A1*, *SLC45A3*	
SFA	HOL	1	5	*RERGL*, *EPS8*, *RERG*, *ARHGDIB*, *GUCY2C*	Freitas et al., 2020
SFA, UFA			14	*LY6D*, *LYNX1*, *LYPD2*, *SLURP1*, *TSNARE1*, *ARC*, *JRK*, *SLC45A4*, *PTK2*, *AGO2*, *TRAPPC9*, *KCNK9*	
SFA, UFA, C18:0	HOL	1	5	*CPM*	Shi et al., 2020
UFA, SFA, C16:0, C18:0	HOL	1 - 4	1	*AGPAT3*	Shi et al., 2021

[1] SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, C16:0 = palmitic acid, C18:0 = stearic acid.
[2] HOL = Holstein-Friesian.
[3] BTA = *Bos taurus* autosome.

Interval from calving to first insemination

Also for the fertility trait ICF SNP close to candidate genes related to immunity, energy, lipid metabolism, and fertility were detected on BTA 4 - 6, 10, 11, 13, 15, 16 - 18, 20, 21, 23, 24, 29 (Table 1.8). Zhang et al. (2019) analyzed genotype-by-environment interaction of fertility traits in Danish Holsteins using a single-step genomic reaction norm model. They identified two genomic regions associated with both fertility traits ICF and the interval from first to last insemination on BTA 23 and on BTA 17. The corresponding candidate genes *IL17*, *IL17F*, and *LIF* were immunity related genes (Table 1.8, Zhang et al., 2019). Candidate genes *IGF1* and *TGFB2* on BTA 5 and 16 contributed to ICF were detected by Minozzi et al. (2013). These genes were associated with milk production, body condition, involved in follicular development and the interaction with reproductive hormones. Chebel and Santos (2011) significantly associated the energy metabolism and feed intake related leptin genotype (located in *LEP* gene) with the proportion of cows classified as oestrous cyclic at 49 ± 3 DIM (Table 1.8). According to Wathes et al. (2013) polymorphisms in many genes including *DGAT1*, *SCD1*, *DECR1*, *CRH*, *CBFA2T1*, *GH*, *LEP*, and *NPY* affecting lipid metabolism also showed associations with fertility traits i.e., ICF. Thus, genomic investigations regarding KET, KET indicator traits and also cows fertility would provide further insights into genomic relationships and shared genes.

Table 1.8. Potential candidate genes for the interval from calving to first insemination.

Breed[1]	Parity	BTA[2]	Candidate gene	Reference
HOL		18	*NDRG4*	Pimentel et al., 2010
sires		20	*CCNB1*	
HOL	1 - 3	23	*TNFa*	Shirasuna et al., 2011
HOL	All	4	*LEP*	Chebel and Santos, 2011
HOL		5	*IGF1*	Minozzi et al., 2013
sires		16	*TGFB2*	
RDC,		4	*ADCY1*	Höglund et al., 2014
JER sires		11	*PPM1B, SLC1A4*	
RDC		6	*KCNIP4*	Höglund et al., 2015
sires		13	*ANKRD60*	
		15	*GRAMD1B*	
HOL		5	*ABCC9*	Nayeri et al., 2016
sires		13	*FAM188A, MRC1*	
		21	*FAM181A, ASB2, SLC24A4, NKX2-1*	
BS sires		29	*PYGM, PLCB3*	Frischknecht et al., 2017
HOL		4	*CHN2*	Liu et al., 2017b
sires		10	*ENSBTAG00000021414, ENSBTAG00000025634*	
		11	*FAM84A, ENSBTAG00000019284, TRIB2, LPIN1*	
		13	*RSU1, SLC39A12, CACNB2*	
		15	*CADM1, BUD13*	
		16	*SLC25A34, PLEKHM2, ENSBTAG00000027809, GPR52*	
		17	*PISD*	
		18	*SIPA1L3*	
		21	*PRKD1, G2E3, NKX2-8, PAX9*	
		24	*AQP4, KCTD1*	
HOL		6	*EREG, AREG*	Zhang et al., 2019
sires		16	*TPR, NMNATI*	
		17	*LIF*	
		23	*IL17, IL17F*	

[1] BS = Brown Swiss, HOL = Holstein-Friesian, JER = Jersey, RDC = Red dairy cattle (Norwegian Red).
[2] BTA = *Bos taurus* autosome.

Prospects of cow reference groups

Cow reference groups offered new prospects in dairy cattle breeding for improved disease resistance by combining phenotypes for novel traits with high-density genetic markers (Naderi et al., 2016). In general, the direct genomic value (**dGV**) was combined with the classical breeding value, based on own and offspring performance, to the combined genomic enhanced

breeding value (**gEBV**) since 2010 for Holsteins and Red Holsteins in Germany. For dGV calculation knowledge about the relationship between marker and the trait is obligatory. For genomic evaluations a formula summarising the marker effects of any animal according to its specific marker information estimated the dGV (VIT, 2021). According to VIT (2021) well proven animals were daughter proven bulls with known genetic based performances. The formula for the genomic estimations was based on those bulls displaying the reference population. The greater the number of animals in the reference population and the more reliable their EBV were the more reliable were the genomic estimations and the dGV. Hence, the reference population was enhanced by genotyped bulls from France, Netherlands, Denmark, Sweden, Finland, and Poland to the EuroGenomics reference population. However, due to the strong preselection of young genotyped bulls in this reference population the number of new daughter proven bulls decreased and the reference bulls did not represent the population anymore.

Thus, the breeding organizations and the IT solutions for animal production (**VIT**) in Germany initiated projects (e.g., KUH-L, KuhVision) to build a cow reference population by genotyping whole German Holstein herds. The reference population is genetically closer to the current selection candidates due to the inclusion of young cows. The lower the genetic differences between the reference population and the selection candidates are, the better are the reliabilities of genomic breeding values of the young selection candidates. Especially, for breeding programs regarding animal health the focus on pure bull reference population was not usable. Selection was based on performance data and recorded health data were not sufficient for effective evaluations with a bull reference population because just a low number of older bulls had enough daughter records regarding animal health (Reinhardt, 2019). As a result, older progeny tested bulls did not have reliable estimated breeding values for new traits. Especially, novel health traits had limited historical data and thus genomic selection regarding those traits was challenging (Heringstad et al., 2018). For health traits a smaller reference population was available to be used in genomic predictions compared with e.g., production traits. According to Heringstad et al. (2018) genotyping cows in herds with reliable health records (e.g., claw trimming records) might have been one strategy to enhance the accuracy of genomic predictions for those traits. The genetic evaluation e.g., for claw health in the Nordic countries included cows in the reference population for several years. In 2014, the addition of 10,000 cows to 7,800 bulls in the reference population resulted in an increase in reliability for Norwegian Red cattle of 0.09 (Heringstad et al., 2018). Hence, a cow reference population including unselected genotyped cows with their own records (inclusive new health traits) will be advantageous in

health evaluations. According to Alkhoder et al. (2017) and Liu et al. (2019) an increase in accuracy of genomic predictions for all regular traits (e.g., milk yield) and also low heritable novel health traits (e.g., disease traits, calf fitness) was found for a combined cow and bull reference population. Therefore, since 2019 the combined reference population has been used for routine genomic breeding value estimation for health traits in German Holsteins (Liu et al., 2019).

Study objectives

The present thesis deals with phenotypic, quantitative genetic and genomic analyses considering a cow reference group to infer relationships between KET, the FPR, novel, innovative milk indicator traits, i.e., ketone body concentrations, the FA profile, and the fertility interval trait ICF.

The aims are summarized as follows:

Chapter 2 analyzes associations between the first test-day FPR and KET in first-lactation German Holstein cows based on a large data set of genotyped cows from large scale co-operator herds. This aim implies the application of generalized linear mixed models to infer phenotypic trait associations, the estimation of pedigree- and SNP-based (co)variance components, GWAS to detect significantly associated SNP marker for KET and the FPR, as well as the annotation and physiological explanation of potential candidate genes.

Chapter 3 investigates associations between KET and first test-day FTIR milk acetone and BHB concentrations in Holstein cows. Further aim was to study associations between acetone, BHB and KET with test-day traits from the very early-lactation period. Against this background, we applied generalized linear mixed models to study phenotypic trait relationships, we estimated genetic (co)variance components, we performed GWAS based on SNP marker data for acetone and BHB in milk, and we identified potential candidate genes influencing related metabolic pathways.

Chapter 4 specifies relationships among milk FA groups (SFA, UFA, MUFA, PUFA) as well as specific C16:0 and C18:0 FA concentrations, KET diagnoses and ICF in a comprehensive ssGBLUP approach. We applied ssGBLUP to estimate genetic (co)variance components for KET, first test-day FTIR milk FA profiles and ICF, and we conducted ssGWAS to identify SNP marker associations and potential candidate genes for the traits.

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CHAPTER 2

Phenotypic relationships, genetic parameters, genome-wide associations, and identification of potential candidate genes for ketosis and fat-to-protein ratio in German Holstein cows

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Abstract

Energy demand for milk production in early lactation exceeds energy intake, especially in high-yielding Holstein cows. Energy deficiency causes increasing susceptibility to metabolic disorders. In addition to several blood parameters, the fat-to-protein ratio (FPR) is suggested as an indicator for ketosis, because a FPR > 1.5 refers to high lipolysis. The aim of this study was to analyze phenotypic, quantitative genetic, and genomic associations between FPR and ketosis. In this regard, 8,912 first-lactation Holstein cows were phenotyped for ketosis according to a veterinarian diagnosis key. Ketosis was diagnosed if the cow showed an abnormal carbohydrate metabolism with increased content of ketone bodies in the blood or urine. At least one entry for ketosis in the first 6 wk after calving implied a score = 1 (diseased); otherwise, a score = 0 (healthy) was assigned. The FPR from the first test-day was defined as a Gaussian-distributed trait (FPRgauss), and also as a binary response trait (FPRbin), considering a threshold of FPR = 1.5. After imputation and quality controls, 40,993 and 41,017 SNP markers from the 8,912 genotyped cows were used for genomic studies for ketosis and FPR, respectively. Phenotypically, an increasing ketosis incidence was associated with significantly higher FPR, and vice versa. Hence, from a practical trait recording perspective, first test-day FPR is suggested as an indicator for ketosis. The ketosis heritability was slightly larger when modeling the pedigree-based relationship matrix (pedigree-based: 0.17; SNP-based: 0.11). For FPRbin, heritabilities were larger when modeling the genomic relationship matrix (pedigree-based: 0.09; SNP-based: 0.15). For FPRgauss, heritabilities were almost identical for both pedigree and genomic relationship matrices (pedigree-based: 0.14; SNP-based: 0.15). Genetic correlations between ketosis with FPRbin and FPRgauss using either pedigree- or genomic-based relationship matrices were in a moderate range from 0.39 to 0.71. Applying genome-wide association studies, we identified the specific SNP rs109896020 (BTA 5, 115,456,438 bp) significantly contributing to ketosis. The identified potential candidate gene *PARVB* in close chromosomal distance is associated with nonalcoholic fatty liver disease in humans. The most important SNP contributing to FPRbin was located within the *DGAT1* gene. Different SNP significantly contributed to ketosis and FPRbin, indicating different mechanisms for both traits genomically.

Key words: ketosis, fat-to-protein ratio, genetic parameter, genome-wide association, potential candidate gene

Introduction

During early lactation, nutrient demand for milk production exceeds the slow increase in DMI, causing a negative energy balance (**NEB**). Energy deficiency is a major component explaining susceptibility to metabolic disorders, such as ketosis (**KET**; Buttchereit et al., 2012). Ketosis induces further cost-intensive diseases (e.g., metritis, mastitis, laminitis, or displaced abomasum; Suthar et al., 2013). During the NEB period, ketone bodies serve as short-term energy sources for several organs (Robinson and Williamson, 1980). However, excessive concentrations of circulating ketone bodies cause ketoacidosis, and an increase of ketone bodies in blood, milk, and urine (Bashir et al., 2016). To overcome energy deficiency periods, fat mobilization from body fat depots is associated with increasing milk fat synthesis. Furthermore, reduced feed intake in the first third of lactation causes insufficient ruminal bacteria protein production, implying a decline in milk protein content (Gürtler and Schweigert, 2005). Hence, a fat-to-protein ratio (**FPR**) larger than 1.5 refers to high lipolysis. The trait FPR is available on the basis of routinely recorded test-day data, and consequently, suggested as a KET indicator (Heuer et al., 1999).

Phenotypically, Kessel et al. (2008) analyzed metabolic processes in high-yielding Red Holstein cows during the transition period. The cows under study were kept under identical housing and feeding systems. They clustered the cows according to their ketone body concentration in plasma, and identified significant cow differences for metabolite concentrations (e.g., BHB, acetone, glucose) and for hormone levels. Such individual differences for cows kept in the same herd environment suggest genetic or epigenetic influence on physiological mechanisms coping with metabolic stress (Kessel et al., 2008).

Quantitative genetic, heritabilities for KET ranged between 0.01 (SE = 0.006) and 0.14 (SE = 0.03) for Holstein cows, depending on the genetic-statistical modeling approach (Pryce et al., 2016). In most studies, clinical KET cases were the trait basis for genetic parameter estimations because clinical cases are quite easy to detect (Pryce et al., 2016). Nevertheless, consideration of more precise phenotypes in genetic analyses (i.e., of subclinical KET cases) contribute to increasing incidence rates and more accurate breeding value estimations. Detection of subclinical KET implies detailed recording of biomarkers (e.g., ketone body concentrations in blood or in milk; König and May, 2018). As an alternative, test-day FPR is suggested for indirect selection strategies on subclinical KET (van Knegsel et al., 2010). Koeck et al. (2013) estimated a heritability of 0.15 (SE = 0.015) for Gaussian FPR, 0.07 (SE = 0.012) for binary FPR, and a favorable genetic correlation between FPR and KET of 0.35 (SE = 0.16).

So far, only a limited number of studies have focused on genome-wide association studies (**GWAS**) and potential candidate gene identification for KET. Parker Gaddis et al. (2018) estimated SNP effects for producer-recorded KET, including 1,750 medium-density genotyped US Jersey cows. They identified SNP significantly associated with KET on BTA 10, 11, 14 and 23. Genes involved in insulin regulation, lipid metabolism, and immune response were located in close distance to the associated SNP markers (Parker Gaddis et al., 2018). The large number of identified significant SNP indicate that KET is a polygenic trait, influenced by numerous genomic regions. These results are confirmed by Kroezen et al. (2018), who identified 6 KET candidate genes on BTA 2, 3, 13, 27, and 29 involved in fatty acid metabolism, gluconeogenesis, and citric acid cycle. Considering 248 genotyped Holstein cows, Tetens et al. (2015) conducted a GWAS for the KET indicator, ratio of glycerophosphocholin to phosphocholine, in milk. Via ongoing candidate gene and sequence analyses, Tetens et al. (2015) identified a QTL on BTA 25 for the ratio of glycerophosphocholin to phosphocholine.

The aim of the present study was to study phenotypic, quantitative genetic and genomic associations between first test-day FPR and KET in first-lactation German Holstein cows, based on a large data set of genotyped cows from large scale co-operator herds. This aim includes 1) the application of generalized linear mixed models to infer phenotypic trait associations, 2) the estimation of pedigree- and SNP-based (co)variance components, 3) GWAS to detect significantly associated SNP markers, and 4) the annotation and physiological explanation of potential candidate genes.

Materials and Methods

Data

Phenotypes

We considered 8,912 first-lactation German Holstein cows kept in 27 large-scale co-operator herds, located in the federal states of Mecklenburg-West Pommerania and Brandenburg, Germany. Calving dates of cows spanned the years 2014 to 2016. Health data recording including KET was accomplished by veterinarians within the first 6 wk after calving, using an electronic recording systems. The recording system has a hierarchical structure with several disease levels (from overall disease categories up to specific diseases), following the International Committee for Animal Recording diagnosis key (Stock et al., 2013). According to the recording guidelines, a KET diagnosis reflects disturbance of carbohydrate metabolism with increased content of ketone bodies in blood or urine (measured via handheld ketometers or urine test strips). Only herds with at least one KET entry in the 6-wk interval were considered.

Cows with at least one KET diagnosis during the 6-wk interval were coded as diseased (KET = 1), whereas cows without any KET diagnosis in the 6-wk interval were coded as healthy (KET = 0). Cows with a culling date within the 6-wk interval have "no further chance" to get the disease. Hence, these cows with no KET entry until the culling date are considered as healthy. This is a general problem with health data (i.e., cullings within the defined time period). The early lactation interval definition was used for other disease categories, such as claw disorders (König et al., 2005). Nevertheless, KET has only a minor effect on statistic or genetic parameter estimations because (1) KET is diagnosed very early after calving (60% within the first 10 d after calving), and (2) the number of cullings is very small within the 6-wk interval after calving. Most of the cullings occur later in lactation. In our data set, only 0.75% of the cows diagnosed as healthy had a culling date before second test-day. Descriptive statistics for KET are given in Table 2.1. Most of the KET-diagnosed cows (~80% of the cows) were diagnosed for another disease, such as claw disorders or mastitis. Test-day records included milk yield, fat percentage, protein percentage, FPR, and SCS from the first test-day after calving. The first test-day was within the period from 5 to 42 DIM. Descriptive statistics for all test-day traits are given in Table 2.2. According to Hein et al. (2018), extremely low values for milk yield (2 kg) were considered, because very strong KET cases might cause a sudden milk yield decline. The data editing threshold for milk yield as used in official genetic evaluations is 5 kg, but only 14 test-day records were in the range from 2 to 5 kg. Somatic cell count was log-transformed into SCS as follows: $SCS = \log_2(SCC/100)+5$ (Ali and Shook, 1980). The number of SCS records was slightly smaller than for milk yield or percentage traits because of a few missing records for SCC.

Furthermore, we calculated lactation persistency, using the formula by Visscher and Mason (2016):

$$\text{Persistency (\%)} = \left[1-\frac{(\text{milk yield (kg) earlier test-day} - \text{milk yield (kg) later test-day}) \times \frac{30 \text{ days}}{\text{days between test-days}}}{\text{milk yield (kg) earlier test-day}}\right]\times 100.$$

In this regard, the earlier test-day was test-day 3, and the later test-day was test-day 5. Test-day 3 was within 50 and 140 DIM, and test-day 5 reflected the lactation period from 150 to 220 DIM. Data editing excluded records with persistency values lower than 65% or larger than 120%. Fat-to-protein ratio was defined as a Gaussian-distributed trait (**FPRgauss**) and as a binary response trait (**FPRbin**). A FPR > 1.5 at the first test-day implied a score of 1 for FPRbin (diseased); otherwise, a score of 0 (healthy) was assigned. The incidences for the binary trait definitions are in Table 2.1.

Table 2.1. Descriptive statistics for ketosis (KET) and fat-to-protein ratio (FPR) of first lactating Holstein cows.

Characteristic	Analyzed data
Total no. of herds	27
Total no. of animals	8,912
Mean no. of cows per herd	330
Healthy animals KET, no. (%)	8,750 (98.18)
Diseased animals KET, no. (%)	162 (1.82)
Healthy animals FPR ≤ 1.5, no. (%)	7,688 (86.27)
Diseased animals FPR > 1.5, no. (%)	1,224 (13.73)

Table 2.2. Descriptive statistics for first official test-day traits of first lactating Holstein cows.

Trait	No. of records	Mean	SD	Minimum	Maximum
Milk yield (kg)	8,912	28.88	6.58	2	53.8
Fat percentage (%)	8,912	4.10	0.78	1.62	9.26
Protein percentage (%)	8,912	3.22	0.32	2.24	4.89
Fat-to-protein ratio	8,912	1.28	0.23	0.43	2.91
Fat-to-protein ratio > 1.5	1,224	1.70	0.21	1.50	2.91
Somatic cell score	8,893	4.85	1.69	0.94	11.64
Persistency (%)	8,549	99.14	5.09	66.87	119.86

Genotypes

A total of 2,374 cows were genotyped with the Illumina BovineSNP50 v2 BeadChip (Illumina Inc., San Diego, CA), and 6,538 cows with the EuroGenomics 10K chip (Illumina Inc., San Diego, CA). Low-density genotypes were imputed by the project partner VIT (Vereinigte Informationssysteme Tierhaltung w.V, Verden, Germany) to the 50K panel, applying the algorithm as outlined by Segelke et al. (2012). The SNP data set considered 45,613 SNP from the 8,912 phenotyped cows. Quality control of the genotype data was performed using the software package PLINK (Purcell et al., 2007). The SNP with more than 1% missing genotype data, with a minor allele frequency lower than 5%, and deviation from Hardy-Weinberg equilibrium (P-value < 10^{-8}), were discarded. Thus, due to different cows in case and control groups for both traits, 40,993 and 41,017 SNP from the 8,912 cows were available for genomic studies for KET and FPRbin, respectively.

Statistical Models

Phenotypic associations between fat-to-protein ratio and ketosis

A generalized linear mixed model (**GLMM**) with a binomial distribution and a logit link function was applied to analyze the effect of FPRbin on binary KET. For this purpose, the Glimmix procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC) was used. The statistical model [1] was:

$$\text{logit}(\pi) = \log[\pi_{rstuv}/(1-\pi_{rstuv})] = \varphi + \text{Herd}_r + \text{CYear}_s + \text{CMon}_t + \text{CAge}_u + \text{FPRbin}_v \quad [1]$$

where π_{rstuv} = probability of a KET occurrence; φ = overall mean effect; Herd_r = fixed herd effect (27 herds); CYear_s = fixed effect of calving year (3 yr, 2014-2016); CMon_t = fixed effect of calving month (12 mo); CAge_u = covariate age at first calving (linear regression); FPRbin_v = fixed effect of FPRbin (2 classes, FPR $\leq$ 1.5 or FPR >1.5).

The recursive effect (i.e., of KET on the Gaussian-distributed test-day traits milk yield, fat percentage, protein percentage, FPR, SCS, and persistency) also was analyzed using the Glimmix procedure in SAS (SAS Institute Inc., Cary, NC). In this regard, a GLMM with an identity link function was defined. The statistical model [2] was:

$$y_{ijklm} = \varphi + \text{Herd}_i + \text{CYear}_j + \text{CMon}_k + \text{CAge}_l + \text{KET} \times \text{Diffdat}_m + e_{ijklm} \quad [2]$$

where y_{ijklm} = observation for the test-day traits; Herd_i = fixed herd effect (27 herds); CYear_j = fixed effect of calving year (3 yr, 2014–2016); CMon_k = fixed effect of calving month (12 mo); CAge_l = covariate age at first calving (linear regression); $\text{KET}\times\text{Diffdat}_m$ = combined fixed effect of KET (healthy or diseased) × period between the first test-date and the KET diagnosis date [9 classes considering the following periods: 31 to 21 d (n = 5 cows), 20 to 11 d (n = 8 cows), 10 to 5 d (n = 13 cows), 4 to 1 d (n = 15 cows) before the diagnosis date; 0 to 4 d (n = 13 cows), 5 to 10 d (n = 33 cows), 11 to 20 d (n = 43 cows) and 21 to 31 d (n = 32 cows) after the diagnosis date; and a "dummy" class for healthy cows (n = 8,750 cows)]; and e_{ijklm} = random residual. A multiple comparison adjustment for the effect of the KET×Diffdat effect was accomplished using the Dunnett correction as implemented in the Glimmix procedure (Dunnett, 1955).

Pedigree- and genomic-based genetic parameter estimation

Pedigree-based variance components and heritabilities for KET and FPR were estimated using the AI-REML algorithm as implemented in the DMU software package (Madsen and Jensen, 2013). The pedigree file comprised 93,446 animals. The GLMM (model [3]) considered the same fixed effects as specified in model [1] and model [2], but we (1) excluded the explanatory

variables FPRbin and KET×Diffdat, and (2) we included the random additive-genetic animal effect.

Genetic correlations between KET and FPRgauss and FPRbin were estimated via bivariate linear animal models.

The SNP-based variance components, heritabilities, and genomic correlations were estimated with GCTA (Yang et al., 2011), using the GREML function. Fixed effects were identical to the pedigree-based analyses, but instead of using the pedigree-based relationship matrix, the genomic relationship matrix (**GRM**) was constructed.

Genome-wide association studies

The data set used for FPRbin included 1,224 cases (FPR > 1.5) and 7,688 controls (FPR ≤ 1.5). Regarding KET, 162 cases (diseased) and 8,750 controls (healthy) were available. Also for GWAS, the GCTA software (Yang et al., 2011) was applied. In GCTA, we used the option mlma-loco (i.e., to perform a mixed linear model via the "leaving one chromosome out" strategy). The model [4] was:

$$\mathbf{y}_{ij} = \mathbf{X\beta} + \mathbf{Zu} + \mathbf{S}_k s_{jk} + \mathbf{e}_{ij} \quad [4]$$

where $\mathbf{y}_{ij}$ represents the vector of phenotypic observations from the ith cow (i = 1 - 8,912) for the jth trait (j = KET or FPRbin), **X** is the incidence matrix relating fixed effects in **β** (as specified in model [3]) with observations in $\mathbf{y}_{ij}$; **Z** is the incidence matrix relating random additive polygenic effects in **u** with observations in $\mathbf{y}_{ij}$, $\mathbf{S}_k$ is the vector of genotypes for the kth SNP across all animals; s_{jk} represents the additive effect of the kth SNP on the jth trait; and $\mathbf{e}_{ij}$ is the vector of random residual effects.

For the trait FPRbin, Manhattan plots from model [4] suggested a strong effect of the single SNP rs109421300 which is located in the *DGAT1* gene. It was our intention to account for a major *DGAT1* gene effect on the traits of interest. Hence, as an extension of model [4] an additional model [5] was chosen for both traits KET and FPRbin. In model [5] we considered the genotype for the *DGAT1* SNP rs109421300 as a further fixed effect.

The effective number of independent SNP (N_{SNP} = 29,548) was calculated using the software Genetic type 1 Error Calculator (Li et al., 2012), to define the genome-wide significance level according to Bonferroni (**pBF** = 0.05 / N_{SNP} = 1.69e-06). In addition, a less conservative normative significance threshold was used to identify potential candidate SNP, considering **pCD** = 1e-04 (Kurz et al., 2018). Annotated genes located in 250 kb upstream or downstream from the significantly associated candidate SNP were detected using Ensembl release 91 (Zerbino et al., 2018).

Results and Discussion

Phenotypic associations between ketosis and test-day traits

Phenotypically, increasing KET incidences were associated with significantly higher FPR ($P < 0.001$, model [1]). The probability for KET occurrence was 3-fold higher for cows with a FPR larger than 1.5 (predicted incidence = 0.050, SE = 0.022) compared to cows with a FPR lower or equal 1.5 (predicted incidence = 0.017, SE = 0.007). Vice versa, we also identified a significant influence of a KET diagnosis on FPR, but depending on the intervals between the test date and the diagnosis date (model [2]). Table 2.3 addresses the influence from KET diagnoses recorded before and after the test date. Cows with a KET diagnosis had a significant higher first test-day FPR and fat percentage compared with healthy cows. Differences between sick and healthy cows were most obvious for diagnosis days in close distance to the test-day (i.e., test-day 4 to 1 d before and 0 to 10 d after the diagnosis date). Hence, FPR is a very useful KET indicator for a dense data structure (i.e., in the optimal case for records on a daily level from automatic milking systems; Santos et al., 2018). Increased FPR and fat percentage for cows with a KET diagnosis reflect the increased milk fat synthesis caused by elevated body fat mobilization during the energy deficiency period (Bell, 1995).

Milk yield was significantly lower for KET-diagnosed cows in the period from 10 d before to 20 d after the diagnosis date (Table 2.3). Accordingly, Koeck et al. (2013) confirmed the decline in milk yield during the disease period for cows with a KET diagnosis. In our study, protein percentage was significantly lower from d 11 to 31 after the diagnosis date (Table 2.3), compared with protein percentage of healthy cows. The decrease of test-day protein percentage after the KET diagnosis date is in agreement with Duffield et al. (2009). The insufficient intake of carbohydrates in the state of NEB decreases the microbial protein synthesis, causing the decline in milk protein percentage (Gürtler and Schweigert, 2005).

Phenotypically, in our study, there was no significant effect of KET on SCS and persistency.

Table 2.3. Differences for least squares means (LSM) of first test-day traits for the cow group comparison of ketosis healthy cows minus ketosis diseased cows [considered time periods: test-day dates before (b) or after (a) the ketosis diagnosis date].

		Time period							
		31 to 21 d		20 to 11 d		10 to 5 d		4 to 0 d[2]	
Trait[1]		Difference of LSM	*P*-value	Difference of LSM	*P*-value	Difference of LSM	*P*-value	Difference of LSM	*P*-value
MY (kg)	b	0.96	NS	4.00	NS	4.69	*	7.45	***
	a	-1.58	NS	2.60	*	3.46	**	6.90	***
F (%)	b	-0.66	NS	-0.64	NS	-0.63	*	-1.39	***
	a	0.41	*	0.14	NS	-0.43	**	-0.84	***
P (%)	b	-0.01	NS	-0.11	NS	-0.17	NS	0.00	NS
	a	0.21	**	0.17	**	0.11	NS	0.07	NS
FPR	b	-0.20	NS	-0.15	NS	-0.13	NS	-0.45	***
	a	0.04	NS	-0.04	NS	-0.20	***	-0.30	***
SCS	b	-0.69	NS	-0.31	NS	-0.51	NS	-1.05	NS
	a	0.36	NS	-0.07	NS	-0.40	NS	0.09	NS
PER (%)	b	1.21	NS	-2.53	NS	-0.37	NS	-0.57	NS
	a	0.62	NS	0.96	NS	0.35	NS	-1.30	NS

[1]MY = Milk yield, F = fat percentage, P = protein percentage, FPR = fat-to-protein ratio, SCS = somatic cell score, PER = persistency.

[2]4 to 0 d: 4 to 1 d before the ketosis diagnosis date, 0 to 4 d after the ketosis diagnosis date.

*** $P \leq 0.001$; ** $P \leq 0.01$; *$P \leq 0.05$; NS = nonsignificant.

Heritabilities and genetic correlations

Heritabilities were 0.17 and 0.11 for KET, 0.09 and 0.15 for FPRbin, and 0.14 and 0.15 for FPRgauss, when applying pedigree-based or genomic models, respectively (Table 2.4, model [3]). In other studies using producer data, pedigree-based heritabilities for KET ranged between 0.02 and 0.14 (Heringstad et al., 2005; Koeck et al., 2014). In general, low heritabilities for KET might be due to the complex disease trait character, showing the phenomenon of the missing heritability (Blanco-Gómez et al., 2016). The pathogenesis of complex diseases is often regulated by intermediate phenotypes with quantitative inheritance. Blanco-Gómez et al. (2016) argued that not-detectable intermediate phenotypes explain a major part of the missing

heritability. An application of more precise continuous phenotypes for KET, such as Fourier transform mid-infrared blood or milk BHB concentration, may lead to detection of subclinical cases, higher disease incidences, and higher heritability estimates (Belay et al., 2017). For KET we estimated smaller SNP-based heritabilities than pedigree-based heritabilities, indicating that the 40,993 SNP marker panel did not fully explain the genetic trait variation. Accordingly, Yang et al. (2017) assumed smaller SNP heritabilities for complex diseases for low- or medium-density SNP chips because not all causal variants are in linkage disequilibrium with the available SNP. McNeel et al. (2017) defined KET as a wellness trait and suggested a reduction of disease incidences and improvements of farm profitability through the implementation of direct genetic selection strategies on low heritability wellness traits.

In analogy to KET, heritabilities for FPRbin and FPRgauss were quite small (Table 2.4). Koeck et al. (2013) estimated a pedigree heritability of 0.15 for first test-day FPRgauss, and of 0.07 for FPRbin. We detected slight differences between the SNP- and pedigree-based heritability for FPRbin. Opposite to KET, the pedigree heritability for FPRbin (0.09) was lower than the SNP-based estimation (0.15). Low estimates for pedigree-based heritabilities point to erroneous or missing pedigree information. However, the same pedigree was used for KET and FPR genetic analyses. On the other hand, FPRgauss heritabilities were almost identical for both pedigree and genomic relationship matrices, probably due to the Gaussian trait character (Golan et al., 2014; Ge et al., 2017).

Genetic correlations between KET and FPRgauss and between KET and FPRbin are given in Table 2.4. Genetic correlations from different methods (i.e., SNP- versus pedigree-based) ranged from moderate to high levels. Genetic correlations between KET and FPRgauss were 0.39 (SNP-based) and 0.52 (pedigree-based), and 0.50 (SNP-based) and 0.71 (pedigree-based) between KET and FPRbin (model [3]). Genetic correlations based on the GRM were lower than pedigree-based genetic correlations. This refers to the missing correlation phenomenon, caused by the fact that the covariance between KET and FPR was not fully explained by the used SNP marker (Momen et al., 2017). As estimates for complex traits may differ between pedigree-based and genomic approaches, Momen et al. (2017) suggested utilization of both information sources simultaneously.

Table 2.4. Heritabilities for ketosis (KET), binary distributed fat-to-protein ratio (FPRbin) and Gaussian-distributed fat-to-protein ratio (FPRgauss; diagonal elements), and their genetic correlations (off-diagonal elements) considering pedigree-based or genomic-based relationship matrices (corresponding SE in parentheses).

Method	Trait	KET	FPRbin	FPRgauss
Pedigree-based	KET	0.17 (0.08)	0.71 (0.18)	0.52 (0.16)
	FPRbin		0.09 (0.02)	
	FPRgauss			0.14 (0.02)
Genomic-based	KET	0.11 (0.06)	0.50 (0.21)	0.39 (0.17)
	FPRbin		0.15 (0.02)	
	FPRgauss			0.15 (0.01)

Genome-wide association studies

Ketosis

Applying GWAS, we identified 5 suggestively associated candidate SNP on BTA 5, 8, 9, and 15 contributing to KET (Figure 2.1, model [4]). Twenty-three genes in the interval of 250 kb surrounding the suggestive SNP were retrieved from the Ensembl database (Zerbino et al., 2018; Table 2.5). The SNP rs109896020 with strongest association was located on BTA 5, and 4 potential candidate genes involved in physiological processes were located in the defined interval: EF-hand calcium binding domain 6 (*EFCAB6*), parvin beta (*PARVB*), parvin gamma (*PARVG*) and shisa like 1 (*SHISAL1*/*KIAA1644*). Eckel-Passow et al. (2014) identified downregulations of *EFCAB6* expressions in obese individuals. In their study, *EFCAB6* expression profiles were associated with obesity-related renal cell carcinoma in humans, suggesting a relationship with mechanisms of the lipid metabolism. The *PARVB* gene, located in close chromosomal distance (61,382 bp) to the SNP rs109896020, contributes to nonalcoholic fatty liver disease in humans (Kitamoto et al., 2013). Applying a genome-wide case-control association study, a haplotype in the *PARVB* gene was strongly associated with nonalcoholic fatty liver disease, suggesting an involvement of *PARVB* in the lipid metabolism. In addition, *PARVB* encodes a member of the parvin family of actin-binding proteins which inhibits the activity of integrin-linked kinase (NCBI, 2017). Integrins in turn play a crucial role regarding the progression of fibrosing liver diseases (Patsenker and Stickel, 2011). The increased influx of fatty acids in the liver, caused by the elevated body fat mobilization in the state of NEB, exceeds the metabolic capacity. An increase of the metabolic capacity contributes

to hepatocyte accumulations of triglycerides and causes the fatty liver disease. Ketosis-diseased cows often have fatty livers (White, 2015), suggesting *PARVB* as a potential candidate gene regulating KET processes. Similarly, Wu et al. (2016) detected an association between polymorphisms in the *PARVB* gene with the fatty liver disease syndrome in humans.

Interestingly, the only suggestively associated marker on BTA 5 for KET is the SNP rs109896020. The Manhattan plot for KET (Figure 2.1) indicates that no other polymorphism close to SNP rs109896020 surpasses the candidate threshold. Probably, variation generated by the causal variants is not fully explained by genotyped SNP from the medium-density chip. Cow genotyping using a denser SNP panel, or utilization of sequence data, might contribute to the identification of a larger number of significantly associated genetic markers within this specific chromosomal segment.

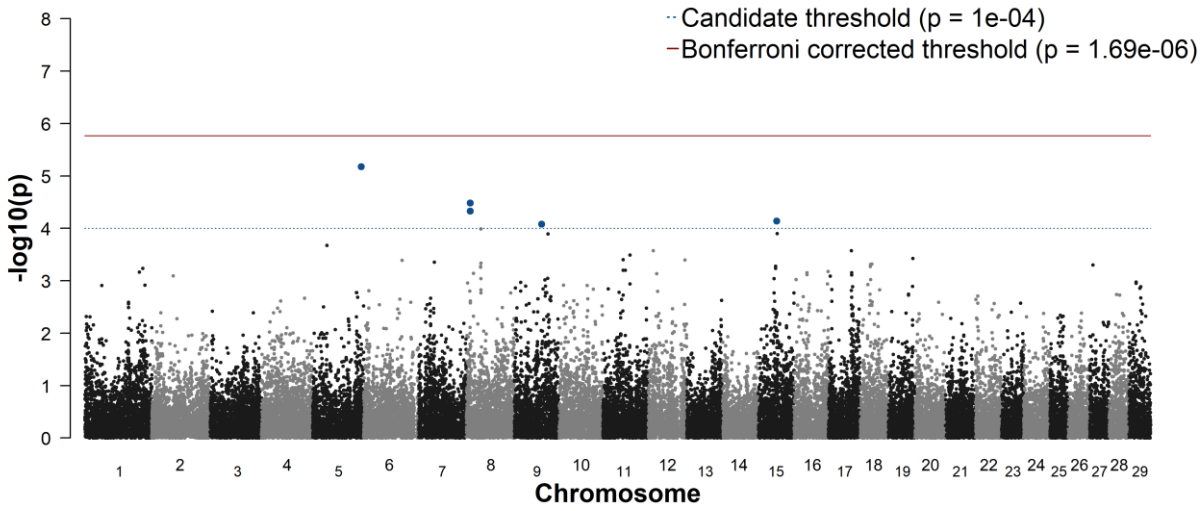

Figure 2.1. Manhattan plot for SNP effects for ketosis of first-lactation Holstein cows.

On BTA 8, the SNP rs41860668 and rs41859808 surpassed the candidate threshold. One of the 3 genes including these two SNP is the homeobox containing 1 (*HMBOX1*) gene. In humans, Chen et al. (2006) identified associations between *HMBOX1* polymorphisms and type-2 diabetes. Furthermore, Dai et al. (2011) showed that *HMBOX1* is highly expressed in the hepatic tissue, which plays an important role in the glycometabolism (Rui, 2014). Moreover, a mutation in the HNF1 homeobox A (*HNF1A*) gene, also referring to the homeoboxes gene family, was associated with insulin-dependent diabetes mellitus and maturity-onset diabetes (Owen and Hattersley, 2001). Ketosis is closely related to diabetes since diabetes is the most common pathological cause of elevated blood ketones (Preeti and Sushil, 2016). Due to the

impact of *HMBOX1* on diabetes, and due to the associations between diabetes with NEB and KET, we hypothesize that *HMBOX1* also plays a crucial role in the progression of KET.

On BTA 9, SNP rs42858549 was suggestively associated with KET. One of the 3 genes including this specific SNP is protein coding *ENSBTAG00000011330*. A disease associated orthologue of *ENSBTAG00000011330* was significantly increased in diabetic rats and in consequence, associated with diabetes mellitus (Schmatz et al., 2009).

On BTA 15, 13 genes were located in the defined interval in close distance to the candidate SNP rs109536046. The genes tripartite motif containing 66 (*TRIM66*), serine/threonine kinase 33 (*STK33*), 60S ribosomal protein L27a (*RPL27A*) and suppression of tumorigenicity 5 (*ST5*) influence diabetes mellitus and body mass in humans. Lau et al. (2017) focused on next generation sequencing and they identified genetic susceptibility to type-2 diabetes. In humans, functional annotations were close to the potential candidate genes *TRIM66* and *STK33*. Furthermore, Rask-Andersen et al. (2013) reported effects of the *STK33* polymorphism rs4929949, located within a 47 kb haplotype block, on the body mass index in children. The *STK33* is located in close distance to *TRIM66*, *RPL27A*, and *ST5*, which are all located within a 200 kb segment surrounding the marker rs4929949. Obesity or a large body mass index are associated with diabetes, and with high frequency, obesity and diabetes occur together (Golay and Ybarra, 2005). Recently, Kroezen et al. (2018) identified 6 genes involved in lipid, ketogenic, glucose metabolism, or in the citric acid cycle. The SNP and genes identified in our study were different from those detected by Kroezen et al. (2018).

Table 2.5. Position and description of potential candidate genes[1] located in the interval of 250 kb surrounding suggestive associated SNP for ketosis in first-lactation Holstein cows.

BTA	Start (bp)	End (bp)	Ensembl Gene ID	Description
5	115,127,019	115,149,183	ENSBTAG00000030189	*EFCAB6*, EF-hand calcium binding domain 6
5	115,517,920	115,572,329	ENSBTAG00000021978	*PARVB*, β-parvin
5	115,583,627	115,608,935	ENSBTAG00000000805	*PARVG*, parvin gamma
5	115,683,472	115,697,601	ENSBTAG00000003574	*KIAA1644*
8	9,276,105	9,481,183	ENSBTAG00000017232	*KIF13B*, kinesin family member 13B
8	9,500,635	9,691,300	ENSBTAG00000010819	*HMBOX1*, homeobox containing 1
8	9,692,513	9,781,880	ENSBTAG00000008845	*INTS9*, integrator complex subunit 9
9	64,879,264	64,929,935	ENSBTAG00000011330	*ENSBTAG00000011330*
9	65,241,868	65,241,949	ENSBTAG00000047184	*ENSBTAG00000047184*
9	65,003,566	65,003,656	ENSBTAG00000044914	*U6*, U6 spliceosomal RNA
15	44,335,342	44,460,360	ENSBTAG00000005356	*ST5*, suppression of tumorigenicity 5
15	44,175,366	44,203,114	ENSBTAG00000013366	*NRIP3*, nuclear receptor interacting protein 3
15	44,199,004	44,199,141	ENSBTAG00000038873	*U3*, Small nucleolar RNA U3
15	44,215,628	44,234,314	ENSBTAG00000008310	*TMEM9B*, TMEM9 domain family member B
15	44,237,476	44,237,790	ENSBTAG00000004771	*ENSBTAG00000004771*
15	44,246,871	44,247,423	ENSBTAG00000032339	*ASCL3*, achaete-scute family bHLH transcription factor 3
15	44,250,291	44,260,216	ENSBTAG00000001917	*C11orf16*, chromosome 11 open reading frame 16
15	44,260,212	44,267,703	ENSBTAG00000001922	*AKIP1*, A-kinase interacting protein 1
15	44,469,327	44,472,127	ENSBTAG00000005349	*RPL27A*, 60S ribosomal protein L27a
15	44,469,600	44,469,728	ENSBTAG00000042335	*SNORA3*, small nucleolar RNA SNORA3/SNORA45 family
15	44,470,841	44,470,970	ENSBTAG00000042354	*SNORA3*, small nucleolar RNA SNORA3/SNORA45 family
15	44,500,912	44,530,712	ENSBTAG00000020890	*TRIM66*, tripartite motif containing 66
15	44,552,624	44,726,438	ENSBTAG00000011910	*STK33*, serine/threonine kinase 33

[1] Ensembl release 91 (Zerbino et al., 2018).

Fat-to-protein ratio

In total, 24 genome-wide significant SNP (pBF = 1.69e-06) and 30 suggestive SNP (pCD = 1e-04) were detected for FPRbin on different chromosomes [i.e., 1 SNP on BTA 3, BTA 4, BTA 24, BTA 28, and BTA 29, 2 SNP on BTA 13, 3 SNP on BTA 9, 4 SNP on BTA 27, and 40 SNP on BTA 14; Figure 2.2, model [4]]. The Manhattan plot (Figure 2.2) illustrates that the majority of the SNP was located on BTA 14. Due to the large number of detected SNP for FPRbin, we

only focused on gene annotations for significantly associated SNP. As expected, the most significant associated SNP rs109421300 ($-\log_{10}(P)$ = 17.19) was located within the diacylglycerol O-acyltransferase 1 (*DGAT1*) gene. The *DGAT1* and genes in the *DGAT1* cluster [e.g., scratch family transcriptional repressor 1 (*SCRT1*), heat shock transcription factor 1 (*HSF1*), protein phosphatase 1 regulatory subunit 16A (*PPP1R16A*), forkhead box H1 (*FOXH1*), cysteine and histidine rich 1 (*CYHR1*), plectin (*PLEC*), lymphocyte antigen 6 family member H (*LY6H*), rhophilin Rho GTPase binding protein 1 (*RHPN1*), chromosome 14 open reading frame, human C8orf33 (*C14H8orf33*), kinesin family member C2 (*KIFC2*), and tonsoku like, DNA repair protein (*TONSL*)] are associated with milk yield, fat or protein percentage, and fatty acid composition in Holstein cows (Li et al., 2014; Bovenhuis et al., 2016). Results from random regression analyses (Oikonomou et al., 2009) implied significant effects of a polymorphism in the *DGAT1* gene on energy and metabolic traits in Holstein cows. The influenced traits in first-lactation cows were BCS, energy balance and serum levels of fatty acids during the first 4 wk after calving.

The 2 SNP rs110519353 [$-\log_{10}(P)$ = 8.60] and rs109599512 [$-\log_{10}(P)$ = 6.88] on BTA 27 surpassed the Bonferroni corrected threshold, and are located in a segment for a QTL influencing milk fat percentage on BTA 27 (Littlejohn et al., 2014). Variants of the gene glycerol-3-phosphate acyltransferase 4 (*AGPAT6*; *AGPAT6* includes the SNP rs110519353) are associated with milk fat percentage in Holstein-Friesian x Jersey crossbreed indicating involvement in lipid metabolism (Littlejohn et al., 2014). In addition, the SNP rs43088681 on BTA 13 (46,239,050 bp) was significantly associated with FPRbin. A polymorphism in the adenosine deaminase RNA-specific B2 (*ADARB2*) gene, located in the specified interval surrounding this SNP, is significantly associated with diabetes-related traits in humans (Oguro et al., 2012). Individuals with a particular genotype for *ADARB2* polymorphism showed high serum triglyceride and serum adiponectin levels, implying a contribution of *ADARB2* in energy metabolism.

The quantile-quantile (**QQ**) plot for FPRbin (Figure 2.3) illustrates a strong deviation from the diagonal, possibly due to the strong effect of the most significant associated SNP rs109421300 located in the *DGAT1* gene.

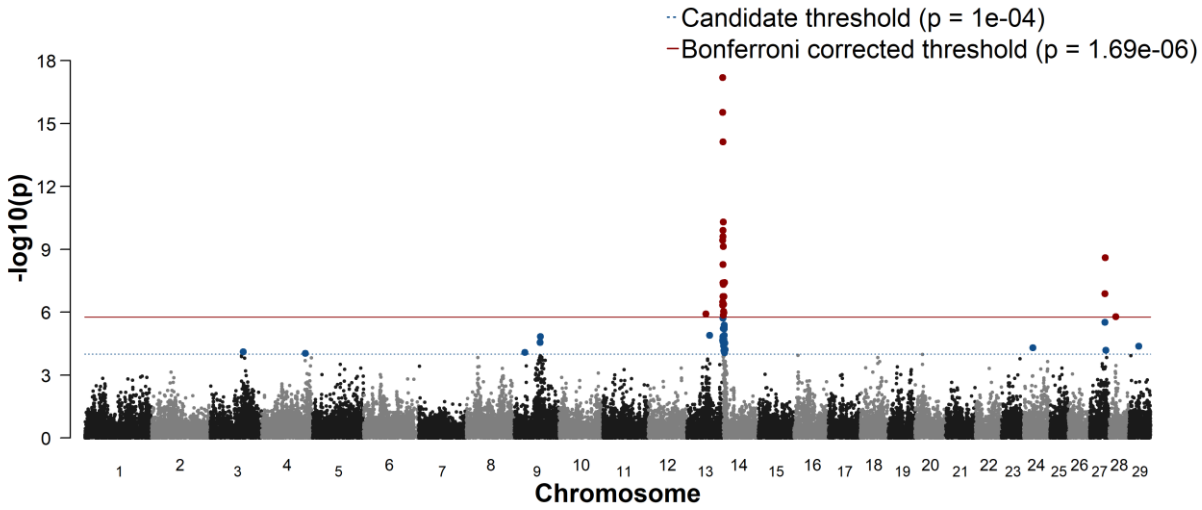

Figure 2.2. Manhattan plot for SNP effects for binary distributed first test-day fat-to-protein ratio of first-lactation Holstein cows.

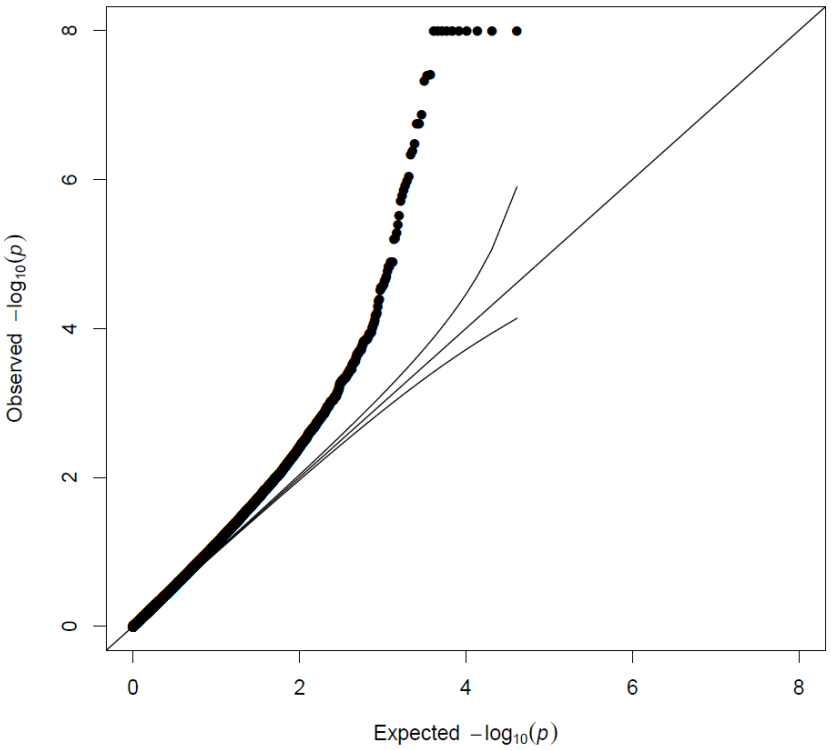

Figure 2.3. Quantile-Quantile plot for binary distributed first test-day fat-to-protein ratio.

In consequence, to verify this hypothesis, we included the SNP genotype for rs109421300 as an additional fixed effect in our association analyses for FPRbin (see model [5]). The Manhattan and QQ plots for FPRbin after *DGAT1* correction display the expected results. Previously detected significant associated SNP on BTA 14 were eliminated due to the *DGAT1* correction (Figure 2.4). Accordingly, the QQ plot indicates less deviation from the diagonal (Figure 2.5).

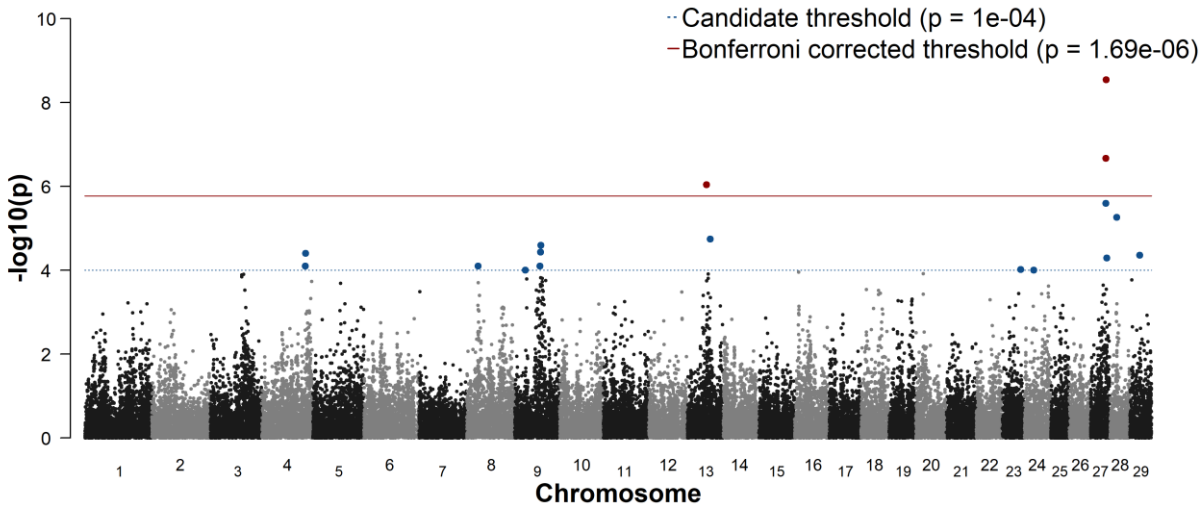

Figure 2.4. Manhattan plot for SNP effects for binary distributed first test-day fat-to-protein ratio after *DGAT1* correction.

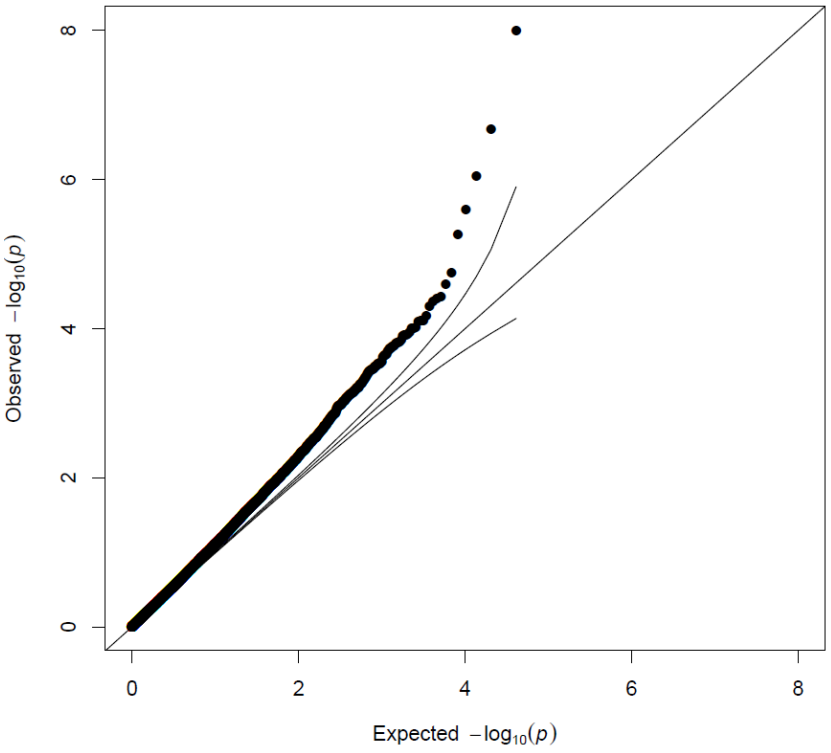

Figure 2.5. Quantile-Quantile plot for binary distributed first test-day fat-to-protein ratio after *DGAT1* correction.

In a next step, we additionally considered the SNP genotype for rs109421300 as a fixed effect in the association analysis for KET (model [5]). The Manhattan and QQ plots for KET with or without *DGAT1* correction display no differences, suggesting that this SNP only has substantial effect on FPR, but not on KET. This finding indicates that rs109421300 is not involved in milk

fat synthesis processes in the states of NEB and KET (i.e., transformations from mobilized body fat depots into milk fat), but contributes to the *DGAT1* effect on high fat percentages.

Overall, we identified five suggestively associated candidate SNP (pCD = 1e-04) influencing KET. For FPRbin (without *DGAT1* correction), we detected 24 significant SNP (pBF = 1.69e-06) and 30 suggestive SNP beyond the candidate threshold. For FPRbin (with *DGAT1* correction), we detected 3 significantly associated (pBF = 1.69e-06) and 15 suggestive SNP (pCD = 1e-04). Despite the significant phenotypic associations and the moderate quantitative genetic correlations between FPRbin and KET, different SNP were significantly associated with KET and FPR genomically.

Conclusions

Phenotypically, we detected strong associations between first test-day FPR and KET, as increasing KET incidences were significantly associated with higher FPR, and vice versa. In addition, we estimated moderate genetic correlations between KET and FPR using pedigree- or genomic-based relationship matrices. Results lead us to suggest the utilization of FPR from the first test-day as an indicator for genetic KET predictions. Heritabilities for KET, FPRgauss, and FPRbin were very similar. Interestingly, for KET the estimate was slightly higher when modeling was based on the pedigree-based relationship matrix, but for FPRbin, higher estimates were achieved using the GRM. For FPRgauss, heritabilities were almost identical for both pedigree and genomic relationship matrices. Genomically, different SNP significantly contributed to KET and FPR, indicating different genetic mechanisms for both traits. We identified potential candidate genes being associated with diabetes and lipid metabolism, and which are involved in KET progression. Also, significantly associated SNP for FPR were in close distance to lipid and energy metabolism genes, influencing the state of NEB in early lactation.

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CHAPTER 3

Genetic and nongenetic profiling of milk β-hydroxybutyrate and acetone and their associations with ketosis in Holstein cows

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Abstract

Ketosis is a metabolic disorder of increasing importance in high-yielding dairy cows, but accurate population-wide binary health trait recording is difficult to implement. Against this background, proper Gaussian indicator traits, which can be routinely measured in milk, are needed. Consequently, we focused on the ketone bodies acetone and β-hydroxybutyrate (BHB), measured via Fourier-transform infrared spectroscopy (FTIR) in milk. In the present study, 62,568 Holstein cows from large-scale German co-operator herds were phenotyped for clinical ketosis (KET) according to a veterinarian diagnosis key. A sub-sample of 16,861 cows additionally had first test-day observations for FTIR acetone and BHB. Associations between FTIR acetone and BHB with KET and with test-day traits were studied phenotypically and quantitative genetically. Furthermore, we estimated SNP marker effects for acetone and BHB (application of genome-wide association studies) based on 40,828 SNP markers from 4,384 genotyped cows, and studied potential candidate genes influencing body fat mobilization. Generalized linear mixed models were applied to infer the influence of binary KET on Gaussian-distributed acetone and BHB (definition of an identity link function), and vice versa, such as the influence of acetone and BHB on KET (definition of a logit link function). Additionally, linear models were applied to study associations between BHB, acetone and test-day traits (milk yield, fat percentage, protein percentage, fat-to-protein ratio and somatic cell score) from the first test-day after calving. An increasing KET incidence was statistically significant associated with increasing FTIR acetone and BHB milk concentrations. Acetone and BHB concentrations were positively associated with fat percentage, fat-to-protein ratio and somatic cell score. Bivariate linear animal models were applied to estimate genetic (co)variance components for KET, acetone, BHB and test-day traits within parities 1 to 3, and considering all parities simultaneously in repeatability models. Pedigree-based heritabilities were quite small (i.e., in the range from 0.01 in parity 3 to 0.07 in parity 1 for acetone, and from 0.03 - 0.04 for BHB). Heritabilities from repeatability models were 0.05 for acetone, and 0.03 for BHB. Genetic correlations between acetone and BHB were moderate to large within parities and considering all parities simultaneously (0.69 to 0.98). Genetic correlations between acetone and BHB with KET from different parities ranged from 0.71 to 0.99. Genetic correlations between acetone across parities, and between BHB across parities, ranged from 0.55 to 0.66. Genetic correlations between KET, acetone, and BHB with fat-to-protein ratio and with fat percentage were large and positive, but negative with milk yield. In genome-wide association studies, we identified SNP on BTA 4, 10, 11, and 29 significantly influencing acetone, and on BTA 1 and 16 significantly influencing BHB. The identified potential candidate genes *NRXN3*,

ACOXL, *BCL2L11*, *HIBADH*, *KCNJ1*, and *PRG4* are involved in lipid and glucose metabolism pathways.

Key words: ketosis, acetone, β-hydroxybutyrate, genetic parameter, genome-wide associations

Introduction

The period of negative energy balance after calving implies an imbalance between glucose supply and glucose requirement. In consequence, adipose tissue is mobilized and fatty acids are converted to ketone bodies, especially to acetone and BHB (David Baird, 1982). From a physiological perspective, ketones are fuel for the brain, the heart and for muscle activities, but excessive amounts of ketone body levels cause the metabolic disorder ketosis (**KET**). In the context of farm economy, KET implies substantial economic losses due to effect on other diseases, reduced milk production in the ongoing lactation, and veterinary costs (Berg et al., 2002; Mostert et al., 2018). In most of the genetic and genomic studies, putative clinical cases of binary KET (producer records for either healthy or diseased cows) were considered because clinical cases are easier to detect than subclinical cases without any visible signs (Parker Gaddis et al., 2018). Nevertheless, additional consideration of subclinical KET cases in genetic and genomic analyses might contribute to improved prediction accuracies of genetic parameters, due to higher incidences. Incidences for clinical KET were lower than 2%, but a large number of cows were diagnosed for subclinical KET (up to 47%; Berge and Vertenten, 2014; Vanholder et al., 2015). Furthermore, subclinical KET was strongly associated with the occurrence of cost-intensive diseases and with decreased milk content (Suthar et al., 2013). However, for the detection of subclinical KET, detailed recording of proper traits, such as ketone body concentrations (acetone or BHB) in blood or in milk, is imperative (van Knegsel et al., 2010).

Phenotypic correlations between blood and milk ketone body concentrations were very strong for acetone (0.96), and moderate for BHB (0.66; Enjalbert et al., 2001). The phenotypic correlation between milk acetone and milk BHB was 0.68 (Enjalbert et al., 2001). Santschi et al. (2016) evaluated Fourier-transform infrared spectroscopy (**FTIR**) for the detection of ketone bodies in milk. Fourier-transform infrared spectroscopy is a cost-efficient, fast, and reliable alternative to blood ketone analysis and can be implemented in the routine process of monthly milk recording (Santschi et al., 2016). Grelet et al. (2016) used FTIR for the prediction of milk BHB and acetone concentrations. Prediction accuracies were quite large with 71% and 73%, respectively.

With regard to quantitative genetic studies in Holstein cows, the genetic correlation between producer-recorded KET and first test-day milk FTIR measurements for BHB was 0.70

(Koeck et al., 2016). Lee et al. (2016) estimated heritabilities for FTIR milk BHB and acetone in parities 1 to 3 in Holstein cows. Early-lactation heritability estimates ranged between 0.04 and 0.10 for BHB and from 0.05 to 0.18 for acetone, depending on the lactation number. Häggman et al. (2019) also estimated low heritabilities of 0.07 for both milk BHB traits with linear models, and of 0.12 with threshold models on the underlying liability scale. Weigel et al. (2017) used pedigree and SNP marker data and estimated a heritability of 0.07 for blood BHB. Heritability estimates for binary KET were small in the range from 0.01 to 0.08 (Belay et al., 2017; Parker Gaddis et al., 2018; Freebern et al., 2020).

In molecular genetic analyses, Kroezen et al. (2018) focused on the detection of potential candidate genes for producer-recorded KET and identified 6 genes influencing the lipid, ketogenic and glucose metabolism. Kroezen et al. (2018) applied a case-control design for binary KET, but they also discussed the reduced statistical power of such a design for Gaussian-distributed BHB. Freebern et al. (2020) applied a GWAS and fine mapping to identify potential candidate genes related to disease traits in Holstein cattle. They identified one important segment (including the *DGAT1* gene) on BTA 14 for KET. A first GWAS for FTIR BHB in milk was conducted by Nayeri et al. (2019). They identified significantly associated SNP markers on BTA 6, 14, and 20. The identified SNP on BTA 14 was located within the *DGAT1* gene. The *DGAT1* is a candidate gene influencing inflammatory response and lipid metabolism in dairy cattle (Mach et al., 2012).

So far, a few studies separately addressed physiological, quantitative genetics, and genomic associations for BHB, acetone and KET. However, for a deeper understanding, it seems to be imperative to study physiological mechanisms on different scales (i.e., phenotypically, genetically, and genomically) simultaneously using the same cow data set. Consequently, the aim of this study was to infer statistic, quantitative genetic and genomic associations between binary clinical KET and first test-day FTIR milk acetone and BHB concentrations in Holstein cows. Further aim was to study associations between acetone, BHB and KET with test-day traits from the very early-lactation period. Against this background, we applied generalized linear mixed models to study phenotypic trait relationships, we estimated genetic (co)variance components, we performed GWAS based on SNP marker data for acetone and BHB in milk, and we identified potential candidate genes influencing metabolic pathways.

Materials and Methods

Cow traits

The present study considered 65,777 Holstein cows from parities 1 to 3, kept in 62 large-scale co-operator herds from the German federal states of Mecklenburg-West Pommerania and Brandenburg. Calving ages ranged from 20 to 40 mo in parity 1, from 30 to 56 mo in parity 2, and from 44 to 75 mo in parity 3. First test-day records after calving included milk yield, fat percentage, protein percentage, fat-to-protein ratio (**FPR**), SCS [transformed somatic cell count according to Ali and Shook (1980)], as well as acetone and BHB milk concentrations. The interval for the first official test-day after calving comprised DIM 5 to 42. Acetone and BHB milk concentrations (in m*M*) were determined using FTIR (Foss Analytical, Hillerød, Denmark). All test-day traits followed a Gaussian distribution, based on residual analyses according to Villemereuil (2018). Veterinarians and herd manager phenotyped the cows for clinical KET based on a central disease diagnosis key (Stock et al., 2013). According to the recording guidelines, a KET score = 1 for diseased cows was assigned for cows with at least one diagnosis for fever and increased content of ketone bodies in blood or urine (measured via handheld ketometers or urine test strips) in a 6-wk interval after calving. Hence, a score of 0 was assigned for healthy cows without any KET entry during this period. The number of Holstein cows and herds with acetone and BHB measurements and with clinical KET observations are shown in Table 3.1. The descriptive statistics for first test-day milk traits including acetone and BHB milk concentrations by parity are shown in Table 3.2.

Table 3.1. Number of Holstein cows (no. of herds in parentheses) with acetone and β-hydroxybutyrate (BHB) measurements and diagnoses for clinical ketosis.

	Parity			
Trait	1	2	3	No. of records[1]
Acetone, BHB	8,965 (32)	6,399 (25)	4,289 (24)	19,653 (32)
Ketosis				
Healthy	35,842 (36)	32,889 (47)	22,199 (49)	90,930 (52)
Diseased	491 (36)	761 (47)	921 (49)	2,173 (52)
Ketosis, acetone, BHB	5,874 (18)	5,604 (19)	3,791 (20)	15,269 (22)

[1]No. of observations from all 3 parities.

Table 3.2. Descriptive statistics for first test-day milk traits including acetone and β-hydroxybutyrate concentrations in parities 1, 2, and 3.

Trait	Parity	Mean	SD	Minimum	Maximum
Milk yield (kg)	1	28.18	6.67	2.10	51.70
	2	38.40	8.13	3.00	62.40
	3	40.41	8.74	2.30	68.00
Fat (%)	1	4.20	0.81	1.61	10.06
	2	4.34	0.82	1.83	10.34
	3	4.43	0.89	1.60	9.52
Protein (%)	1	3.32	0.36	2.13	6.40
	2	3.34	0.39	2.23	5.02
	3	3.31	0.39	2.30	6.23
Fat-to-protein ratio	1	1.27	0.23	0.37	3.69
	2	1.30	0.23	0.50	3.26
	3	1.34	0.26	0.46	3.28
Somatic cell score	1	2.75	1.78	-2.06	9.64
	2	2.17	1.89	-3.64	9.64
	3	2.49	2.08	-1.64	9.64
Acetone (m*M*)	1	0.05	0.10	0	2.05
	2	0.05	0.09	0	1.54
	3	0.06	0.12	0	2.89
β-hydroxybutyrate (m*M*)	1	0.01	0.05	0	1.61
	2	0.02	0.06	0	1.80
	3	0.02	0.07	0	1.20

Genotypes

A subset of 858 cows was genotyped with the Illumina BovineSNP50 v2 BeadChip (Illumina Inc., San Diego, CA), and 3,526 cows were genotyped with the EuroGenomics 10K chip (Illumina Inc., San Diego, CA). Genotyping was accomplished at the end of the project, with focus on first parity cows, implying that most of the genotyped cows had no phenotypic records for later lactations. Low-density 10K genotypes were imputed by the project partner VIT (Vereinigte Informationssysteme Tierhaltung w.V, Verden, Germany) to the 50K panel, applying the imputation design as described by Segelke et al. (2012). The SNP data set

considered 45,613 SNP from 4,384 genotyped cows with phenotypic records for acetone and BHB. Quality control of the genotype data was performed using the software package PLINK (Purcell et al., 2007). The SNP with more than 1% missing genotype data, with a minor allele frequency lower than 5%, and deviation from Hardy-Weinberg equilibrium ($P < 10^{-8}$) were discarded. Thus, 40,828 SNP from the 4,384 cows were available for genomic studies.

Statistical Models

Statistical associations between clinical ketosis, acetone and β-hydroxybutyrate concentrations

Generalized linear mixed models (**GLMM**) with an identity link function for Gaussian-distributed traits were applied to analyze the effect of clinical KET on the acetone and BHB concentrations from the first official test-day. In this regard, the Glimmix procedure as implemented in SAS (version 9.4, SAS Institute Inc., Cary, NC), was used. Associations were studied for parities 1 to 3 using a repeatability model. In this regard, we considered the cows with measurements for all 3 traits KET, acetone and BHB (Table 3.1). The corresponding statistical model [1] was defined as follows:

$$y_{ijklmno} = \varphi + Herd_i + CYear_j + DIM_k + CMon_l + CAge_m + KET \times Parity_n + Cow_o + e_{ijklmno} \quad [1]$$

where $y_{ijklmno}$ = first test-day observation for acetone or BHB; φ = overall mean; $Herd_i$ = fixed herd effect; $CYear_j$ = fixed effect of calving year (2015-2017); DIM_k = linear regression on DIM; $CMon_l$ = fixed effect of calving month (12 months); $CAge_m$ = linear regression on calving age; $KET \times Parity_n$ = combined fixed effect of clinical KET (healthy or diseased) and parity (1, 2, or 3); Cow_o = random cow effect due to repeated measurements from different parities; $e_{ijklmno}$ = random residual effect.

Model [2] was applied to analyze the effect of first test-day acetone and BHB concentrations on the disease probability for KET. In this regard, we used a GLMM with a logit link function for binary KET. Model [2] was:

$$\text{logit}(\pi) = \log[\pi_{rstuvwxy}/(1-\pi_{rstuvwxy})] = \varphi + Herd_r + CYear_s + DIM_t + CMon_u + CAge_v + Parity_w + ACEcl_x \text{ or } BHBcl_x + Cow_y \quad [2]$$

where $\pi_{rstuvwxy}$ = probability of a KET occurrence; $Parity_w$ = fixed parity effect; $ACEcl_x$ or $BHBcl_x$ = fixed effect of acetone (2 classes: < 0.15 m*M*; ≥ 0.15 m*M*) or BHB concentration [2 classes: < 0.10 m*M*; ≥ 0.10 m*M* as suggested by de Roos et al. (2007)]; and further effects as specified in model [1].

Model [3] was defined to study the detailed influence of KET from different diagnosis dates during the early-lactation period on acetone and BHB. Model [3] was:

$$y_{ijklmnop} = \varphi + Herd_i + CYear_j + DIM_k + CMon_l + CAge_m + Parity_n + KET \times Diffdat_o + Cow_p + e_{ijklmnop} \text{ [3]}$$

where $y_{ijklmnop}$ = first test-day records for acetone or BHB concentration; KET × $Diffdat_o$ = combined fixed effect of KET (healthy or diseased) × period between the first test date and the KET diagnosis date (8 classes considering the following periods: 37 - 21 d, 20 - 11 d, 10 - 5 d, 4 - 1 d before the diagnosis date; and 0 - 4 d, 5 - 10 d, 11 - 20 d and 21 - 37 d after the diagnosis date); a dummy class for healthy cows; and further effects as specified in models [1] and [2]. Adjustment of *P*-values for multiple testing was done using the Dunnett statement in the Glimmix procedure (Dunnett, 1955).

Statistical associations between test-day production traits with acetone and β-hydroxybutyrate concentrations

The effects of acetone and BHB on the test-day traits milk yield, fat percentage, protein percentage, FPR and SCS were estimated using a GLMM with an identity link function. The statistical model [4] was:

$$y_{ijklmnop} = \varphi + Herd_i + CYear_j + DIM_k + CMon_l + CAge_m + Parity_n + ACE_o \text{ or } BHB_o + Cow_p + e_{ijklmnop} \text{ [4]}$$

where $y_{ijklmnop}$ = observations for test-day traits, ACE_o or BHB_o = linear regression on the acetone or BHB milk concentration (modeled via at-statements in SAS according to König et al., 2005), respectively, and further effects as specified in model [1] and [2].

Estimation of genetic parameters

Pedigree-based (co)variance components and heritabilities for acetone and BHB were estimated using the AI-REML algorithm as implemented in the DMU software package (Madsen and Jensen, 2013). Heritabilities for acetone and BHB were estimated in single-trait animal models separately for parities 1, 2, and 3. Furthermore, we considered acetone and BHB from all 3 parities in single-trait repeatability models. Genetic statistical modeling considered the same fixed effects as specified in model [1], but we excluded the combined KET × Parity effect. With regard to random effects, we included the random additive-genetic animal effect and the permanent environmental effect in repeatability models. In matrix notation, the genetic statistical model [5] was:

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Zu} + \mathbf{Wpe} + \mathbf{e} \quad [5]$$

where **y** was an observation vector for acetone or BHB; **β** was a vector of fixed effects including same effects as specified in model [1] (without KET × Parity effect, and with parity effect in the repeatability model); **u** was a vector of additive-genetic effects, with $\mathbf{u} \sim N(0, \mathbf{A}\sigma_u^2)$, where **A** was the pedigree relationship matrix considering animals back to the birth year 1941, and σ_u^2 was the additive-genetic variance; **pe** was a vector of permanent environmental effects in the repeatability model, with $\mathbf{pe} \sim N(0, \mathbf{I}\sigma_{pe}^2)$; **e** was a vector of random residual effects, with $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$; **I** being identity matrices for the number of cows and number of observations, respectively; and **X**, **Z**, and **W** were incidence matrices for **β**, **u**, and **pe**, respectively.

Genetic correlations among KET, acetone, BHB, and milk production traits, between acetone from different parities, and between BHB from different parities, were estimated via bivariate linear animal models (within parities) and via bivariate linear repeatability models (including records from different parities simultaneously). Hence, also binary KET was analyzed in bivariate linear-linear models. As proved by Vinson and Kliewer (1976), genetic correlations from bivariate linear and bivariate threshold models are expected to be the same. The model was defined as follows:

$$\begin{bmatrix}\mathbf{y_1}\\ \mathbf{y_2}\end{bmatrix} = \begin{bmatrix}\mathbf{X_1} & 0\\ 0 & \mathbf{X_2}\end{bmatrix}\begin{bmatrix}\boldsymbol{\beta}_1\\ \boldsymbol{\beta}_2\end{bmatrix} + \begin{bmatrix}\mathbf{Z_1} & 0\\ 0 & \mathbf{Z_2}\end{bmatrix}\begin{bmatrix}\mathbf{u_1}\\ \mathbf{u_2}\end{bmatrix} + \begin{bmatrix}\mathbf{W_1} & 0\\ 0 & \mathbf{W_2}\end{bmatrix}\begin{bmatrix}\mathbf{pe_1}\\ \mathbf{pe_2}\end{bmatrix} + \begin{bmatrix}\mathbf{e_1}\\ \mathbf{e_2}\end{bmatrix} \quad [6]$$

where $\mathbf{y_1}$ and $\mathbf{y_2}$ were vectors of observations for the 2 traits; $\mathbf{X}_1$ and $\mathbf{X}_2$ were incidence matrices relating each observation to the fixed effects as specified for the single-trait model [5] in $\boldsymbol{\beta}_1$ and $\boldsymbol{\beta}_2$; $\mathbf{Z}_1$ and $\mathbf{Z}_2$ were incidence matrices relating the random additive-genetic effects of animals in $\mathbf{u_1}$ and $\mathbf{u_2}$ to each observation, $\mathbf{pe_1}$ and $\mathbf{pe_2}$ were the vectors for permanent environmental effects in repeatability models with corresponding incidences matrices $\mathbf{W}_1$ and $\mathbf{W}_2$; and $\mathbf{e_1}$ and $\mathbf{e_2}$ were the vectors for random residual effects for the 2 traits.

Genome-wide association studies

A GWAS for acetone and BHB considering the 4,384 genotyped cows was performed using the software package GCTA (Yang et al., 2011). In this regard, we applied a linear mixed model and we specified the option of leaving one chromosome out (Yang et al., 2014). The corresponding model [7] was:

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Zu} + \mathbf{Ss} + \mathbf{e} \quad [7]$$

where **y** was the vector including records for acetone and BHB; **β** was a vector of fixed effects including the same effects specified in model [5] (with parity); **u** was a vector of polygenic random effects of all SNP excluding those on the chromosome that carries a candidate SNP, with $\mathbf{u} \sim N(0, \mathbf{G}\sigma_u^2)$, with **G** denoting the genetic similarity matrix among individuals, and σ_u^2 the polygenic variance; **s** was the vector for fixed effects of the SNP tested for association coded as 0, 1, or 2 according to the respective allele dosage; **e** was a vector of random residual effects with $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$; and **X**, **Z**, and **S** were incidence matrices for **β**, **u**, and **S**, respectively. A small fraction of genotyped cows had phenotypic records in first lactations. Due to failed convergence when considering repeated measurements from these cows, we ignored the random permanent environmental effect in genomic analyses.

The effective number of independent SNP (N_{SNP} = 29,548) was calculated using the software Genetic type 1 Error Calculator (Li et al., 2012), in order to define the genome-wide significance level according to Bonferroni (**pBF** = 0.05 / N_{SNP} = 1.69e-06). In addition, a less conservative normative significance candidate threshold was used, considering **pCD** = 1e-04 (Kurz et al., 2018). Genes located in a chromosomal window 250 kb upstream or downstream from the significantly associated SNP were detected using Ensembl, release 98, on the basis of the *Bos taurus* ARS1.2 genome assembly (Zerbino et al., 2018).

Results and Discussion

Statistical associations between clinical ketosis with acetone and β-hydroxybutyrate

A clinical KET diagnosis was associated with significantly higher first test-day acetone and BHB milk concentrations ($P < 0.001$, model [1]). The acetone and BHB concentrations for cows with a KET diagnosis was at least 2-fold higher compared to estimates for healthy cows. This was the case for all 3 lactations (Table 3.3). Nevertheless, the parity effect was highly significant ($P < 0.001$), showing a decrease of least squares means for acetone and BHB with increasing lactation number. Inferred associations reflect the physiological processes. In the stage of a negative energy balance and especially for cows with a KET diagnosis, fatty acid oxidation from body fat depots and ongoing ketone production in the liver implies an accumulation of ketone bodies in blood and in milk (Andersson and Lundström, 1984). Results from Table 3.3 clearly indicate that FTIR milk ketones are useful indicators for the detection of clinical KET in early lactation. Similarly, using the same threshold for acetone (≥ 0.15 m*M*) and BHB (≥ 0.10 m*M*), de Roos et al. (2007) recommended FTIR acetone and BHB for the early detection of subclinical KET cases. Grelet et al. (2016) focused on cross-validations for

KET detection, and could clearly distinguish between diseased and healthy cows based on FTIR spectrometry data.

Table 3.3. Least squares means (corresponding SE in parentheses) of first test-day acetone and β-hydroxybutyrate concentrations in milk (m*M*) for cows with the absence (0) or the presence (1) of a ketosis diagnosis.

		Ketosis diagnosis	
Trait	Parity	0	1
Acetone	1	0.08 (0.00)[a]	0.15 (0.01)[b]
	2	0.03 (0.00)[a]	0.10 (0.01)[b]
	3	< 0.00 (0.00)[a]	0.06 (0.01)[b]
β-hydroxybutyrate	1	0.03 (0.00)[a]	0.06 (0.01)[b]
	2	0.01 (0.00)[a]	0.05 (0.00)[b]
	3	< 0.00 (0.00)[a]	0.04 (0.01)[b]

[a, b] Different superscripts indicate significant differences within parity ($P < 0.001$).

Inversely, we detected a significant ($P < 0.001$) effect of acetone and BHB concentrations on KET incidences (results from model [2]). The probability for a clinical KET diagnosis was generally low, but significantly higher in cows with an acetone concentration above the threshold of 0.15 m*M*, and with a BHB concentration above the threshold of 0.10 m*M*, compared with the respective healthy cow group. Specifically, probabilities for a KET diagnosis were 4-fold higher for the cows allocated to the high acetone concentration (≥ 0.15 m*M*) or to the high BHB concentration (≥ 0.10 m*M*) group.

Due to the possible time lag between calving and first test-day date, a more frequent milk sampling in the critical period after calving could help to detect KET as early as possible. Acetone and BHB concentrations were significantly increased for KET diagnosed cows in the period from 37 to 20 d before the diagnosis date (Table 3.4), indicating that cows are suffering from elevated ketone body concentrations long time before clinical signs are obvious. Accordingly, van der Drift et al. (2012) detected the highest prevalence for hyperketonemia (defined as plasma BHB 1,200 µmol/L) in the very early lactation between 5 and 10 DIM. Results suggest utilization of earliest test-day milk samples to determine milk ketone bodies, and to consider results for acetone and BHB levels in preventive health management strategies. Addressing the period after a KET diagnosis, acetone substantially increased within the period of 20 to 37 d, and BHB within the period of 11 to 20 d (Table 3.4). Klein et al. (2019) studied

time lagged effects of KET diagnoses on KET indicator traits, and they confirmed the importance of the period comprising 20 to 30 d for metabolic disorders.

Table 3.4. Least squares means (with corresponding SE in parentheses) of first test-day acetone and β-hydroxybutyrate (BHB) milk concentrations (m*M*) for ketosis healthy cows and ketosis diseased cows (considered time periods: test-day dates before or after the ketosis diagnosis date).

			Diseased cows			
Trait	Healthy cows	Before or after diagnosis	37 to 21 d	20 to 11 d	10 to 5 d	4 to 0 d[1]
Acetone	0.03 (0.00)	Before	0.15 (0.04)*	0.16 (0.02)***	0.15 (0.02)***	0.29 (0.02)***
		After	0.07 (0.01)**	0.08 (0.01)***	0.07 (0.01)*	0.18 (0.02)***
BHB	0.01 (0.00)	Before	0.10 (0.02)**	0.06 (0.01)**	0.08 (0.01)***	0.15 (0.01)***
		After	0.02 (0.01)	0.04 (0.01)***	0.05 (0.01)***	0.11 (0.01)***

[1] 4 to 1 d before the ketosis diagnosis date, 0 to 4 d after the ketosis diagnosis date.

*$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; adjusted *P*-values (Dunnett adjustment) for least squares means differences between ketosis diseased and ketosis healthy cows.

Statistical associations between acetone and β-hydroxybutyrate with test-day production traits

Milk BHB and acetone significantly ($P \leq 0.001$) affected milk yield (Figure 3.1), fat and protein percentage (Figure 3.2), FPR, and SCS (Figure 3.3). In agreement with the results from the present study, Chandler et al. (2018) and Santschi et al. (2016) identified a milk yield and protein percentage decline with increasing BHB and acetone concentrations. The decline in milk yield and protein percentage is due to impaired production processes requiring energy. Osorio et al. (2016) identified that especially protein synthesis in the mammary gland is a highly energy demanding process. Thus, due to energy deficiency in early lactation, protein synthesis might be restricted. As expected, fat percentage and FPR significantly increased with increasing ketone body concentrations in milk. The fat percentage and FPR increase is due to the strong body fat mobilization in the period of a negative energy balance, and the utilization of BHB for fatty acid synthesis in the mammary gland (Dodds et al., 1981). SCS increased with increasing BHB and acetone concentration. A possible explanation addresses immune responses in the mammary gland (i.e., an increasing production of somatic cells in response to excessive ketone body levels; Hillreiner et al., 2016). Santschi et al. (2016) created 3 classes for FTIR BHB

concentrations (< 0.15 m*M*, 0.15 - 0.19 m*M*, and ≥ 0.20 m*M*). Holstein cows allocated to the extreme group with a BHB concentration ≥ 0.20 m*M* had substantial lower milk yield and protein percentage, higher fat percentage, FPR, and SCS compared with cows from the 2 other groups. Furthermore, we detected a significant association between acetone and BHB milk concentrations. The BHB concentration increased linear with increasing acetone concentration in milk (not shown).

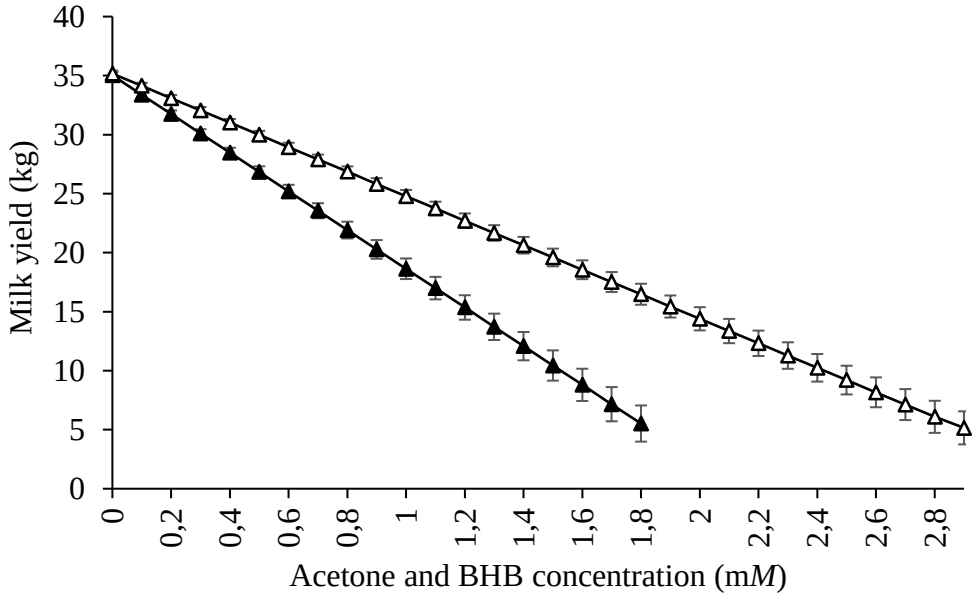

Figure 3.1. Least squares means with corresponding SE for first test-day milk yield in dependency of acetone (Δ, SE = 0.26-1.40) and β-hydroxybutyrate (BHB, ▲, SE = 0.03-1.53) milk concentrations.

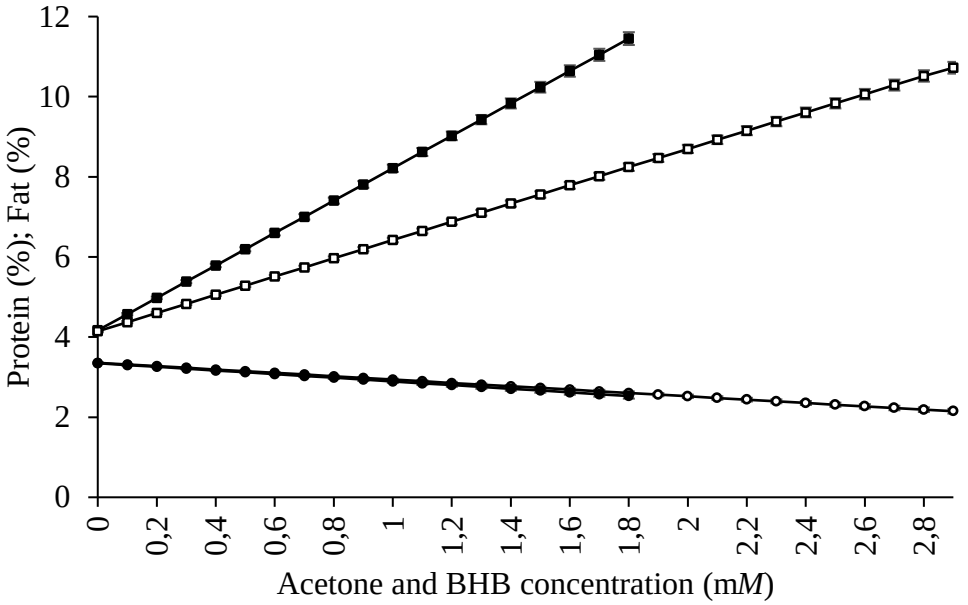

Figure 3.2. Least squares means with corresponding SE for first test-day protein percentage (○, SE = 0.01-0.06; ●, SE = 0.01-0.07) and fat percentage (□, SE = 0.03-0.05; ■, SE = 0.03-0.06) in dependency of acetone (white symbols) and β-hydroxybutyrate (BHB, black symbols) milk concentrations.

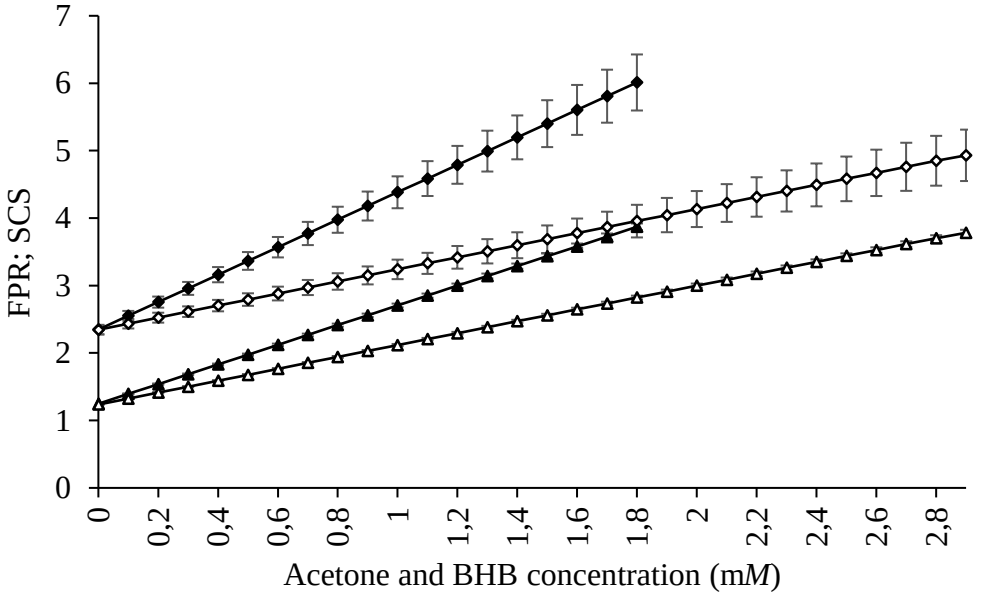

Figure 3.3. Least squares means with corresponding SE for first test-day fat-to-protein ratio (FPR; Δ, SE = 0.01-0.04; ▲, SE = 0.01-0.05) and somatic cell score (SCS, ◊, SE = 0.07-0.28; ♦, SE = 0.07-0.32) in dependency of acetone (white symbols) and β-hydroxybutyrate (BHB, black symbols) milk concentrations.

Heritabilities for acetone and β-hydroxybutyrate and genetic correlations among acetone, β-hydroxybutyrate, ketosis and test-day production traits

Heritabilities for milk ketone body concentrations from the first test-day are shown in Table 3.5. Heritabilities for acetone were 0.07 (SE = 0.02) in first and second lactations, 0.01 (SE = 0.01) in the third lactation, and 0.05 (SE = 0.01) from the repeatability model. For BHB, heritabilities were 0.04 (SE = 0.01), 0.04 (SE = 0.02) and 0.03 (SE = 0.02) in lactations 1, 2 and 3, respectively. The heritability for BHB from the repeatability model was 0.03 (SE = 0.01). Due to the small permanent environmental variance component, repeatabilities for acetone and for BHB were smaller than 10%. Repeatabilities for same test-day production traits from different lactations were generally small, but quite large for different test-days within lactation (Gernand et al., 2007). In consequence, in most of the official national genetic evaluations, same test-day traits from different lactations are considered as different traits in multiple-trait models.

The very small heritabilities for acetone and BHB in third-parity cows are due to a substantial increase of the residual variance component, especially for acetone. Nevertheless, the quite small heritabilities are in agreement with estimates by Weigel et al. (2017) and Häggman et al. (2019). Weigel et al. (2017) used blood BHB concentrations between d 5 and 18 postpartum, measured with a handheld blood ketone meter in 1,453 Holstein cows. They defined 3 different BHB traits: BHB_{MAX} [maximum BHB blood concentration (mmol/l) from twice weekly sampling between 5 - 18 d postpartum], BHB_{SQRT} (square root of BHB_{MAX}) and BHB_{BIN} (binary scale, 1 if $BHB_{MAX} \geq 1.2$ mmol/l; 0 if $BHB_{MAX} < 1.2$ mmol/l). Heritabilities for the 3 traits ranged between 0.06 (SE = 0.04) and 0.07 (SE = 0.05). Häggman et al. (2019) grouped BHB measurements into 3 classes and estimated heritabilities with linear animal models. Heritabilities for BHB in the different classes ranged from 0.07 to 0.09. Lee et al. (2016) focused on FTIR milk ketones from different lactation stages after calving (30 DIM, 150 DIM, 250 DIM) in parities 1 to 3. According to our results, heritability estimates for acetone and BHB slightly decreased with increasing parity. Satoła and Ptak (2019) detected larger permanent environmental and residual variances in later lactations than in first-parity cows, indicating stronger nongenetic influence on trait variability with aging. The quite large heritability estimates up to 0.36 for acetone and 0.14 for BHB in the studies by Lee et al. (2016) and Ranaraja et al. (2018) are possibly due to pronounced phenotypic trait variations and a smaller residual component in later lactation stages.

Genetic correlations between acetone and BHB with KET were in the range from 0.71 to 0.99 for the different parities, with moderate to large SE (0.07 - 0.27) (Table 3.5). The large

genetic correlations per parity between clinical KET and ketone concentrations suggest consideration of milk acetone and milk BHB in health selection indices to improve resistance against metabolic disorders (König and May, 2018). Genetic correlations between acetone and BHB with KET from the repeatability model were lower and ranged from 0.22 to 0.37 (SE = 0.12 - 0.14; Table 3.5). Koeck et al. (2016) defined different traits for FTIR milk BHB based on means, averages and SD from repeated measurement data. Genetic correlations between the different BHB definitions with producer-recorded KET ranged from 0.64 to 0.82. In contrast, Belay et al. (2017) estimated low to moderate genetic correlations between KET and BHB traits in the range from 0.18 and 0.47, whereas correlations decreased with DIM. In the present study, genetic correlations between acetone measurements from different parities and between BHB measurements from different parities ranged from 0.55 to 0.66. The moderate to large correlations suggest early selection on FTIR profiles in first-parity cows. Nevertheless, SE of genetic correlation estimates were quite large (0.18 - 0.30). Genetic correlations between acetone and BHB for the different parities were in the range from 0.69 to 0.98 (SE = 0.13 - 0.33).

Table 3.5. Pedigree-based heritabilities (h^2; SE in parentheses), additive-genetic (σ^2_a) ($\times 10^{-3}$), permanent environmental (σ^2_{pe}) ($\times 10^{-3}$) and residual (σ^2_e) ($\times 10^{-3}$) variances for acetone and β-hydroxybutyrate milk concentrations, and their genetic correlations (r_g) with clinical ketosis (KET; SE in parentheses).

			Variance			
Trait	Parity	h^2	σ^2_a	σ^2_{pe}	σ^2_e	r_g with KET
Acetone	1	0.07 (0.02)	0.71		8.81	0.82 (0.17)
	2	0.07 (0.02)	0.45		6.07	0.96 (0.07)
	3	0.01 (0.01)	0.20		13.98	0.99 (0.27)
	All[1]	0.05 (0.01)	0.40	0.36	8.80	0.22 (0.12)
β-hydroxybutyrate	1	0.04 (0.01)	0.09		2.40	0.71 (0.21)
	2	0.04 (0.02)	0.13		3.00	0.99 (0.10)
	3	0.03 (0.02)	0.13		4.23	0.71 (0.15)
	All	0.03 (0.01)	0.09	< 0.00	3.04	0.37 (0.14)

[1]All = consideration of records from all 3 parities simultaneously in a repeatability model.

Genetic correlations between acetone, BHB, KET with test-day traits (Table 3.6) reflect the phenotypic associations. First test-day milk yield and protein percentage were negatively correlated with acetone, BHB and KET, whereas correlations between fat percentage, FPR, and SCS with acetone, BHB, and KET were positive (Table 3.6). This was the case for both modeling strategies (i.e., the analyses within parities or repeated measurement applications).

Table 3.6. Genetic correlations between clinical ketosis (KET), acetone, and β-hydroxybutyrate (BHB) milk concentrations with first test-day traits from parities 1, 2, and 3 (corresponding SE in parentheses).

Trait	Parity	Acetone	BHB	KET
Milk yield (kg)	1	-0.51 (0.06)	-0.57 (0.07)	-0.52 (0.08)
	2	-0.73 (0.05)	-0.67 (0.08)	-0.74 (0.04)
	3	-0.89 (0.06)	-0.84 (0.08)	-0.82 (0.03)
	All[1]	-0.11 (0.10)	-0.18 (0.12)	-0.08 (0.06)
Fat (%)	1	0.78 (0.03)	0.86 (0.03)	0.68 (0.08)
	2	0.85 (0.02)	0.85 (0.03)	0.70 (0.06)
	3	0.91 (0.03)	0.91 (0.02)	0.74 (0.05)
	All	0.42 (0.08)	0.65 (0.08)	0.15 (0.06)
Protein (%)	1	-0.67 (0.06)	-0.48 (0.10)	-0.67 (0.09)
	2	-0.74 (0.06)	-0.60 (0.10)	-0.59 (0.10)
	3	-0.78 (0.10)	-0.63 (0.13)	-0.53 (0.07)
	All	-0.54 (0.08)	-0.32 (0.10)	-0.37 (0.04)
Fat-to-protein ratio	1	0.88 (0.02)	0.91 (0.02)	0.86 (0.06)
	2	0.92 (0.02)	0.92 (0.02)	0.84 (0.04)
	3	0.92 (0.02)	0.92 (0.02)	0.83 (0.04)
	All	0.73 (0.06)	0.82 (0.06)	0.39 (0.05)
Somatic cell score	1	0.38 (0.10)	0.61 (0.11)	0.42 (0.14)
	2	0.33 (0.14)	0.46 (0.17)	0.39 (0.13)
	3	0.99 (0.22)	0.67 (0.21)	0.04 (0.11)
	All	0.13 (0.11)	0.24 (0.13)	0.12 (0.07)

[1]All = consideration of records from all 3 parities simultaneously in a repeatability model.

The negative genetic correlations between acetone and BHB with milk yield and protein percentage are in line with results from previous studies (Koeck et al., 2013; Lee et al., 2016; Belay et al., 2017). The positive genetic correlations between fat percentage and FPR with milk ketone bodies support results by Lee et al. (2016), Ranaraja et al. (2018) and Mehtiö et al. (2020). The positive genetic correlations between SCS with milk ketone body concentrations or with KET indicate that KET and mastitis are genetically related in the early-lactation period (Heringstad et al., 2005). Again, correlations from the repeatability model were smaller compared to estimates within lactation. The generally lower genetic correlations from the repeatability model lead us to conclude that consideration of repeated measurements from different lactations with very low repeatabilities might bias genetic parameter estimates.

Genome-wide association studies for acetone and β-hydroxybutyrate

We identified 9 SNP significantly associated with acetone and BHB FTIR concentrations in milk according to the candidate threshold (Supplemental Table S3.1, https://doi.org/10.3168/jds.2019-18339). The $-\log_{10}$ *P*-values of the tested SNP are shown in Figure 3.4 (for acetone) and in Figure 3.5 (for BHB) as Manhattan plots. In total, 28 positional candidate genes in the interval of 250 kb surrounding the significant SNP (Supplemental Table S3.2, https://doi.org/10.3168/jds.2019-18339) were retrieved from the Ensembl database (Zerbino et al., 2018). In the following, we focused on inferring potential candidate genes possibly related to acetone or BHB due to their functional or physiological background.

Acetone

Significantly associated SNP were identified on BTA 4, 10, 11, and 29 (Figure 3.4, Supplemental Table S3.1). The SNP rs29021343 on BTA 10 (90,924,914 bp) with the highest $-\log_{10}$ *P*-value is located in the gene neurexin 3 (*NRXN3*, 90,495,258 - 91,099,930 bp). Zhang et al. (2017) applied a genetic pleiotropic conditional false discovery rate approach and discovered loci associated with obesity and type-2 diabetes. In their study, a SNP variant associated with obesity and diabetes in humans was located in the *NRXN3* gene. Because diabetes and obesity are strongly related to KET and energy deficiency in early lactation (Hayirli, 2006), *NRXN3* is a potential candidate gene for metabolic disorders. As described by Hayirli (2006), cows with clinical KET have a low responsiveness to insulin due to ketoacidosis. Ketosis symptoms and associated physiological mechanisms in cows show similarities to diabetes in humans. In this regard, Lucy (2004) identified similar characteristics when comparing diabetic states in humans and low-insulin states postpartum in cows (e.g., low

insulin levels or insulin resistance). Insulin inhibits lipolysis, regulates ketone body output from the liver, and enhances ketone utilization in peripheral tissues (Fukao et al., 2004). In causality, low insulin levels initiate adipocyte lipolysis and liver accumulation with fatty acids.

The SNP rs110395452 on BTA 11 (1,283,185 bp) is localized in the gene acyl-CoA oxidase like (*ACOXL*, 1,237,943 - 1,576,166 bp), and also located in close chromosomal distance to the gene BCL2 like 11 (*BCL2L11*, 1,187,496 - 1,233,256 bp). According to Fang et al. (2017) and Hasstedt et al. (2013), *ACOXL* and *BCL2L11* are associated with diabetes in humans. Genome-wide expression profiling (Fang et al., 2017) showed significant up-regulation of *BCL2L11* in type-1 diabetes mellitus samples compared with healthy controls. These results suggest an involvement of *BCL2L11* in cell death of islet β-cells in the pathogenesis of diabetes. As reported by Hasstedt et al. (2013), the lipid metabolism gene *ACOXL* contributed to diabetes as well. Also, Hayirli (2006) clearly described the physiological interactions between KET and insulin regulations, and stretched the insulin resistance phenomenon in postpartum dairy cows. The second significantly associated SNP rs109102963 on BTA 11 (75,410,689 bp) is located in the gene kelch like family member 29 (*KLHL29*, 75,240,502 - 75,572,709 bp) and in close chromosomal distance to the gene ATPase family AAA domain containing 2B (*ATAD2B*, 75,091,973 - 75,213,880 bp). Both genes *KLHL29* and *ATAD2B* are putative candidate genes for milk protein composition traits in Chinese Holstein cows (Zhou et al., 2019).

The gene 3-hydroxyisobutyrate dehydrogenase (*HIBADH*, 68,473,794 - 68,614,954 bp) in the defined interval surrounding SNP rs109374730 (68,808,125 bp) on BTA 4 is associated with KET related fatty liver disease and negative energy balance in early-lactation cows (McCarthy et al., 2010; Sejersen et al., 2012). McCarthy et al. (2010) indicated that *HIBADH* was downregulated in cows with severe negative energy balance. Sejersen et al. (2012) described significant influence of fatty liver disease on *HIBADH* expression. Cows with high accumulation of liver triglycerides indicating fatty liver disease showed a significant downregulation of *HIBADH* in the liver. Additionally, *HIBADH* was correlated with plasma BHB and bilirubin. Apart from that, downregulated *HIBADH* in humans' adipose tissue was associated with insulin resistance (Wiklund et al., 2016). Furthermore, the gene *HIBADH* regulates fatty acid transport, enhances lipid accumulation and was increased in mice and human with diabetes (Jang et al., 2016).

On BTA 29, the gene potassium voltage-gated channel subfamily J member 1 (*KCNJ1*, 32,214,439 - 32,244,810 bp) is located in close chromosomal distance to the significantly associated SNP rs41651011 (32,373,604 bp). Karnes et al. (2013) reported that *KCNJ1* is

associated with abnormal blood sugar levels and diabetes. Alterations of blood sugar levels and a state of insulin resistance are characteristics for the period around calving, because insulin depended transfer of glucose into the mammary gland is prioritized (De Koster and Opsomer, 2013).

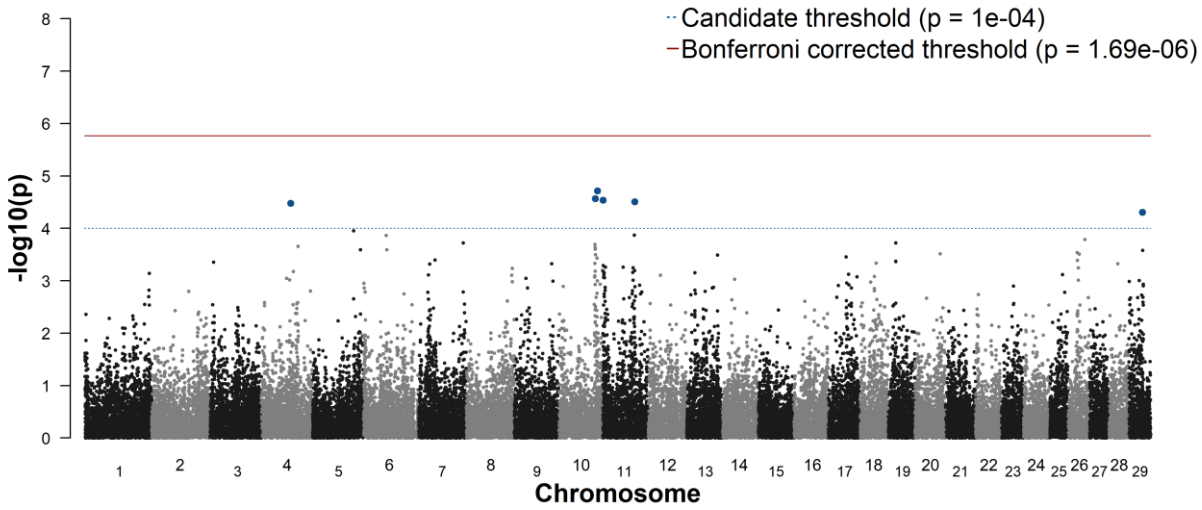

Figure 3.4. Manhattan plot for -$\log_{10}$ *P*-values of SNP effects for first test-day milk acetone concentration (m*M*) in Holstein cows.

β-hydroxybutyrate

Three potential candidate SNP were identified for the FTIR BHB concentration (Figure 3.5, Supplemental Table S3.1). The marker rs109480845 (67,532,667 bp) with the strongest association is located on BTA 16, in close chromosomal distance to the gene proteoglycan 4 (*PRG4*, 67,381,599 - 67,398,481 bp). Geyer et al. (2016) identified impact of *PRG4* on the glucose and lipid metabolism. Nahon et al. (2019) described the influence of *PRG4* on the glucose intolerance and fatty liver disease in mice. For the SNP rs109224751 (68,984,620 bp) on BTA 16 and the SNP rs111002696 (47,765,383 bp) on BTA 1, no potential candidate genes explaining metabolic diseases, were identified.

Genome-wide associations based on producer-recorded KET in Holstein cows (Huang et al., 2019) and US Jerseys (Parker Gaddis et al., 2018) detected significant SNP markers on BTA 10 and 11, with annotated potential candidate genes involved in insulin regulation and lipid metabolism. These chromosomal segments were in a distance of approximately 1 Mb to our significantly associated SNP on BTA 10 and 11. Applying a single SNP regression mixed linear model and enrichment analysis, Nayeri et al. (2019) detected significantly associated SNP on BTA 6, 14, and 20. Ongoing pathway analyses inferred associations with the lipid

metabolism and immune functions. In the present study, we did not find signals on BTA 14 indicating the *DGAT1* gene. Accordingly, GWAS in a previous study (Klein et al., 2019) with different statistical approaches suggested significant SNP within *DGAT1* for FPR, but not for KET. The authors concluded that these SNP from the *DGAT1* segment influence milk fat synthesis, but not contribute to the mobilization of body fat depots in the stage of negative energy balance. Tetens et al. (2012) mentioned the narrow correlations between FPR and energy balance indicators, but they did not identify quantitative trait loci affecting both traits simultaneously. Also in their study, *DGAT1* was only relevant for FPR.

The significantly associated SNP and annotated potential candidate genes from different chromosomes suggest a strong polygenic effect on milk acetone and BHB. The number of SNP surpassing the candidate threshold (pCD = 1e-04) was limited (9 SNP) and -$\log_{10}$ *P*-values of significantly associated SNP were quite low. As stated by Goddard et al. (2016), complex traits are more complex than presumed and influenced by thousands of SNP with very small effects. This is also true for the KET indicators acetone and BHB. All SNP effects were too small to be significant when considering the strict Bonferroni corrected *P*-value. McCarthy et al. (2008) proposed to enlarge the sample size to improve the statistical power in GWAS for complex traits with low incidences. Furthermore, cow genotyping with a denser SNP chip (as done by Freebern et al., 2020) might influence significance tests in GWAS.

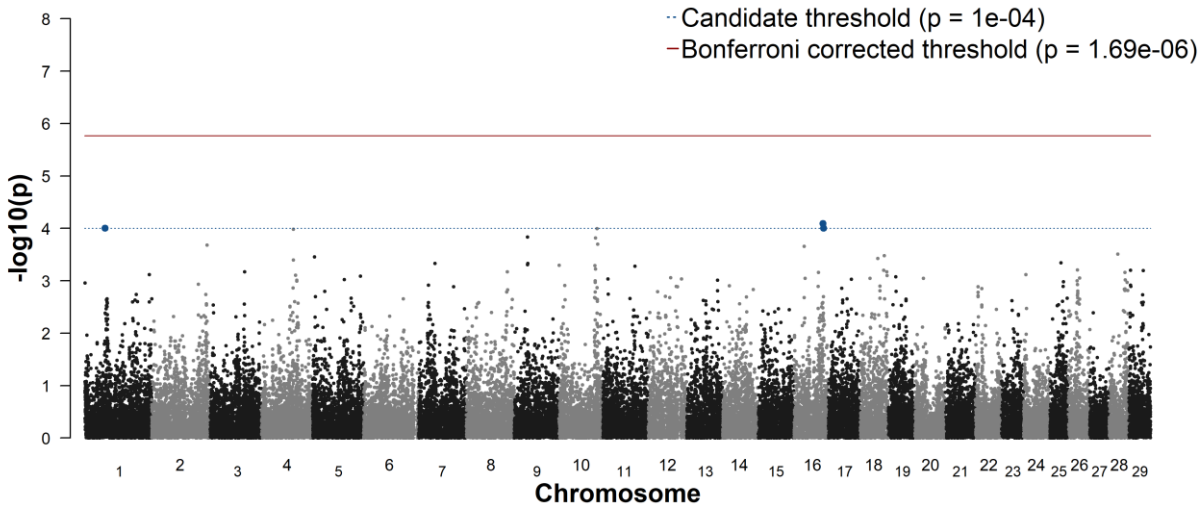

Figure 3.5. Manhattan plot for -$\log_{10}$ *P*-values of SNP effects for first test-day milk β-hydroxybutyrate concentration (m*M*) in Holstein cows.

Conclusions

The strong phenotypic associations between first test-day FTIR acetone and FTIR BHB with KET suggest routinely determination and utilization of ketone bodies in milk, to improve the dairy cow health management. Furthermore, we identified pronounced favorable genetic correlations among KET, FTIR acetone, FTIR BHB, and FPR within parities 1 to 3, and moderate genetic correlations from the repeatability model.

Hence, from a breeding perspective, results suggest consideration of milk ketone bodies in selection indices for metabolic disorders. The strong genetic correlations between FTIR acetone and BHB from first lactation with the respective traits in later lactations indicate the usefulness of early selection in first-parity cows. Pedigree-based heritabilities for acetone and BHB were quite small. Nevertheless, we identified a small number of significantly associated SNP markers on 6 different chromosomes. The identified potential candidate genes in close chromosomal distance, (i.e., *NRXN3*, *ACOXL*, *BCL2L11*, *HIBADH*, *KCNJ1* and *PRG4*) are involved in human diabetes, lipid and glucose metabolism pathways.

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Appendix

Supplemental Tables

Table S3.1. Candidate SNP associated with first test-day acetone and β-hydroxybutyrate (BHB) concentration in milk.

Trait	BTA	SNP	Position (bp)	MAF[1]	SNP effect	*P*-value	-log$_{10}$(*p*)
Acetone	10	rs29021343	90,924,914	0.18	0.01	< 0.001	4.71
	10	rs111009767	86,564,854	0.08	0.02	< 0.001	4.57
	11	rs110395452	1,283,185	0.15	0.01	< 0.001	4.54
	11	rs109102963	75,410,689	0.25	-0.01	< 0.001	4.51
	4	rs109374730	68,808,125	0.14	0.01	< 0.001	4.48
	29	rs41651011	32,373,604	0.50	-0.01	< 0.001	4.31
BHB	16	rs109480845	67,532,667	0.19	0.01	< 0.001	4.09
	1	rs111002696	47,765,383	0.42	-0.01	< 0.001	4.00
	16	rs109224751	68,984,620	0.14	0.01	< 0.001	4.00

[1] MAF = Minor allele frequency.

Table S3.2. Genes[1] located in the interval of 250 kb surrounding associated SNP for first test-day acetone and β-hydroxybutyrate (BHB) in Holstein cows.

Trait	BTA	Position (bp)	Ensembl Gene ID	Gene Name
Acetone	4	68,473,794 - 68,614,954	ENSBTAG00000001036	*HIBADH*
	4	68,797,645 - 68,801,018	ENSBTAG00000020919	*EVX1*
	4	68,842,500 - 68,844,385	ENSBTAG00000014735	*HOXA13*
	10	90,495,258 - 91,099,930	ENSBTAG00000025324	*NRXN3*
	10	86,319,206 - 86,327,460	ENSBTAG00000013983	*ZC2HC1C*
	10	86,332,450 - 86,367,622	ENSBTAG00000016612	*NEK9*
	10	86,374,541 - 86,407,514	ENSBTAG00000005694	*TMED10*
	10	86,488,645 - 86,492,108	ENSBTAG00000004322	*FOS*
	10	86,610,233 - 86,655,611	ENSBTAG00000009451	*JDP2*
	10	86,713,963 - 86,736,092	ENSBTAG00000025405	*BATF*
	10	86,764,090 - 86,898,356	ENSBTAG00000040078	*FLVCR2*
	11	1,187,496 - 1,233,256	ENSBTAG00000024105	*BCL2L11*
	11	1,237,943 - 1,576,166	ENSBTAG00000004297	*ACOXL*
	11	75,091,973 - 75,213,880	ENSBTAG00000017255	*ATAD2B*
	11	75,240,502 - 75,572,709	ENSBTAG00000021969	*KLHL29*
	29	32,051,549 - 32,184,256	ENSBTAG00000008283	*FLI1*
	29	32,214,439 - 32,244,810	ENSBTAG00000000008	*KCNJ1*
	29	32,262,390 - 32,290,687	ENSBTAG00000006902	*KCNJ5*
	29	32,317,429 - 32,318,160	ENSBTAG00000024731	-
	29	32,330,486 - 32,459,266	ENSBTAG00000015905	*ARHGAP32*
BHB	16	67,285,178 - 67,288,013	ENSBTAG00000054031	-
	16	67,381,599 - 67,398,481	ENSBTAG00000011932	*PRG4*
	16	67,448,413 - 67,448,793	ENSBTAG00000047559	*histone H2B type 1-L-like*
	16	67,461,867 - 67,498,396	ENSBTAG00000011946	*ODR4*
	16	67,521,135 - 67,525,396	ENSBTAG00000004176	*PDC*
	16	68,929,292 - 68,991,907	ENSBTAG00000024449	*CENPF*
	16	69,034,474 - 69,223,706	ENSBTAG00000021553	*PTPN14*
	16	69,234,217 - 69,287,670	ENSBTAG00000013166	*SMYD2*

[1] Ensembl release 98 (Zerbino et al., 2018).

CHAPTER 4

Single-step genomic best linear unbiased predictor genetic parameter estimations and genome-wide associations for milk fatty acid profiles, interval from calving to first insemination, and ketosis in Holstein cattle

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Abstract

Milk fatty acids (FA) have been suggested as biomarkers for early-lactation metabolic diseases and for female fertility status. The aim of the present study was to infer associations between FA, the metabolic disorder ketosis (KET) and the interval from calving to first insemination (ICF) genetically and genomically. In this regard, we focused on a single-step genomic BLUP approach, allowing consideration of genotyped and ungenotyped cows simultaneously. The phenotypic data set considered 38,375 first-lactation Holstein cows, kept in 45 large-scale co-operator herds from 2 federal states in Germany. The calving years for these cows were from 2014 to 2017. Concentrations in milk from the first official milk recording test-day for saturated, unsaturated (UFA), monounsaturated (MUFA), polyunsaturated, palmitic, and stearic (C18:0) FA were determined via Fourier-transform infrared spectroscopy. Ketosis was defined as a binary trait according to a veterinarian diagnosis key, considering diagnoses within a 6-wk interval after calving. A subset of 9,786 cows was genotyped for 40,989 SNP marker. Variance components and heritabilities for all Gaussian-distributed FA and for ICF, and for binary KET were estimated by applying single-step genomic BLUP single-trait linear and threshold models, respectively. Genetic correlations were estimated in series of bivariate runs. Genomic breeding values for the single-step genomic BLUP estimations were dependent traits in single-step GWAS. Heritabilities for FA were moderate in the range from 0.09 to 0.20 (standard error = 0.02 - 0.03), but quite small for ICF (0.08, standard error = 0.01) and for KET (0.05 on the underlying liability scale, posterior standard deviation = 0.02). Genetic correlations between KET and UFA, MUFA, C18:0 were large (0.74 to 0.85, posterior standard deviation = 0.14 - 0.19), and low positive between KET and ICF (0.17, posterior standard deviation = 0.22). Genetic correlations between UFA, MUFA, and C18:0 with ICF ranged from 0.34 to 0.46 (standard error = 0.12). In single-step GWAS, we identified a large proportion of overlapping genomic regions for the different FA, especially for UFA and MUFA, and for saturated and palmitic FA. One identical significantly associated SNP was identified for C18:0 and KET on BTA 15. However, there was no genomic segment simultaneously significantly affecting all trait categories ICF, FA, and KET. Nevertheless, some of the annotated potential candidate genes *DGKA*, *IGFBP4*, and *CXCL8* play a role in lipid metabolism and fertility mechanisms, and influence production diseases in early lactation. Genetic and genomic associations indicate that Fourier-transform infrared spectroscopy FA concentrations in milk from the first official test-day are valuable predictors for KET and for ICF.

Key words: ketosis, FTIR milk fatty acids, fertility, single-step genomic evaluation, candidate genes

Introduction

Phenotypically, ketosis (**KET**) as well as milk fatty acid (**FA**) profiles were associated with reproductive performance in dairy cows (Stádník et al., 2015; Rutherford et al., 2016). Holstein cows with increased levels of blood BHB (KET indicator) showed a significantly delayed interval from calving to first oestrus and interval from calving to first insemination (**ICF**; Rutherford et al., 2016). The ICF is a female fertility interval trait mainly indicating the start of the cycle activity after calving, and independent from further effects such as service sire or semen quality, which are relevant for female fertility traits reflecting a successful insemination. In this regard, Lucy (2019) elaborated the physiological background explaining associations between FA concentrations and cycle activity, and they emphasized that a negative energy balance (**NEB**) depresses follicle growth and follicle functionality. Accordingly, in the stage of a NEB, the increase of circulating ketone bodies, especially of BHB, and of FA, caused impaired oocyte fertility and immune dysfunctions with detrimental impact on uterine recovery (Wathes et al., 2009). Stádník et al. (2015) identified favorable associations between concentrations of milk monounsaturated FA (**MUFA**) and female fertility interval traits, whereas opposite observations were made for saturated FA (**SFA**). In addition, with regard to genetic evaluation improvements, Gernand and König (2017) emphasized the positive genetic trend in female fertility since explicitly considering ICF. Hence, it is imperative to focus on female interval traits displaying close functional relationships with NEB and with metabolic disorders.

Genetic correlations between early-lactation milk ketone bodies and nonesterified FA concentration with ICF were positive with 0.38 and 0.39, respectively (Mehtiö et al., 2020). Similarly, correlations between KET breeding value with breeding values for fertility traits varied from 0.26 and 0.33, indicating fewer KET cases with an improved fertility status (Vosman et al., 2015). Genomically, detected shared SNP markers, potential candidate genes, and metabolic pathways indicated influence of the lipid metabolism on cow fertility and on KET (Wathes et al., 2013; Nayeri et al., 2019).

The single-step genomic BLUP (**ssGBLUP**) approach, combining genomic and pedigree relationship matrices (e.g., Legarra et al., 2009; Aguilar et al., 2010), contributed to increased reliabilities of breeding values for production traits of young animals, when compared with traditional BLUP methods (Oliveira et al., 2019). So far, ssGBLUP has been applied to study FA profiles in many livestock species, and the advantages over traditional BLUP have been reported for sheep, goats, and cattle (Cesarani et al., 2019; Gebreyesus et al., 2019; Freitas et al., 2020). Gain in prediction accuracies from single-step applications were especially

observed for low heritability traits (Ismael et al., 2017; Guarini et al., 2018). In consequence, ssGBLUP is currently implemented into routine genomic evaluations for many livestock species (Misztal et al., 2020).

So far, no study addressed genetic and genomic relationships among milk FA groups (SFA, UFA, MUFA, and polyunsaturated FA (**PUFA**)) as well as specific palmitic (**C16:0**) and stearic (**C18:0**) FA concentrations, KET diagnoses, and ICF in a comprehensive ssGBLUP approach. Thus, we applied ssGBLUP to estimate genetic (co)variance components for KET, first test-day Fourier-transform infrared spectroscopy (**FTIR**) milk FA profiles, and ICF, and we applied single-step GWAS to identify SNP marker associations and potential candidate genes for these traits.

Materials and Methods

Cow traits

The present study considered 38,375 first-lactation Holstein cows, kept in 45 large-scale co-operator herds from the German federal states of Mecklenburg-West Pomerania and Brandenburg. These cows calved from 2014 to 2017. Ages at first calving ranged from 20 to 39 mo, and the first official test-day conducted by the milk recording organization was between 5 d to 42 d after calving. The FA milk concentrations in g/100 g of milk from the first official test-day including UFA, MUFA, PUFA, SFA, C16:0, and C18:0 were determined in the laboratory of the milk recording organization using FTIR (Foss Analytics, Hillerød, Denmark). Data editing of FA measurements excluded test-day records with one or several missing FA fractions, and FA test-day records where MUFA concentrations were equal or lower than PUFA concentrations. For ICF, we deleted records smaller than 21 or larger than 250 d.

Clinical KET was diagnosed by veterinarians and herd managers considering the definitions from the central disease diagnosis key (Stock et al., 2013). Accordingly, a KET score = 1 for diseased cows implied at least one observation for increased content of ketone bodies in blood or urine (measured via handheld ketometers or urine test strips) in a 6-wk interval after calving. Hence, a score = 0 was assigned for healthy cows without any KET entry during the early-lactation period. The numbers of first-lactation Holstein cows and herds with FA measurements, clinical KET, and ICF records are given in Table 4.1. The descriptive statistics for first test-day FA milk concentrations and ICF are shown in Table 4.2.

Table 4.1. Number of phenotyped and genotyped first-lactation Holstein cows (no. of herds in parentheses) with fatty acid measurements, diagnosis for clinical ketosis, and interval from calving to first insemination (ICF) records, and with overlapping records.

	No. of cows (no. of herds)	
Trait	Phenotyped	Genotyped
Fatty acid concentration	5,920 (17)	1,858 (17)
Ketosis		
Healthy	35,842 (36)	8,565 (27)
Diseased	491 (36)	163 (27)
ICF	18,511 (45)	7,624 (39)
Ketosis and fatty acid concentration	3,878 (8)	800 (5)
Ketosis and ICF	17,132 (36)	6,758 (27)
Fatty acid concentration and ICF	4,154 (17)	1,523 (17)

Table 4.2. Descriptive statistics for first test-day fatty acid milk concentrations (in g/100 g of milk) and for the interval from calving to first insemination (ICF, in days) in first-lactation Holstein cows.

Trait	Mean	SD	Minimum	Maximum
Saturated fatty acids	2.47	0.46	0.72	4.96
Unsaturated fatty acids	1.47	0.40	0.42	3.60
Monounsaturated fatty acids	1.19	0.35	0.03	3.01
Polyunsaturated fatty acids	0.16	0.04	0.00	0.33
Palmitic acid	1.08	0.21	0.32	2.25
Stearic acid	0.42	0.11	0.00	1.01
ICF	72.37	23.44	21	250

Genotypes

A subset of 2,322 cows was genotyped with the Illumina BovineSNP50 v2 BeadChip (Illumina Inc., San Diego, CA), and 7,464 cows were genotyped with the EuroGenomics 10K chip (Illumina Inc., San Diego, CA). Low-density 10K genotypes were imputed by the project partner VIT (Vereinigte Informationssysteme Tierhaltung w.V, Verden, Germany) to the 50K panel, applying the algorithm as described by Segelke et al. (2012). The SNP data set considered

45,613 SNP from 9,786 genotyped cows with phenotypic records for KET, ICF, or FA concentration (Table 4.1). Quality control of the genotype data was performed using the software package PLINK (Purcell et al., 2007). The SNP with more than 1% missing genotype data, with a minor allele frequency lower than 5%, and deviation from Hardy-Weinberg equilibrium (P-value < 10^{-8}), were discarded. Thus, 40,989 SNP from the 9,786 cows were available for genomic studies.

Statistical Models

ssGBLUP genetic parameter estimations

For the estimation of heritabilities and variance components for Gaussian-distributed milk FA concentrations and ICF, the genetic-statistical single-trait animal model [1] was defined as follows:

$$\mathbf{y}=\mathbf{X\beta} + \mathbf{Za} +\mathbf{e} \text{ [1]}$$

where **y** was a vector including observations for ICF (in days) or first test-day FA concentrations (in g/100 g of milk) for SFA, UFA, MUFA, PUFA, C16:0, and C18:0; **β** was a vector of fixed effects including herd (45 herds for ICF, 17 herds for FA), calving year (4 yr from 2014 - 2017), calving month (12 mo), age at first calving (linear regression, 20 - 39 mo), and a linear regression on DIM (5 - 42 DIM) for FA concentrations; **a** was a vector of additive-genetic effects, with $\mathbf{a} \sim N(0, \mathbf{H}\sigma^2_a)$, where σ^2_a was the additive-genetic variance; **e** was the vector of random residual effect with $\mathbf{e} \sim N(0, \mathbf{I}\sigma^2_e)$, where σ^2_e was the residual variance; **X** and **Z** were the incidence matrices for fixed and random effects, respectively. The combined inverse of the **H** matrix was computed by blending the pedigree relationship matrix **A** and the genomic relationship matrix ($\mathbf{G}_w$; Legarra et al., 2009). The $\mathbf{G}_w$ was calculated as follows: $\mathbf{G}_w = (0.95 \times \mathbf{G} + 0.05 \times \mathbf{A}_{22})$, where $\mathbf{A}_{22}$ was the submatrix of the pedigree-based relationship matrix for genotyped animals and **G** was genomic relationship matrix (VanRaden, 2008). The pedigree relationship matrix considered ancestors back to birth year 1941, and at least 3 generations backward were available for the cows with phenotypes.

For binary KET, the genetic-statistical single-trait threshold model [2] was:

$$\mathbf{l} =\mathbf{X\beta} + \mathbf{Za} + \mathbf{e} \text{ [2]}$$

where **l** was a vector of underlying liabilities for KET occurrence; **β** was a vector of fixed effects including herd (45 herds), calving year, calving month, the covariate days in milk (linear regression), and the covariate age at first calving (linear regression); **e** was the vector of random

residual effects with a residual variance of 1. The remaining specifications are the same as defined for model [1].

For the estimation of genetic correlations among FA concentrations, ICF, and KET bivariate animal models were applied for all trait combinations. The bivariate model [3] was:

$$\begin{bmatrix}\mathbf{y_1}\\ \mathbf{y_2}\end{bmatrix} = \begin{bmatrix}\mathbf{X_1} & \mathbf{0}\\ \mathbf{0} & \mathbf{X_2}\end{bmatrix}\begin{bmatrix}\boldsymbol{\beta}_1\\ \boldsymbol{\beta}_2\end{bmatrix} + \begin{bmatrix}\mathbf{Z_1} & 0\\ 0 & \mathbf{Z_2}\end{bmatrix}\begin{bmatrix}\mathbf{a_1}\\ \mathbf{a_2}\end{bmatrix} + \begin{bmatrix}\mathbf{e_1}\\ \mathbf{e_2}\end{bmatrix} \quad [3]$$

where $\mathbf{y_1}$ and $\mathbf{y_2}$ were the observation vectors for a trait 1 and a trait 2; $\boldsymbol{\beta}_1$ and $\boldsymbol{\beta}_2$ were the vectors of fixed effects for trait 1 and 2, respectively, as defined above; $\mathbf{X_1}$ and $\mathbf{X_2}$ were corresponding incidence matrices for the fixed effects; $\mathbf{a_1}$ and $\mathbf{a_2}$ were the vectors of additive-genetic random effects for trait 1 and 2, respectively; $\mathbf{Z_1}$ and $\mathbf{Z_2}$ were the corresponding incidence matrices for the random additive-genetic effects; and $\mathbf{e_1}$ and $\mathbf{e_2}$ were the vectors of random residual effects for the 2 traits.

Assumptions for the variance-covariance structure of additive-genetic effects were:

$$\begin{bmatrix}\mathbf{a_1}\\ \mathbf{a_2}\end{bmatrix} \sim N\left(\begin{pmatrix}0\\ 0\end{pmatrix}, \mathbf{H} \otimes \begin{pmatrix}\sigma^2_{a_1} & \sigma_{a_1a_2}\\ \sigma_{a_1a_2} & \sigma^2_{a_2}\end{pmatrix}\right)$$

where $\sigma^2_{a_1}$ and $\sigma^2_{a_2}$ were the additive-genetic variances for the 2 traits and $\sigma_{a_1a_2}$ the additive-genetic covariance between both traits, and $\otimes$ denoting the Kronecker product. Assumptions for the residual effects were:

$$\begin{bmatrix}\mathbf{e_1}\\ \mathbf{e_2}\end{bmatrix} \sim N\left(\begin{pmatrix}0\\ 0\end{pmatrix}, \mathbf{I} \otimes \begin{pmatrix}\sigma^2_{e_1} & \sigma_{e_1e_2}\\ \sigma_{e_1e_2} & \sigma^2_{e_2}\end{pmatrix}\right)$$

where $\mathbf{I}$ was an identity matrix, $\sigma^2_{e_1}$ and $\sigma^2_{e_2}$ were the residual variances for the 2 traits, and $\sigma_{e_1e_2}$ was the residual covariance between both traits. For bivariate runs including binary KET, the (co)variance structure of residual effects was:

$$\begin{bmatrix}\mathbf{e_1}\\ \mathbf{e_2}\end{bmatrix} \sim N\left(\begin{pmatrix}0\\ 0\end{pmatrix}, \mathbf{I} \otimes \begin{pmatrix}\sigma^2_{e_1} & \sigma_{e_1e_2}\\ \sigma_{e_1e_2} & 1\end{pmatrix}\right)$$

where 1 was the residual variance for binary KET.

Variance components from single-trait linear and bivariate linear-linear models including only Gaussian FA traits and ICF were estimated using the AI-REML algorithm as implemented in the AIREMLF90 program (Misztal et al., 2018). For all runs including binary KET, THRGIBBS1F90 (Misztal et al., 2018) was applied to account for the binary trait

structure. In this regard, 200,000 samples were generated, with the first 30,000 samples discarded as burn-in. Every 10th sample was saved. The POSTGIBBSF90 program (Misztal et al., 2018) was used to calculate posterior means for all estimates. The length of the burn-in period and the length of the sampling period were determined according to Raftery and Lewis (1992), which is implemented in the BOA software package (Smith, 2005). For the determinations, we used the first 10,000 iterations of a Gibbs chain for genetic covariances. Furthermore, we visually inspected the trace plots.

Single-step genome-wide association study

Single-step GWAS (**ssGWAS**) for the estimation of SNP marker effects and corresponding *P*-values based on the estimates of genomic breeding values as obtained from ssGBLUP analyses. The back solving was done using "OPTION snp_p_value" as implemented in POSTGSF90 (Aguilar et al., 2019).

The effective number of independent SNP (N_{SNP} = 29,101) was calculated using the software Genetic Type I Error Calculator (Li et al., 2012), in order to define the genome-wide significance level according to Bonferroni (**pBF** = 0.05 / N_{SNP} = 1.72e-06). The Genetic Type I Error Calculator was developed to address multiple-testing issues with dependent SNP. In this regard, SNP markers are divided into linkage disequilibrium blocks. By examining the eigenvalues obtained from decomposition of the linkage disequilibrium correlation matrix, Genetic Type I Error Calculator estimates the effective number of independent SNP in the blocks. In addition, a normative significance threshold was used to identify potential candidate SNP, considering **pCD** = 1e-04 (Kurz et al., 2018). According to Manca et al. (2020), annotated genes located in 250 kb upstream or downstream from the significantly associated candidate SNP were detected using Ensembl, release 102, on the basis of the *Bos taurus* ARS1.2 genome assembly (Zerbino et al., 2018).

Results and Discussion

Heritabilities for fatty acid concentrations, ketosis and the interval from calving to first insemination

Variance components and heritabilities from the ssGBLUP approach for first test-days milk FA concentrations are given in Table 4.3. The moderate FA heritabilities, ranging between 0.09 (SE = 0.02) for PUFA and 0.20 (SE = 0.03) for C16:0, are in agreement with estimates from Narayana et al. (2017) and Fleming et al. (2018), who considered only pedigree relationships. Accordingly, heritability estimates for SFA with 0.19 (SE = 0.03) and C16:0 with 0.20 (SE =

0.03) were larger than for UFA with 0.13 (SE = 0.02). This might be due to the fact that most SFA in milk originate from de novo synthesis in the mammary gland, whereas long chain UFA are derivatives from preformed circulating blood lipids and from body fat mobilization (Grummer, 1991; Bastin et al., 2012). The metabolic enzymes involved in de novo synthesis seems to be under stronger genomic control (Bastin et al., 2011). However, in the present study, heritabilities for UFA, MUFA (0.12, SE = 0.02), and C18:0 (0.13, SE = 0.02) were smaller than for SFA and for C16:0, but still in a moderate range (Table 4.3). Narayana et al. (2017) identified FA heritability alterations dependent on the lactation stage. Smaller early lactation heritabilities were due to the increased residual variance (Narayana et al., 2017). In contrast to milk FA, we estimated quite small heritabilities of 0.05 (SE = 0.02) for KET, and of 0.08 (SE = 0.01) for ICF (Table 4.3). The small heritabilities for KET and ICF reflect estimates from previous studies (e.g., Mehtiö et al., 2020). Recently, Shabalina et al. (2021) compared female fertility and metabolic disorder heritabilities based on **A** (pedigree relationships), **G** (genomic relationships), and **H** (combined relationships) matrices. The estimates were very similar and close to zero.

Table 4.3. Heritabilities (h^2), additive genetic (σ_a^2) ($x10^{-2}$), and residual variances (σ_e^2) ($x10^{-2}$) for fatty acid milk concentration, interval from calving to first insemination (ICF), and clinical ketosis. Corresponding SE are given in parenthesis.

Trait	h^2 (SE)	σ_a^2	σ_e^2
Saturated fatty acids	0.19 (0.03)	3.28	13.64
Unsaturated fatty acids	0.13 (0.02)	0.13	9.22
Monounsaturated fatty acids	0.12 (0.02)	0.97	7.31
Polyunsaturated fatty acids	0.09 (0.02)	0.01	0.13
Palmitic acid	0.20 (0.03)	0.69	2.80
Stearic acid	0.13 (0.02)	0.10	0.74
ICF	0.08 (0.01)	4,078.8	45,914
Ketosis	0.05 (0.02)[1]	5.43	100.03

[1] Posterior standard deviation.

Genetic correlations between ketosis, fatty acid concentration, and interval from calving to first insemination

Genetic correlation estimates among KET, FA concentrations, and ICF (Table 4.4) are in agreement with phenotypic estimates (Nogalski et al., 2015; Rutherford et al., 2016). However,

for some trait combinations, SE of genetic correlations were quite large (Table 4.4). We detected strong genetic correlations between KET with UFA (0.85), MUFA (0.74), and C18:0 (0.74), but correlations between KET with SFA (-0.05), PUFA (-0.14), and C16:0 (-0.10) were close to zero. The genetic correlation between KET and ICF was low positive (0.17), but indicating genetic improvement in ICF due to the selection on metabolic stable cows.

The FA displaying strong genetic correlations with KET also showed moderate positive genetic correlations with ICF (i.e., 0.46 for UFA, 0.42 for MUFA, and 0.34 for C18:0). The positive genetic correlations (Table 4.4) indicate a longer period from calving to first insemination due to increased concentrations of UFA, MUFA, or C18:0 in milk. In contrast, genetic correlations with ICF were close to zero for SFA (-0.04), PUFA (0.15), and C16:0 (-0.08).

Table 4.4. Genetic correlations between clinical ketosis (KET) and the interval from calving to first insemination (ICF) with fatty acid milk concentrations, and between ICF and KET for first-lactation Holstein cows.

Trait	KET[1]	ICF[2]
Saturated fatty acids	-0.05 (0.20)	-0.04 (0.11)
Unsaturated fatty acids	0.85 (0.14)	0.46 (0.12)
Monounsaturated fatty acids	0.74 (0.19)	0.42 (0.12)
Polyunsaturated fatty acids	-0.14 (0.22)	0.15 (0.14)
Palmitic acid	-0.10 (0.21)	-0.08 (0.11)
Stearic acid	0.74 (0.18)	0.34 (0.12)
ICF	0.17 (0.22)	

[1]Posterior standard deviations in parentheses.

[2]SE in parentheses.

Accordingly, Bastin et al. (2012) estimated very similar genetic correlations from pedigree-based approaches between early lactation UFA, MUFA, C18:0 with female fertility interval traits. The strong positive genetic correlations between UFA, MUFA, and C18:0 with KET can be explained in a physiological context in early lactation. A NEB and KET cause catabolism of adipose tissue, and thus, the release of particular long-chain FA from mobilized tissue (Kay et al., 2005). Consequently, Nogalski et al. (2015) detected an increase in UFA, MUFA, and C18:0 concentrations in milk in the stage of a NEB. Approximately 25% of milk FA are MUFA with oleic acid (**C18:1**), accounting for 23.8% of the total FA in dairy cattle milk (Lindmark Månsson, 2008). The fact that C18:1 and C18:0 are predominant in adipocytes and are released

during lipolysis (Kay et al., 2005) may explain the strong and positive correlations between MUFA and C18:0 with KET.

Bastin et al. (2012) reported slightly negative genetic correlations between C16:0 and days open, reflecting the estimate in our study (r_g = -0.06 between ICF and C16:0). Furthermore, we estimated slightly negative genetic correlations between KET with SFA and C16:0. Park et al. (2020) observed low levels of SFA in the stage of NEB in early lactation, but SFA levels increased with increasing DIM. Grummer (1991) clearly described the associations between energy intake on FA syntheses, demonstrating the opposite effects on C16:0 and C18:0. The different physiological pathways may be an explanation for the differing genetic correlations between C18:0 and C16:0 with KET. Accordingly, Stádník et al. (2015) reported antagonistic relationships between milk MUFA and female fertility, but they identified opposite effects for SFA. Demeter et al. (2009) associated increasing concentrations of milk C18:0 FA with impairments in calving rates. Results from our genetic study as well as the physiological mechanisms as outlined above indicate detrimental effect of KET and of increasing UFA, MUFA, and C18:0 on ICF. Hence, genetic selection on lower UFA, MUFA, and C18:0 milk concentrations early in lactation will genetically contribute to an improved metabolic health and fertility status regarding ICF.

ssGWAS for milk fatty acid profile, interval from calving to first insemination, and ketosis

We identified SNP significantly associated with the FA groups SFA, UFA, MUFA, PUFA (Figure 4.1), with the specific FA C16:0 and C18:0, with ICF and with KET (Figure 4.2). All significantly or suggestively associated SNP marker according to the Bonferroni (pBF = 1.72e-06, -$\log_{10}$ *P*-value = 5.76) and the candidate threshold (pCD = 1e-04, -$\log_{10}$ *P*-value = 4.0), as well as annotated positional candidate genes, are provided in Supplemental Table S4.1 (https://jlupub.ub.uni-giessen.de//handle/jlupub/99). The quantile-quantile plots for FA are given in the Supplemental Figure S4.1 (https://jlupub.ub.uni-giessen.de//handle/jlupub/99), and in Supplemental Figure S4.2 (https://jlupub.ub.uni-giessen.de//handle/jlupub/99) for ICF and KET. In the following, we focus on a deeper discussion including potential candidate genes and SNP for FA concentrations, KET, or ICF, which are involved in the respective physiological pathways.

Saturated fatty acids

In total, 34 SNP were significantly associated with first test-day milk SFA concentration (Figure 4.1, Supplemental Table S4.1). All 19 significantly associated SNP according to Bonferroni are

located on BTA 14, including the strongest associated SNP rs109421300 (609,870 bp) in an intronic region of the diacylglycerol O-acyltransferase 1 (*DGAT1*) gene. *DGAT1* is involved in the synthesis of triacylglycerol, explaining the large effect on milk fat percentage (Grisart et al., 2002). The segment including *DGAT1* and surrounding genes such as forkhead box H1 (*FOXH1*) and protein phosphatase 1 regulatory subunit 16A (*PPP1R16A*) is a candidate region for milk SFA and C16:0 in Holstein cows (Palombo et al., 2018; Cruz et al., 2019). In our study, further suggestively associated SNP according to the candidate threshold are located on BTA 1, 19, 22, and BTA 27. The strongest suggestively associated SNP rs110519353 (36,466,414 bp) on BTA 27 is located in gene GINS complex subunit 4 (*GINS4*). Positional candidate genes within a segment of 36 Mbp on BTA 27 [i.e., golgin A7 (*GOLGA7*), secreted frizzled related protein 1 (*SFRP1*), glycerol-3-phosphate acyltransferase 4 (*GPAT4*, synonymous *AGPAT6*)] are involved in differential milk fat synthesis and affected levels of specific SFA (Littlejohn et al., 2014). Littlejohn et al. (2014) confirmed the association between the high-fat percentage 'C' allele of rs110519353 with increased proportions of SFA and C16:0, and with decreasing UFA levels in Holstein-Friesian x Jersey crossbreeds. The SNP rs43682200 (45,350,130 bp) on BTA 1, rs109477972 (29,784,751 bp) on BTA 19, and rs41993977 (5,531,843 bp) on BTA 22 were suggestively associated with SFA. Positional candidate genes were not associated with SFA.

Unsaturated fatty acids

For UFA, we identified 10 suggestively associated SNP according to the candidate threshold on BTA 5, 8, 10, 12, 14, and 28 (Figure 4.1, Supplemental Table S4.1). In contrast to SFA (with strongest effects of SNP markers on BTA 14), the most important segment for UFA including SNP rs110176023 (111,284,112 bp) with the highest $-\log_{10}$ *P*-value is located on BTA 8.

On BTA 5, the gene ubiquitin specific peptidase 15 (*USP15*) is located within the 250 kb distance to the significantly associated SNP rs41625419 (51,366,141 bp). Orthologue of *USP15* contributed to a decline of body fat and circulating glucose levels in mice (Ensembl, 2020). Alterations of body fat and glucose levels were identified in the state of NEB and in dairy cows diagnosed for KET (Yang et al., 2019).

The potential candidate genes C-X-C motif chemokine ligand 12 (*CXCL12*), rho GTPase activating protein 22 (*ARHGAP22*), Jumonji domain containing 1C (*JMJD1C*), located within the 250 kb distance to the SNP rs109278212 (45,261,673 bp), rs110222344 (43,155,287 bp), and rs109839180 (19,620,469 bp) on BTA 28, are related to diabetes, insulin resistance, fatty liver disease, and lipogenesis (Li et al., 2016; Viscarra et al., 2020). Additionally, gene *CXCL12*

is involved in diverse cellular functions, such as immune surveillance, inflammation response, and tissue homeostasis (Janssens et al., 2018). The receptor of *CXCL12*, C-X-C motif chemokine receptor 4 (*CXCR4*), was increasingly expressed in cows suffering from severe NEB (McCarthy et al., 2010). The segment on BTA 28 at 43 Mbp was associated with subclinical KET (Soares, 2020), and gene *JMJD1C* at 19 Mbp was identified as a candidate for metabolic body weight in Holstein cows (Hardie et al., 2017). On BTA 14, the 3 SNP rs109421300, rs109350371, rs109146371 were significantly associated with UFA. The SNP from the same genomic region were detected for SFA (as described above, Supplemental Table S4.1).

Monounsaturated fatty acids

For MUFA, we identified 9 suggestively associated SNP according to the candidate threshold on BTA 5, 8, 9, 10, 14, and 28 (Figure 4.1, Supplemental Table S4.1). The associated SNP, except for SNP rs109137030 (6,218,345 bp) on BTA 9, overlapped with the SNP detected for UFA (Supplemental Table S4.1). The high genetic correlation of 0.95 between MUFA and UFA (Penasa et al., 2015) indicates a similar genomic background.

Polyunsaturated fatty acids

In total, 14 suggestively SNP according to the candidate threshold were detected for PUFA on BTA 3, 4, 13, 14, 19, 24, and 28 (Figure 4.1, Supplemental Table S4.1). The strongest associated SNP rs42854990 (2,669,298 bp) is located on BTA 28, but no potential candidate gene was annotated. The SNP rs109839180 on BTA 28 was also suggestively associated with UFA and MUFA in our study (Supplemental Table S4.1). The SNP rs109823489 (56,044,735 bp) on BTA 21 is located in gene coiled-coil domain containing 88C (*CCDC88C*), which was declared as a candidate gene for cow livability, indicating the overall robustness of a cow (Freebern et al., 2020).

The SNP rs41593945 (117,293,280 bp) on BTA 4 is located within the defined distance to the gene insulin induced gene 1 (*INSIG1*). Gene expression analyses (Fan et al., 2020) revealed that *INSIG1* is involved in the mammary lipid synthesis in ruminants. Associated SNP on BTA 3 were rs110239426 (48,613,050 bp), rs109621977 (48,432,235 bp), and rs42945878 (46,563,516 bp). The SNP rs109621977 is located in gene ALG14 UDP-*N*-acetylglucosaminyltransferase subunit (*ALG14*), which regulates plasma FA levels in humans (Yuan and Larsson, 2020) and levels of milk composition traits in Holstein cows (Lin et al., 2019).

On BTA 13, the SNP rs43705561 (70,050,255 bp) is located within the defined interval of 250 kb to candidate gene lipin 3 (*LPIN3*), playing key roles in hepatic metabolic adaptations to NEB, especially in adipose tissue lipolysis and hepatic FA oxidation (Loor et al., 2007). On BTA 19, the associated SNP rs110933534 (40,505,729 bp) and rs41644917 (40,381,198 bp) are located close to the potential candidate gene insulin like growth factor binding protein 4 (*IGFBP4*), which is related to NEB and female fertility in dairy cows. In this regard, Wathes et al. (2011) hypothesized that alterations in early lactation insulin signalling levels may delay uterine repair mechanisms and impair fertility. Interestingly, the 2 SNP close to *IGFBP4* were also associated with C18:0 in our study. We estimated strong genetic correlations between C18:0 with KET, and moderate genetic correlations with ICF, supporting the overlapping causal genomic mechanisms. The candidate gene lipase G (*LIPG*) located within the 250 kb to SNP rs41570441 (49,057,452 bp) on BTA 24 is involved in triglyceride metabolism, yielding glycerol and free FA, and was upregulated in liver in metabolically imbalanced Holstein cows in early lactation (Wathes et al., 2021).

Palmitic acid

In total, 17 SNP on BTA 14 and 1 SNP on BTA 27 were significantly associated according to Bonferroni, and 18 SNP on BTA 1, 6, 14, 19, 22, and BTA 27 were suggestively associated with C16:0 (Figure 4.2, Supplemental Table S4.1). Significantly associated SNP on BTA 14 widely overlapped with SFA associations, with the strongest effect of the SNP rs109421300 located in *DGAT1*. Moreover, the suggestive SNP on BTA 1, 19, 22, and 27 were the same as detected for SFA. The SNP rs110351063 (65,323,234 bp) on BTA 15 is located in close distance to the candidate gene pyruvate dehydrogenase complex component X (*PDHX*). *PDHX* catalyzes the conversion of pyruvate into acetyl-coenzyme A and was annotated for subclinical KET in first-lactation Holstein cows (Soares, 2020). The SNP associations for C16:0 and SFA overlapped to a large extent. With a fraction of 30%, C16:0 is the most important SFA (Lindmark Månsson, 2008).

Stearic acid

The strongest association was identified for SNP rs109421300, located on BTA 14 in the *DGAT1* gene (Figure 4.2, Supplemental Table S4.1). Additionally, 9 suggestively associated SNP were identified on BTA 1, 5, 8, 12, 15, and 19. The SNP rs41625419 (BTA 5), rs110176023 (BTA 8), and rs41577805 (BTA 12) also displayed significant effects on UFA and MUFA. Furthermore, the SNP rs43682200 (BTA 1) was significantly associated with SFA

and C16:0. The SNP rs110933534 and rs41644917 on BTA 19 were the same as detected for PUFA (Supplemental Table S4.1). The SNP rs110508416 (37,224,652 bp) close to gene INSC spindle orientation adaptor protein (*INSC*) on BTA 15 additionally was detected for KET (Figure 4.2, Supplemental Table S4.1).

We observed a high proportion of overlapping important genomic regions for the different groups of FA. Identified genomic regions for milk FA showed strong overlaps for SFA and C16:0, and for UFA, MUFA, and partly C18:0. Detected overlapping genomic regions may be due to their common origin (i.e., from the blood from mobilized body tissue or de novo synthesis in the mammary gland). The SNP rs109421300 located in *DGAT1* on BTA 14 was associated with all FA, apart from PUFA. Annotated positional candidate genes including *DGAT1*, *GPAT4*, *CXCL12*, *ARHGAP22*, *INSIG1*, *LPIN3*, *LIPG*, and *PDHX* are related to lipid metabolism, insulin resistance, inflammation response, NEB, subclinical KET, and also to female fertility traits.

Generally, a large proportion of the significant SNP for most of the FA are located on BTA 14. In a previous GWAS for fat-to-protein ratio (Klein et al., 2019), we run models with and without correction for the effect of *DGAT1* gene on BTA 14. The model with *DGAT1* correction contributed to a reduction of significantly associated SNP. Due to the overlapping physiological background with fat-to-protein ratio, similar effects are expected for FA. Accordingly, in a GWAS for FA, Cruz et al. (2019) additionally fitted models including the *DGAT1* gene as covariate. Such modeling strategy implied a smaller number of detected potential candidate genes. We did not focus on such model comparisons in the present study, but the strong impact of *DGAT1* on FA is obvious.

Interval from calving to first insemination

In total, we identified 5 suggestively associated SNP according to the candidate threshold on BTA 5, 10, 11, and 17 for ICF (Figure 4.2, Supplemental Table S4.1). The strongest associated SNP rs41599470 (36,317,694 bp) is located on BTA 17. Tenghe et al. (2016) identified a genomic region on BTA 17 for endocrine fertility traits in dairy cattle close to SNP rs41599470. On BTA 5, we detected two SNP rs29018280 (57,356,420 bp) and rs108956573 (57,282,611 bp) in neighbouring distance. The intron variant rs29018280 is positioned in diacylglycerol kinase alpha (*DGKA*). Diacylglycerol kinases are key enzymes in the lipid metabolism. In beef cattle, gene expressions of *DGKA* in endometrial tissue were related to embryo survival (Beltman et al., 2010). Polymorphisms in the annotated potential candidate gene Erb-b2 receptor tyrosine kinase 3 (*ERBB3*) showed significant associations with diabetes susceptibility

in humans and hormonal disorders causing the polycystic ovary syndrome (Welt and Duran, 2014). Accordingly, Opsomer et al. (1999) displayed relationships between cystic ovarian disease in high-yielding dairy cows and insulin resistance mechanisms. Furthermore, we identified significantly associated SNP on BTA 10. Cai et al. (2019) and Minozzi et al. (2013) reported SNP associations on BTA 10 for fertility traits in Holsteins, but genomic regions differed from the detected significant SNP positions in our study.

Ketosis

We identified one significant SNP association according to Bonferroni and 42 suggestively associated SNP according to the candidate threshold (Figure 4.2, Supplemental Table S4.1). The significantly associated SNP rs110942910 (27,440,587 bp) and 6 suggestively associated SNP are located on BTA 18.

The Manhattan plot for KET revealed 4 suggestively associated SNP on BTA 5 (Supplemental Table S4.1). One of these SNP [i.e., rs109896020 (114,222,945 bp)] showed strongest associations for KET when performing GWAS on the basis of the pure genomic relationship matrix (Klein et al., 2019). The corresponding region surrounding 114 Mbp includes several positional candidate genes contributing to subclinical KET (Soares, 2020). Loor et al. (2007) observed that KET implied downregulation of triosephosphate isomerase 1 (*TPI1*), a gene involved in glycolysis and gluconeogenesis, closely located to the SNP rs109046936 (103,549,759 bp) on BTA 5. Further potential candidate genes surrounding a segment at 103 Mbp are protein tyrosine phosphatase non-receptor type 6 (*PTPN6*), G protein subunit beta 3 (*GNB3*), and prolyl 3-hydroxylase 3 (*P3H3*), which influenced metabolic body weight in first-lactation Holstein cows (Hardie et al., 2017).

Five SNP from BTA 15 were suggestively associated with KET: rs41632691 (83,673,161 bp), rs109932511 (83,710,700 bp), rs110944919 (78,017,138 bp), rs110508416 (37,224,652 bp), and rs109138685 (37,720,295 bp). The corresponding genomic region surrounding 83 Mbp includes the potential candidate genes galactosidase β 1 like 2 (*GLB1L2*), beta-1,3-glucuronyltransferase 1 (*B3GAT1*), galactosidase β 1 like 3 (*GLB1L3*), and acyl-CoA dehydrogenase family member 8 (*ACAD8*), which are involved in physiological pathways contributing to subclinical KET (Soares, 2020). The annotated gene phosphodiesterase 3B (*PDE3B*), located within 250 kb to rs109138685, plays a crucial role in lipolysis and cell energy homeostasis (Degerman et al., 2011), suggesting its influence in KET progression. The significantly associated SNP rs110508416 on BTA 15 was additionally associated with C18:0 in our study.

The genomic segment on BTA 10 comprising the SNP rs41586492 (17,746,328 bp), rs110844686 (34,985,737 bp), and rs43710033 (43,625,368 bp) with the annotated potential candidate genes mitogen-activated protein kinase kinase kinase kinase 5 (*MAP4K5*), atlastin GTPase 1 (*ATL1*), abhydrolase domain containing 12B (*ABHD12B*), glycogen phosphorylase L (*PYG*L), thrombospondin 1 (*THBS1*), and fibrous sheath interacting protein 1 (*FSIP1*) was associated with subclinical KET and DMI in Holstein cows (Hardie et al., 2017; Nayeri et al., 2019; Soares, 2020).

The SNP rs109946603 (9,419,987 bp) on BTA 20 was significantly associated with KET according to the candidate threshold. The corresponding genomic region includes the candidate genes microtubule associated protein 1B (*MAP1B*) and mitochondrial ribosomal protein S27 (*MRPS27*), which influenced clinical and subclinical KET (Soares, 2020). The segment including the significantly associated SNP rs41654962 (88,739,008 bp) on BTA 6 encompasses the genes albumin (*ALB*), Ras association domain family member 6 (*RASSF6*), and C-X-C motif chemokine ligand 8 (*CXCL8*), contributing to inflammatory response (Ha et al., 2017), metabolic body weight (Hardie et al., 2017), and metabolic disorders (Nayeri et al., 2019; Soares et al., 2021). Additionally, *CXCL8* was differently expressed in healthy cows and in cows with subclinical endometritis, indicating a potential role of this gene in female fertility mechanisms (Bonsale et al., 2018). The segment including the 2 significantly associated SNP rs41994020 and rs41994761 on BTA 22 is in the defined distance to SNP markers, which were significantly associated with SFA and C16:0 in our study. The significantly associated SNP rs41647957 (8,009,249 bp) is located on BTA 27. The inferred potential candidate gene vascular endothelial growth factor C (*VEGFC*) is involved in inflammatory responses (Zhang et al., 2014).

We detected chromosomal segments and annotated potential candidate genes such as *PDE3B*, *PYGL*, *THBS1*, *ACAD8*, and *CXCL8*, which contributed to KET occurrence, and which are involved in glycolysis, lipolysis, insulin resistance, and inflammatory response. The results from our study confirm the polygenic background of KET, influenced by many genomic regions with probably small effects. According to Aguilar et al. (2019), ssGWAS is an efficient method in QTL detection and *P*-value determination, especially in complex data sets including genotyped and ungenotyped animals. The simultaneous consideration of phenotypic, pedigree and genomic information in ssGWAS contributed to a larger number of SNP associations for KET compared with previous pure genomic approaches (Klein et al., 2019).

We identified genomic regions influencing both trait categories FA and female fertility, especially the segment on BTA 15 with an effect on C18:0 and KET. As outlined in detail

above, several genomic regions identified for FA concentrations have been related to KET in previous studies. However, there was no genomic segment simultaneously significantly affecting all trait categories ICF, FA, and KET. Nevertheless, some of the inferred potential candidate genes (e.g., *DGKA*, *IGFBP4*, *CXCL8*) play a role in lipid metabolism and fertility mechanisms, and influence production diseases in early lactation.

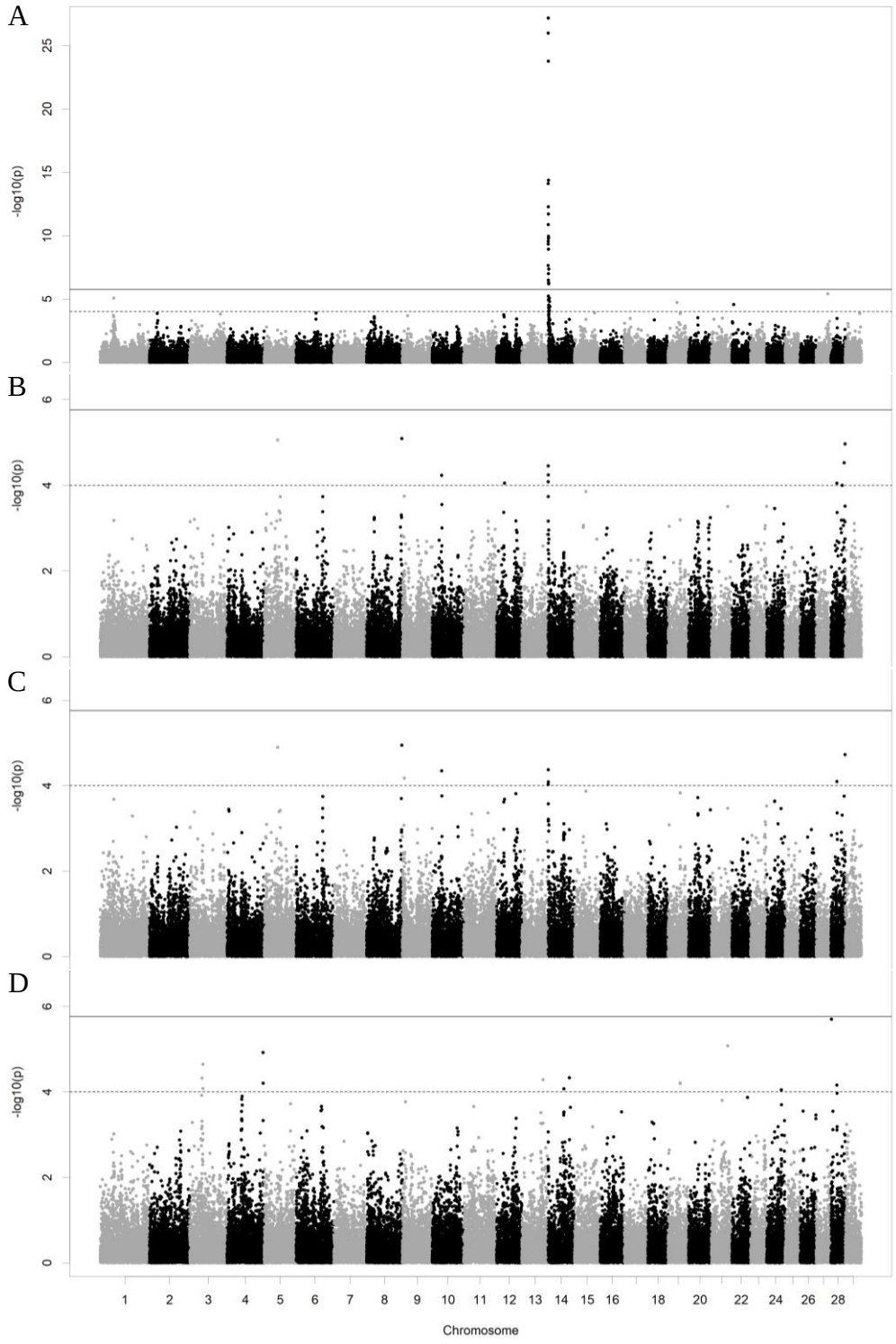

Figure 4.1. Manhattan plot for -log$_{10}$ *P*-values of SNP effects for first test-day (A) saturated, (B) unsaturated, (C) monounsaturated, and (D) polyunsaturated fatty acid concentrations in first-lactation Holstein cows. The dotted line indicates the candidate threshold (pCD = 1e-04, -log$_{10}$ *P*-value = 4.0) and the solid line indicates the Bonferroni corrected threshold (pBF = 1.72e-06, -log$_{10}$ *P*-value = 5.76).

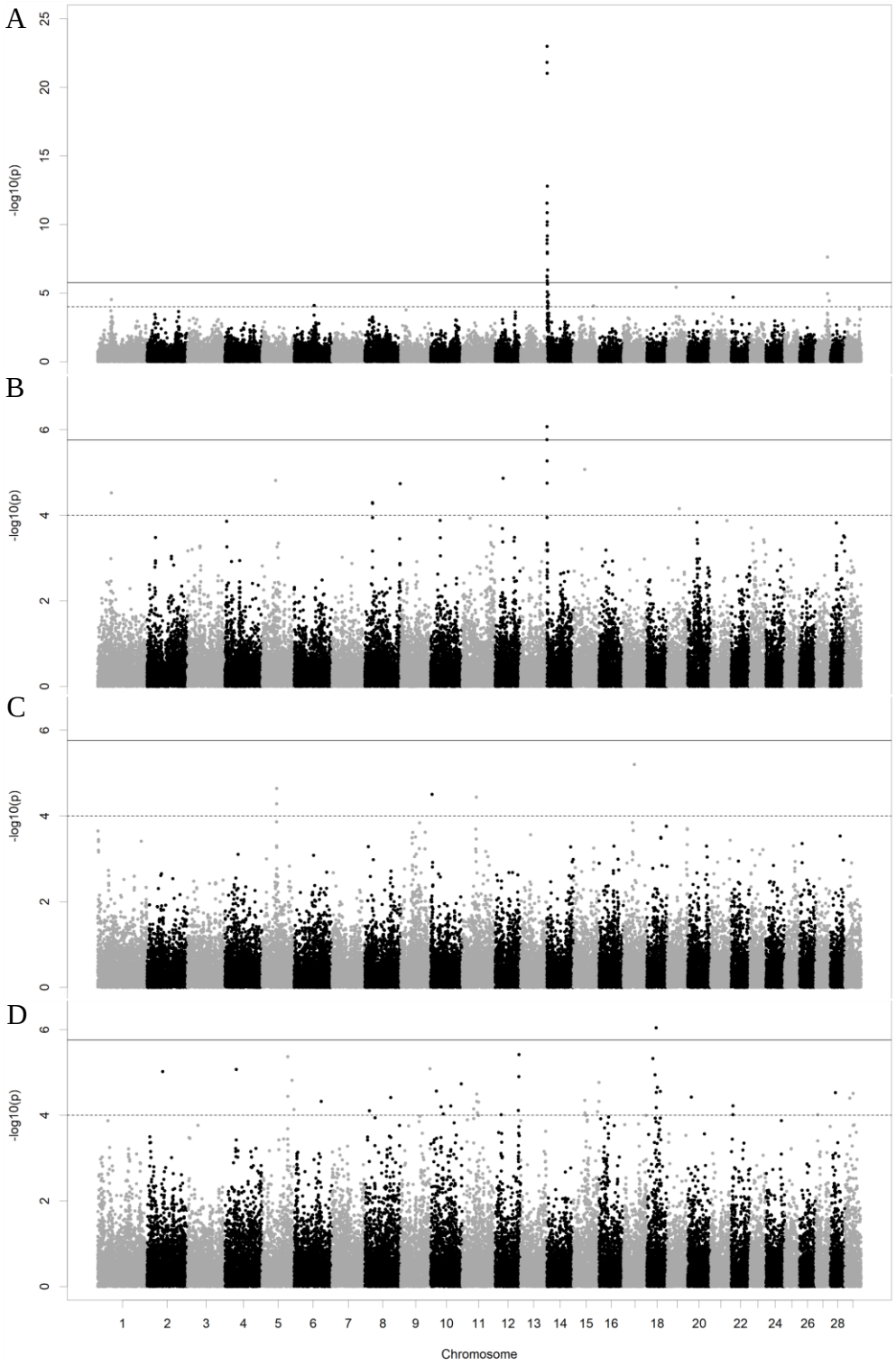

Figure 4.2. Manhattan plot for -log$_{10}$ *P*-values of SNP effects for first test-day (A) palmitic, (B) stearic fatty acid concentration, (C) interval from calving to first insemination, and (D) ketosis in first-lactation Holstein cows. The dotted line indicates the candidate threshold (pCD = 1e-04, -log$_{10}$ *P*-value = 4.0) and the solid line indicates the Bonferroni corrected threshold (pBF = 1.72e-06, -log$_{10}$ *P*-value = 5.76).

Conclusions

We estimated quite large genetic correlations between KET with UFA, MUFA, and C18:0, and low to moderate genetic correlations between KET, UFA, MUFA, C18:0 with ICF. The results indicate that first test-day FTIR FA concentrations in milk are valuable predictors for KET and for ICF. Furthermore, the estimated moderate heritabilities for FTIR FA concentrations suggest consideration of FA in selection indices for female fertility trait ICF and health. Genomically, we identified significantly associated SNP and annotated potential candidate genes indicating shared physiological mechanisms on FA concentrations, KET and ICF. Candidate genes are mainly involved in carbohydrate and lipid metabolism, inflammatory response, diabetes, and fertility. In conclusion, the application of ssGBLUP genetic parameter estimations and ssGWAS inferred closer genetic mechanisms of the 3 trait categories FA, metabolic disorders, and female fertility trait ICF, compared with previous approaches based on either pure pedigree or pure genomic relationship matrices.

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Appendix

Supplemental Table

Table S4.1. Associated SNP for milk fatty acid concentration on first test-day, ketosis and interval from calving to first insemination with positional candidate genes.

BTA	SNP	SNP position (bp)	Trait[1]	Positional candidate genes[2]
1	rs43682200	45350130	SFA, C16:0, C18:0	*ENSBTAG00000026836, ADGRG7, TFG, ENSBTAG00000053387*
2	rs109328804	50669137	KET	*ENSBTAG00000054108*
3	rs110239426	48613050	PUFA	*SLC44A3, CNN3, ALG14*
3	rs109621977	48432235	PUFA	*ALG14, SLC44A3, CNN3, TLCD4, RWDD3*
3	rs42945878	46563516	PUFA	*DPYD, PTBP2*
4	rs41593945	117293280	PUFA	*CNPY1, INSIG1, EN2, RBM33, SHH*
4	rs41664795	117389110	PUFA	*RBM33, EN2, SHH, CNPY1*
4	rs43387397	38051191	KET	*PCLO, CACNA2D1*
5	rs41625419	51366141	UFA, MUFA, C18:0	*USP15, ENSBTAG00000053892*
5	rs29018280	57356420	ICF	*DGKA, PMEL, RAB5B, CDK2, PYM1, SUOX, RNF41, SMARCC2, MYL6, MYL6B, ESYT1, ZC3H10, PA2G4, ERBB3, RPS26, IKZF4, MMP19, ENSBTAG00000009049, DNAJC14, ORMDL2, SARNP, GDF11, CD63, RDH5, BLOC1S1, ITGA7, METTL7B*
5	rs108956573	57282611	ICF	*RPS26, IKZF4, ERBB3, SUOX, RAB5B, CNPY2, CS, COQ10A, ANKRD52, ENSBTAG00000052361, SLC39A5, NABP2, RNF41, SMARCC2, MYL6, MYL6B, ESYT1, ZC3H10, PA2G4, CDK2, PMEL, DGKA, PYM1, MMP19, ENSBTAG00000009049, DNAJC14, ORMDL2, SARNP, GDF11, CD63*
5	rs109366282	103500479	KET	*PHB2, PTPN6, LPCAT3, EMG1, ATN1, ENO2, LRRC23, C1S, C1R, C1RL, ENSBTAG00000037743, RBP5, CLSTN3, SPSB2, USP5, TPI1, CDCA3, GNB3, P3H3, GPR162, CD4, ENSBTAG00000051680, LAG3, PTMS, MLF2, COPS7A, PIANP*
5	rs109896020	114222945	KET	*MPPED1, ENSBTAG00000053264, SCUBE1, EFCAB6*
5	rs109046936	103549759	KET	*LRRC23, ENO2, ATN1, PTPN6, SPSB2, TPI1, USP5, CDCA3, ENSBTAG00000037743, C1R, C1S, LPCAT3, EMG1, PHB2, GNB3, P3H3, GPR162, CD4, ENSBTAG00000051680, LAG3, PTMS, MLF2, COPS7A, PIANP, ZNF384, ING, ACRBP, LPAR5*
5	rs41657085	118894255	KET	*no gene*
6	rs109163865	60520292	C16:0	*LIMCH1, ENSBTAG00000051208, PHOX2B*
6	rs41654962	88739008	KET	*ALB, AFP, AFM, ENSBTAG00000049436, RASSF6, CXCL8, ENSBTAG00000027534, CXCL5, ENSBTAG00000011961, CXCL2, ENSBTAG00000051891*
8	rs110176023	111284112	UFA, MUFA, C18:0	*ADI1, TRAPPC12, EIPR1, ENSBTAG00000049154, ENSBTAG00000052608*

BTA	SNP	SNP position (bp)	Trait[1]	Positional candidate genes[2]
8	rs42263449	26696264	C18:0	*SH3GL2, CNTLN*
8	rs42263474	26763145	C18:0	*SH3GL2, CNTLN*
8	rs43138756	83720729	KET	*ENSBTAG00000009764, CTSV, ZNF484, IARS1, NOL8, CENPP*
8	rs110717374	17138381	KET	*TEK, IFT74, MOB3B, EQTN, LRRC19, PLAA, CAAP1*
9	rs109137030	6218345	MUFA	*no gene*
9	rs109316317	101969365	KET	*TTLL2*
10	rs43619534	31707885	UFA, MUFA	*no gene*
10	rs109974787	5565263	ICF	*SFXN1, HRH2, DRD1*
10	rs41586492	17746328	KET	*LRRC49, THAP10, LARP6, UACA*
10	rs42231661	68291765	KET	*KTN1, PELI2*
10	rs110844686	34985737	KET	*THBS1, FSIP1*
10	rs43710033	43625368	KET	*NIN, MAP4K5, ATL1, SAV1, ENSBTAG00000054530, ABHD12B, PYGL, ENSBTAG00000048395, TRIM9*
11	rs41659401	39446852	ICF	*no gene*
11	rs109882205	41992469	KET	*no gene*
11	rs29022274	41971708	KET	*no gene*
11	rs109038300	47378387	KET	*RPIA, ENSBTAG00000054154, ENSBTAG00000050329, ENSBTAG00000047029, ENSBTAG00000051611, ENSBTAG00000003408, ENSBTAG00000051342, ENSBTAG00000045514, ENSBTAG00000045659, EIF2AK3, TEX37, FOXI3*
11	rs42587069	30352564	KET	*MSH6, FBXO11*
11	rs110926908	44431373	KET	*SH3RF3, SEPTIN10, EDAR, CCDC138, RANBP2, ENSBTAG00000054181*
12	rs41577805	28834261	UFA, C18:0	*FRY, N4BP2L2, N4BP2L1, BRCA2, ZAR1L*
12	rs110539543	82719169	KET	*FAM155A*
12	rs41629862	82277331	KET	*EFNB2, ARGLU1*
12	rs41672734	81142776	KET	*no gene*
12	rs111012814	20111148	KET	*DLEU7, ENSBTAG00000049315, RNASEH2B*
13	rs43705561	70050255	PUFA	*CHD6, PLCG1, ZHX3, LPIN3, EMILIN3*
14	rs109421300	609870	SFA, UFA, MUFA, C16:0, C18:0	*DGAT1, HSF1, TMEM249, SCRT1, BOP1, ADCK5, SLC52A2, FBXL6, ARHGAP39, C14H8orf82, LRRC24, LRRC14, RECQL4, MFSD3, GPT, PPP1R16A, FOXH1, KIFC2, CYHR1, TONSL, VPS28, ENSBTAG00000053637, SLC39A4, CPSF1, SCX, MROH1, ENSBTAG00000039978, HGH1, WDR97, MAF1, ENSBTAG00000051469, SHARPIN, CYC1, GPAA1, EXOSC4, OPLAH, SPATC1, GRINA, PARP10, PLEC*
14	rs110701587	63925324	PUFA	*SNX31, ANKRD46, ENSBTAG00000054554, RNF19A, ENSBTAG00000050156, SPAG1*
14	rs41630566	46570537	PUFA	*EXT1, MED30*
15	rs110508416	37224652	C18:0, KET	*INSC*
15	rs109138685	37720295	KET	*INSC, CALCB, CALCA, CALCB, ENSBTAG00000048777, CYP2R1, PDE3B*

BTA	SNP	SNP position (bp)	Trait[1]	Positional candidate genes[2]
15	rs41632691	83673161	KET	*GLB1L2, B3GAT1, GLB1L3, ENSBTAG00000053675, NCAPD3, ENSBTAG00000046088, VPS26B, THYN1, ACAD8, ENSBTAG00000012229*
15	rs109932511	83710700	KET	*GLB1L2, B3GAT1, NCAPD3, ENSBTAG00000046088, VPS26B, THYN1, ACAD8, ENSBTAG00000012229, GLB1L3,*
15	rs110944919	78017138	KET	*ENSBTAG00000054640, ENSBTAG00000052223, ENSBTAG00000055007, ENSBTAG00000031119, ENSBTAG00000024788, ENSBTAG00000031030, OR4C3, PTPRJ, ENSBTAG00000051670, ENSBTAG00000049550, ENSBTAG00000031025, ENSBTAG00000053247, OR4X1, ENSBTAG00000031032, ENSBTAG00000053761, ENSBTAG00000053438, ENSBTAG00000054267, ENSBTAG00000048640, ENSBTAG00000050306, ENSBTAG00000053824, ENSBTAG00000053261, ENSBTAG00000051253*
15	rs110351063	65323234	C16:0	*EHF, APIP, PDHX*
17	rs41599470	36317694	ICF	*FSTL5, SNORA72*
18	rs110942910	27440587	KET	*no gene*
18	rs109499402	18077073	KET	*ZNF423, ENSBTAG00000051062, ENSBTAG00000052469, C18H16orf78*
18	rs109375227	24203949	KET	*AMFR, NUDT21, OGFOD1, CES1, ENSBTAG00000001851, MT1A, MT1E, MT2A, MT3, ENSBTAG00000049147, ENSBTAG00000049538, MT4, BBS2, GNAO1*
18	rs110198858	33625498	KET	*no gene*
18	rs29021918	42703808	KET	*ZNF507, DPY19L3*
18	rs41632433	28333748	KET	*no gene*
18	rs110600398	27938774	KET	*no gene*
19	rs109477972	29784751	SFA, C16:0	*PIRT, MYH2, MYH3, SCO1, ADPRM, TMEM220*
19	rs41644917	40381198	PUFA, C18:0	*THRA, MED24, NR1D1, CSF3, ENSBTAG00000045067, MSL1, IKZF3, ZPBP2, GSDMB, ORMDL3, LRRC3C, ENSBTAG00000050854, GSDMA, PSMD3, CASC3, RAPGEFL1, WIPF2, CDC6, RARA, ENSBTAG00000052844, TOP2A*
19	rs110933534	40505729	PUFA, C18:0	*WIPF2, CDC6, RAPGEFL1, ORMDL3, LRRC3C, GSDMA, PSMD3, CSF3, MED24, THRA, NR1D1, MSL1, CASC3, RARA, ENSBTAG00000052844, TOP2A, IGFBP4, TNS4*
20	rs109946603	9419987	KET	*MAP1B, MRPS27, ZNF366, PTCD2, ENSBTAG00000053736*
21	rs109823489	56044735	PUFA	*CCDC88C, GPR68, FRMD5, ENSBTAG00000050803, PPP4R3A*

BTA	SNP	SNP position (bp)	Trait[1]	Positional candidate genes[2]
22	rs41993977	5531843	SFA, C16:0	*GADL1*
22	rs41994020	5499054	KET	*GADL1*
22	rs41994761	5476277	KET	*GADL1*
24	rs41570441	49057452	PUFA	*DYM, C24H18orf32, RPL17, LIPG*
27	rs110519353	36466414	SFA, C16:0	*GINS4, GOLGA7, SFRP1, GPAT4, NKX6-3, ENSBTAG00000027629, ENSBTAG00000054394, ENSBTAG00000003275*
27	rs109734522	36747901	C16:0	*ENSBTAG00000052888, GPAT4, NKX6-3, ENSBTAG00000027629, ENSBTAG00000054394, ENSBTAG00000003275, KAT6A,*
27	rs42138713	41773014	C16:0	*THRB, ENSBTAG00000050025, NR1D2*
27	rs41647957	8009249	KET	*VEGFC, ASB5, SPCS3*
28	rs42854990	2669298	PUFA	*ENSBTAG00000048153*
28	rs109278212	45261673	UFA, MUFA	*CXCL12*
28	rs110222344	43155287	UFA	*WDFY4, ARHGAP22, LRRC18, VSTM4, FAM170B*
28	rs109839180	19620469	UFA, MUFA, PUFA	*REEP3, JMJD1C*
28	rs41586819	16074091	KET	*ENSBTAG00000050189*
29	rs29026721	28190477	KET	*SPA17, SIAE, NRGN, VSIG2, ESAM, ENSBTAG00000054187, ENSBTAG00000051944, ENSBTAG00000051107, ENSBTAG00000054033, ENSBTAG00000048913, PANX3, TBRG1, MSANTD2, ROBO3, ROBO4, HEPACAM, CCDC15*
29	rs109868969	17135246	KET	*TENM4*

[1] SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, C16:0 = palmitic acid, C18:0 = stearic acid, KET = ketosis, ICF = interval from calving to first insemination.

[2] Positional candidate genes located in the interval of 250 kb surrounding associated SNP were retrieved from Ensembl release 102 (Zerbino et al., 2018).

Supplemental Figures

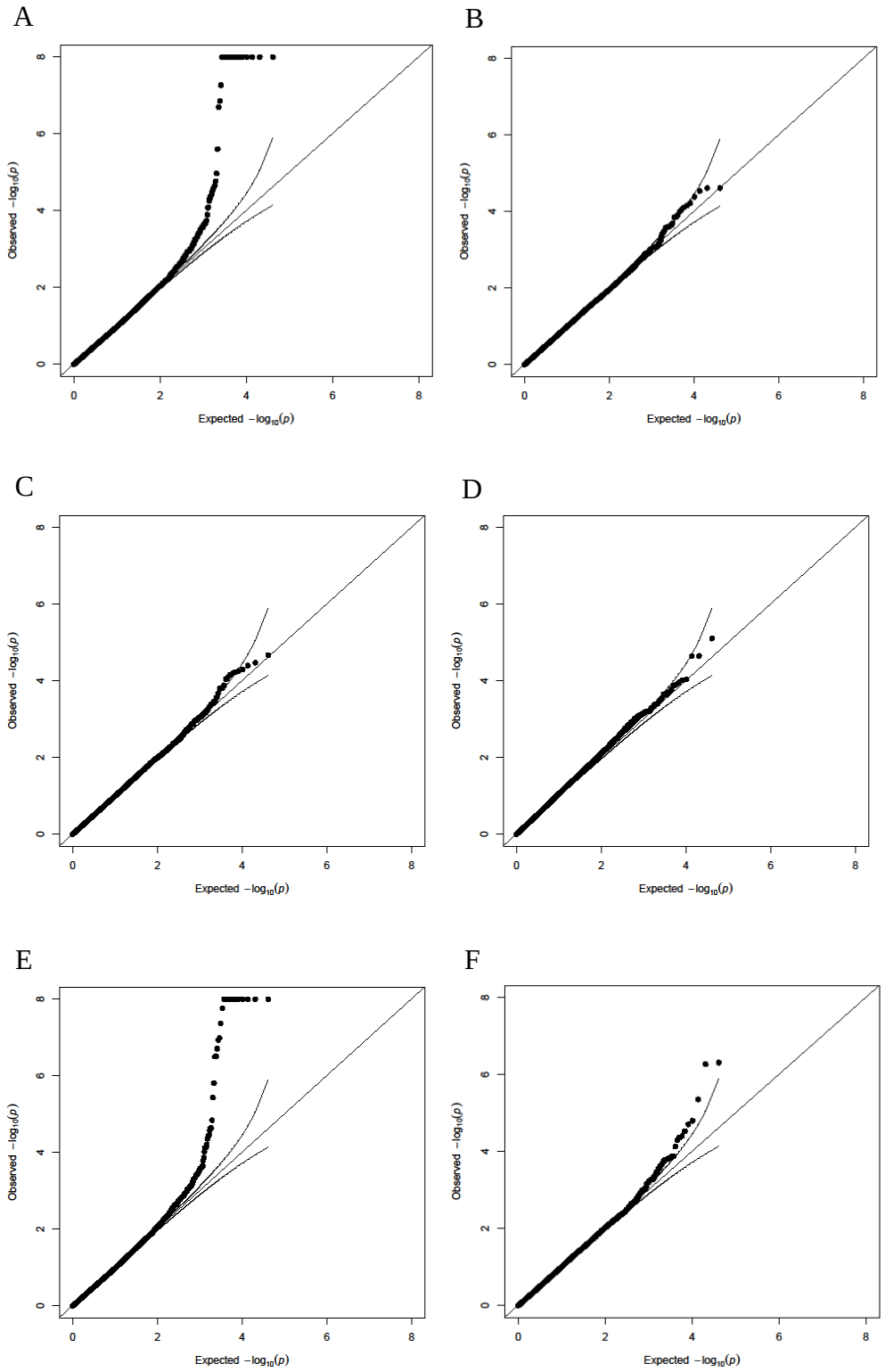

Figure S4.1. Quantile-Quantile plots for first test-day (A) saturated, (B) unsaturated, (C) monounsaturated, (D) polyunsaturated, (E) palmitic, and (F) stearic fatty acid concentration in first-lactation Holstein cows.

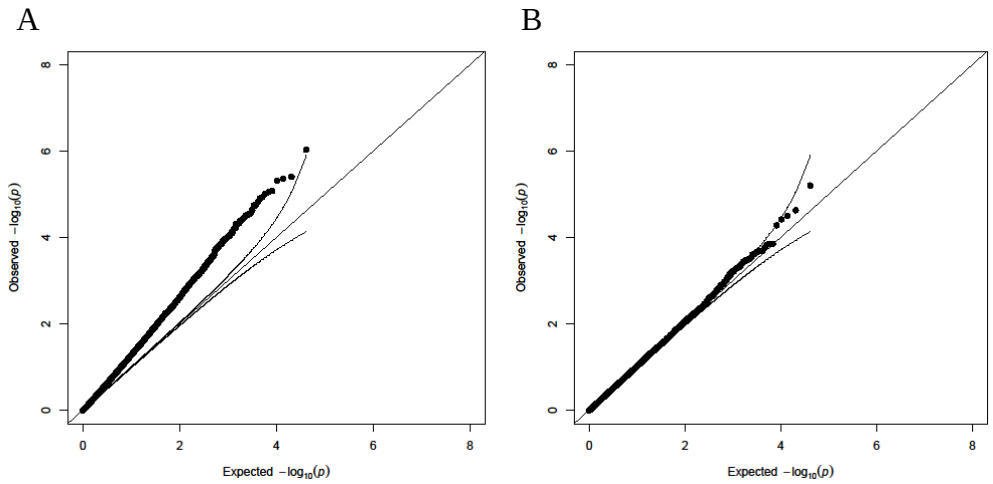

Figure S4.2. Quantile-Quantile plots for (A) ketosis, and (B) interval from calving to first insemination in first-lactation Holstein cows.

CHAPTER 5

General Discussion

Ketosis associations and negative energy balance cow types

Our results suggested strong relationships between KET, the first test-day milk FPR (chapter 2), acetone and BHB concentrations (chapter 3) on phenotypic scales. Cows suffering from KET in early lactation exposed significantly higher FPR, acetone and BHB concentrations than KET healthy cows. Furthermore, we identified negative influence of KET and first test-day milk ketone bodies on milk production traits from the very early-lactation period (chapter 3). The milk yield and the protein percentage was significantly decreased with increasing BHB and acetone concentrations while fat percentage, the FPR, and the SCS increased due to the strong body fat mobilization and immune responses in the mammary gland (Dodds et al., 1981; Hillreiner et al., 2016). We recommend the use of acetone and BHB thresholds for KET monitoring of 0.15 m*M* and 0.10 m*M*, respectively. In our study, those values were already significantly associated with a KET diagnosis, with reduced milk production and changes in milk composition. Previous studies proposed higher thresholds of 0.15 - 0.19 m*M* (milk BHB levels; Santschi et al., 2016; Churakov et al., 2021). Considering our results cows that are in a subclinical stage of KET would be disregarded and would not be detected using those high cut-off values in practice.

Assessing the influence of KET on first test-day FA milk concentration in first-lactation Holsteins, considering the data set described in chapter 4 and a mixed model for FA as implemented in SAS (version 9.4, SAS Institute Inc., Cary, NC, chapter 4, model [1], including KET as fixed effect, excluding additive-genetic effects and relationship matrix) we also identified significant effects of KET on UFA, MUFA, and C18:0 (Table 5.1). First test-day concentrations of UFA, MUFA, and C18:0 were significantly increased due to KET diagnosis. According to Churakov et al. (2021), who associated C18:0 concentration of 0.47 g/100 g of milk with severe NEB our results, especially regarding C18:0, displayed the suggested threshold of 0.47 g/100 g of milk significantly associated with KET (Table 5.1). Churakov et al. (2021) presented this threshold as optimal value that maximizes the sum of sensitivity and specificity to detect cows in NEB. To the best of our knowledge, our phenotypic analysis presented first results on cut-off values for specific first-test-day FA concentrations associated with KET diagnosis (Table 5.1). These findings will have practical implications and will be useful for on-farm detection of metabolic disease KET and severe NEB.

Table 5.1. Least squares means (corresponding SE in parentheses) of first test-day fatty acid concentrations in milk (in g/100 g of milk) for cows with the absence (0) or the presence (1) of a ketosis diagnosis.

	Ketosis diagnosis	
Trait	0	1
Saturated fatty acids	2.49 (0.01)[a]	2.52 (0.09)[a]
Unsaturated fatty acids	1.42 (0.01)[a]	1.72 (0.06)[b]
Monounsaturated fatty acids	1.16 (0.01)[a]	1.41 (0.06)[b]
Polyunsaturated fatty acids	0.16 (0.00)[a]	0.17 (0.01)[a]
Palmitic acid	1.09 (0.00)[a]	1.11 (0.04)[a]
Stearic acid	0.41 (0.00)[a]	0.47 (0.02)[b]

[a, b] Different superscripts indicate significant differences ($P < 0.001$).

Döpfer (2021) provided a reason for a monitoring on NEB impaired cows not exclusively relying on BHB concentration. According to Döpfer (2021) cows differently reacted to the NEB due to different degrees of body fat mobilization, liver damage, immunosuppression, or changes in dry matter intake. The different response patterns reflected five cow types: the athlete cow, clever cow, healthy cow, hyperketonaemic cow, and the poor metabolic adaptation (**PMAS**) cow. The athlete cow represented a cow with high blood BHB concentrations, milk production and FPR, the clever cows had low BHB concentrations and lower FPR, the healthy cows neither showed increased BHB nor elevated NEFA levels and had normal milk production. Elevated BHB values and strongly decreased milk production were present in the hyperketonaemic cows. A PMAS cow revealed low BHB but increased NEFA values, decreased dry matter intake, reduced rumen activity and milk production (Tremblay et al., 2018). Hence, Döpfer (2021) concluded that cows impaired by NEB belonging to category five cows (PMAS cows) will not be detected, if early lactation monitoring just focus on blood BHB values. According to that, we assume that the consideration of milk FA besides milk BHB levels would be advantageous in detecting PMAS cows and cows monitoring in the phase of NEB.

The phenotypic associations were reflected by determined genetic correlations. Also on genetic scales the strong positive correlations between KET, the FPR (chapter 2) and ketone body concentration (chapter 3) hinted to the fact that an inclusion of these indicator traits in breeding programs would lead to metabolic healthier cows at the beginning of lactation. Accordingly, the phenotypic associations between KET, ketone bodies with milk production traits were confirmed by the detected negative genetic correlations of KET and ketone bodies

with first test-day milk yield, protein percentage, and positive genetic correlations with fat percentage, FPR, and the SCS (chapter 3). Additionally, we estimated strong positive genetic correlations between KET and the novel potential milk indicators, the first test-day UFA, MUFA, and C18:0 milk concentrations and low to moderate positive genetic correlations between those FA, KET, and the fertility interval trait ICF (chapter 4). Thus, an inclusion of milk indicators in an index health trait would allow an improvement in early lactation metabolic health, possibly leads to an earlier resumption of oestrus activity and, in that way, shorten the interval from calving to first insemination. In consequence, this might result in lower costs for veterinary treatments regarding KET, related diseases, and lower milk yield losses.

Canada, Denmark, Finland, and Sweden already include KET milk indicators (i.e., BHB milk levels, FPR) in KET breeding value estimations. With regard to our results the consideration of KET milk indicators such as first test-day FPR, acetone, BHB, and the FA concentration in the RZmetabol index in the trait KET is recommended. As shown by Rius-Vilarrasa et al. (2018) the inclusion of indicator traits in breeding value estimations will improve reliabilities of KET breeding values. The use of the information on acetone, BHB, and FA milk concentration could also allow additional subdivision of the KET breeding value into subclinical and clinical KET.

Genomic variant associations and candidate genes for ketosis

Results of the GWAS regarding KET (chapter 2; chapter 4) highlighted the importance of genes related to FA metabolism, immune response, and insulin resistance in KET progression. The suggestively and significantly associated SNP were located close to candidate genes involved in obesity, fatty liver disease (e.g., *EFCAB6*, *PARVB*), insulin resistance, and diabetes (e.g., *HMBOX1*), as well as inflammatory response (e.g., *ALB*, *CXCL8*, *VEGFC*). Also for identified KET milk indicators, FPR, acetone, BHB, and UFA, MUFA, C18:0 FA, positional candidate genes related to these traits were detected (e.g., *ADARB2*, *NRXN3*, *ACOXL*, *BCL2L11*, *HIBADH*, *CXCL12*, *ARHGAP22*, *JMJD1C*, *IGFBP4*, chapter 2, chapter 3, chapter 4). The metabolic diseases KET and fatty liver were strongly related to the NEB and may also be connected with a state of insulin resistance (Herdt, 2000). Insulin a key metabolic hormone plays a major role in lipid metabolism. Insulin blood concentration is influenced by glucose and glucose precursors, stimulates glucose uptake in cells, promotes lipogenesis and inhibits lipolysis in adipose tissue. Thus, insulin supressed the influx of free FA in the blood. In the liver, insulin reduced activity of carnitine palmitoyltransferase I (CPTI, chapter 1, Figure 1.2), hence, decreased the transport of NEFA into mitochondria and supressed ketogenesis (De

Koster and Opsomer, 2013). An insulin-deficient state or insulin resistance caused restricted lipogenesis and supports lipolysis (Hayirli et al., 2006). Within the liver, the NEFA from adipose tissue lipolysis could be metabolized through β-oxidation and to ketone bodies via ketogenesis. However, when the hepatic uptake of NEFA exceeded the oxidation, a fatty liver is developed (Grummer, 1992; Bobe et al., 2004). The excess NEFA were reesterified to triglycerides, accumulated in the liver, impaired metabolic liver functions, and increased the risk for KET (Herdt, 2000). Studies investigating endocrine mediators suggested a close relationship between dairy cows KET, insulin resistance, liver function and oxidative stress (Xu et al., 2014; Cuiyu et al., 2019). For example Cuiyu et al. (2019) examined the relationship between insulin resistance and KET in dairy cows 14 to 21 days postpartum. Glucose tolerance of blood glucose levels in 120 min was used as the cut-off point to divide dairy cows into three groups: the abnormal glucose tolerance KET group (TH), the normal glucose tolerance KET group (TL), and the healthy control group. Results showed that the insulin sensitivity value was significantly lower in TH group. Additionally, liver function abnormalities, determined due to aspartate aminotransferase plasma levels, were more severe in the TH than in the TL and control group. The concentration of BHB, NEFA and oxidative stress was significantly higher in the TH group than in controls. However, insulin resistance can deteriorate lipolysis of adipose tissue and the accumulation of NEFA in turn leads to greater insulin resistance which is associated with health problems such as fatty liver and oxidative stress (Cuiyu et al., 2019).

Generally, if glucose concentrations were abundant, adipose lipogenesis would be favored over lipolysis (Herdt, 2000). This effect might be mediated as described by insulin but could also be related to the effect of glucose in glycerol synthesis. For triglyceride synthesis a source of glycerol is needed. The major precursor of adipose glycerol is glucose, thus, its presence enhances glycerol availability and favors lipogenesis. During NEB glucose concentration is reduced and NEFA mobilization from adipose tissue is stimulated due to the lack of glycerol (Herdt, 2000).

The relationship between KET with inflammatory response genes might be based on the understanding of KET as a response to systemic inflammation in early lactation (Zhang and Ametaj, 2020). Zhang et al. (2016) compared markers for innate immunity activation during the dry-off period (pre-ketotic cows), the week of diagnosis of KET (ketotic cows), and the weeks of recovery from KET (post-ketotic cows). Results showed that both pre-ketotic and ketotic cows had accumulated pro-inflammatory cytokines (e.g., interleukin-6 and tumor necrosis factor), as well as acute phase proteins (e.g., haptoglobin) in the serum compared with healthy controls. Thus, cows with KET experienced a low-grade chronic inflammatory state

before the occurrence of the disease (Zhang et al., 2016). Results were confirmed by Abuajamieh et al. (2016) who found increased acute phase protein serum amyloid A, haptoglobin and lipopolysaccharide binding protein in ketotic Holstein cows post calving. Moreover, cows with subclinical KET showed a higher immune response compared with metabolically healthy individuals based on increased lymphocytes, increasing stimulative properties of peripheral blood mononuclear cell and a relationship between haptoglobin and typically increased values of BHB and NEFA (Schulz et al., 2015).

Sartorelli et al. (2002) reported that ketone body BHB and acetoacetate suppressed non-specific immunity by reducing efficiency of chemotaxis and phagocytosis in neutrophils of ketotic small ruminants. This effect may explain the increased occurrence of infectious diseases during the ketotic state. Thus, on the one hand KET and elevated ketone bodies were related to the activation of the innate immune response but on the other hand, especially, BHB served as a suppressor of inflammatory response, facilitating the development of infectious diseases.

Applying single-step approach in our last GWAS study (chapter 4) identified genomic regions on BTA 5, 6, 10, 15, and 20 associated with KET were common to preceding GWAS results (Nayeri et al., 2019; Soares, 2020; Soares et al., 2021). Thus, the overlapping genomic regions in different studies suggested that these regions should be of great interest in future functional studies analyzing KET progression. Again, positional candidate genes e.g., *PDE3B, PYGL*, *THBS1*, and *CXCL8* were involved in glycolysis, lipolysis, insulin resistance, and inflammatory response. Interestingly, the used single-step approach (chapter 4) analyzing genomic KET and FA associations based on H matrix resulted in a higher number of significantly and suggestively associated SNP regarding producer-recorded KET compared with GWAS results based on G matrix (chapter 2). In the ssGWAS used in chapter 4, all phenotypic information from genotyped and ungenotyped animals as well as their ancestors' information was used simultaneously through common genomic and pedigree information. The main advantage of ssGWAS is the ability to incorporate phenotypes of ungenotyped subjects directly in the association analyses without the necessity to construct pseudo-observations (Wang et al., 2012; Li et al., 2019). Therefore, ssGWAS could be more useful when a large number of phenotyped subjects is not genotyped (Li et al., 2019). Aguilar et al. (2019) confirmed that ssGWAS is an efficient method in QTL detection and *P*-value generation, particularly in complex data sets. The consideration of phenotypic, pedigree and genomic information in ssGWAS and thus higher number of animals used in our study (chapter 4) may have led to the greater number of SNP associations for KET compared with our preceding study results (chapter 2). Especially, regarding highly polygenic traits and the case-control study

design the more information included in the analysis due to bigger sample size considered, the more robust associations could be identified (McCarthy et al. 2008; Li et al., 2019).

In general, GWAS for complex traits helped to explore the relationship between common genome sequence variation and genome-wide disease predisposition (McCarthy et al., 2008). Nevertheless, SNP associations were mostly suggestive and not significant regarding KET (chapter 2, chapter 4) reflecting the polygenic character of the complex disease trait. Polygenic traits are influenced by multiple genomic regions with compared low effects (Kemper and Goddard, 2012). In comparison to commonly used medium-density SNP chip arrays the usage of whole-genome sequence (**WGS**) data in GWAS was confirmed as an effective method in identifying common and also rare genomic variants especially for complex polygenic traits in cattle (Daetwyler et al., 2014; Wolf et al., 2021). According to Wu et al. (2015) who compared association results for udder health in Holsteins using medium-density, high-density SNP chip, and sequence data, the dense genomic information lead to more powerful and reliable GWAS results. The power of association detection significantly increased with ascending marker density. Due to the application of sequence data the number of significantly associated genomic regions for the complex trait udder health was increased and important genomic regions could be defined more precisely (Wu et al., 2015). With increasing SNP density the probability that a SNP is in a perfect linkage disequilibrium with a QTL is enhanced (Meuwissen et al., 2016). Furthermore, using WGS the causative mutations might be present in the data set and genomic selection can use this causative mutation information directly instead of relying on the linkage disequilibrium between marker and causative mutation (Meuwissen et al., 2016). Additionally, compared with WGS data SNP chips are known to lack a substantial proportion of globally rare variants (Geibel et al., 2021).

This leads to the suggestion that the implementation of WGS data in association analyses for KET might have beneficial effects on the GWAS results. The medium-density SNP chip with around 54,000 marker is commonly used for GWAS in dairy cattle. However, during the last decades high-density SNP chip (~ 777,000 marker) were designed and WGS data became available. As sequencing costs continue to decline WGS will be more and more applied in future analyses, in particular, regarding traits with complex genomic architecture (Meuwissen et al., 2016; Pryce et al., 2018).

Impact of epigenetic processes related to negative energy balance and ketosis

Epigenetics comprises the investigation of heritable molecular modifications responsible for the regulation of genome activities and gene expression resulting in phenotypic differences

without alterations to the basic DNA sequence (Wang and Ibeagha-Awemu, 2021). The word epigenetic could be divided into “epi” and “genetic” referring to information beyond encoded DNA sequence (Greally, 2018) and epigenetic processes due to DNA methylation, histone modification, chromatin remodeling, non-coding RNA regulation, affected gene expression, genome function, and stability. These processes form the epigenome being subject to continued changes and thus dynamic during the whole life. Several studies have provided evidence of epigenetic regulation processes involved in livestock health (Wang and Ibeagha-Awemu, 2021).

Van Hoeck et al. (2013) and Desmet et al. (2016) found that DNA methylation changes due to maternal stressors (i.e., metabolic disorders) partly explained poor performances of offsprings. The analysis regarding the exposure of maturing bovine oocytes and developing embryos to elevated NEFA suggested that maternal metabolic disorders can disturb epigenetic programming, i.e., DNA methylation in the offsprings. Oocytes and embryos exposed to pathophysiological concentrations of C18:1, C16:0, and C18:0 FA resulted in blastocysts with different DNA methylation and transcriptomic fingerprints compared to physiological concentrations of these FA. Transcriptomic comparison revealed that 311 genes were differently expressed in blastocysts originated from embryos cultured with high FA concentrations compared to normal conditions. Differently methylated and expressed genes of blastocysts (i.e., *LEP*, *TCR*, *IGF1R*, *LIF*, *PEPCK*) were related to lipid and carbohydrate metabolism, cell death, immune response, and metabolic disorders. Additionally, Van Hoeck et al. (2011) indicated that blastocysts exposed to the high FA concentration displayed glucose intolerant and mitochondrial dysfunction signs (i.e., reduced oxygen, pyruvate and glucose consumption, up-regulated lactate consumption, higher amino acid metabolism). Furthermore, the number of blastocysts from oocytes matured and the number of blastocysts from cleaved zygotes were significantly decreased due to the influence of pathophysiological FA concentrations (Desmet et al., 2016). According to that, Carvalho et al. (2014) showed that Holstein cows significantly losing body weight from calving to three weeks after calving displayed a lower number of viable and transferable embryos after a superovulation around 100 days postpartum. As summarized by Leroy et al. (2017) oocytes and embryos were of questionable quality in females suffering from lipolytic disorders. As stated by Desmet et al. (2016) more research is necessary to examine long-term effects of the epigenetic dysregulation.

Epigenetic effects of the ketone body BHB on histone acetylation (an epigenetic modification), bovine oocytes and embryos were assessed by Sangalli et al. (2018). Cumulus-oocyte complexes were matured without (control group) or supplemented with 2 m*M* of BHB

(BHB group) during in vitro maturation. Additionally, subsequent embryos were incubated with or without 6 m*M* BHB supplement. Using histone acid extraction, H3K9ac, a robust post-transcriptional modification, was measured and no differences in H3K9ac levels were detected in controls or BHB treated oocytes. The BHB treatment stimulated genes associated with ketolysis and metabolism regulators (e.g., *BDH1*, *OXCT1*, *ACAT1*, *PPARA*, *PPARGC1A*, and *SREBF1*) in cells. Also in oocytes treated with BHB gene expression of *PPARA*, a regulator for genes involved in lipid metabolism and ketone body synthesis was upregulated. The expression of *PPARA* is strongly induced during fasting to shift metabolism away from glucose metabolism to lipolysis to provide alternative sources of energy for the organism. Whereas no influence of BHB was detected on H3K9ac levels in oocytes significant influence was identified on H3K9ac levels and gene expression of *FOXO3A*, a gene related to oxidative stress response, in zygotes which maintained until blastocyst stage. Zygotes treated with BHB showed elevated levels of histone acetylation and blastocysts generated from these zygotes presented elevated H3K9ac levels and gene expression of *FOXO3A*. Those findings suggested that the metabolite BHB commonly circulating in cows' blood, was able to affect an epigenetic mark (H3K9ac) in zygotes and blastocysts (Sangalli et al., 2018).

The in vivo study of Chaput and Sirard (2020) analyzing the embryonic response to high BHB levels postpartum dairy cows confirmed the existence of epigenetic processes in this context. Differential expression in response to increased BHB concentrations in the maternal environment due to NEB in early lactation was found for 1,154 genes. 891 genes were downregulated and 335 were upregulated in embryos from cows in metabolic deficit. Genes *FOXP4* and *OPA1* of relevance to energy metabolism and mitochondrial functions showed significant expression differences in morulae from cows with high BHB blood levels postpartum (Chaput and Sirard, 2020). Moreover, Chaput and Sirard (2020) detected hypermethylation in high BHB groups in all regions except for exons. DNA methylation i.e., the chemical modification on a cytosine base, is one of the most widespread epigenetic marks and influences gene transcription. Thus, these findings indicated the appearance of a characteristic epigenetic signature of energy deficit experienced in vivo raised embryos, possibly reflecting an adaption to the maternal metabolic stress (Chaput and Sirard, 2020). Summarized, the in vivo and in vitro results suggested an influence of NEB, KET, elevated ketone bodies, and FA on cows' fertility, oocyte maturation and blastocyst development due to epigenetic processes and altered gene expression.

A recent study by Wu et al. (2020) addressed clinical KET associated alterations of gene expression in Holstein cows during the transition period. Comparison of gene expression of

healthy and KET diseased cows pre- and postpartum revealed 75 and four differently expressed genes between sick and healthy cows at post- and prepartum, respectively. Subsequent functional analyses exposed one gene *STX1A* associated with stress stimulations significantly higher expressed in sick cows pre- and postpartum. The potential biological effect of *STX1A* on KET has to be investigated (Wu et al., 2020).The presented studies indicated an epigenetic influence of the state of NEB and KET in dairy cows, especially, in reproductive organs in postpartum cattle not detectable with common GWAS. Future gene expression and epigenetic analysis probably provides insights on how KET might also affect the offspring by altering gene expression due to epigenetic processes during early embryonic stage.

Usefulness of FTIR measurements

In our studies we confirmed the usefulness of first test-day FTIR measurements of FPR and ketone body concentrations in early lactation KET monitoring. On the one hand ketone body concentrations provide an indication of the disease relatively early and as our analyses showed up to several weeks before KET was diagnosed (chapter 3). The FPR on the other hand showed significant changes only shortly before and on the actual diagnosis day (chapter 2). Therefore, it would be advantageous for dairy cattle farmer, if milk sampling with respect to KET indicators took place more frequently in the first weeks after calving.

The FTIR data used in our studies were generated by the same analysis instruments in the milk recording organization. Thus, a standardization of data was not necessary. However, milk recording organizations around the globe use different analyzers for FTIR predictions. In that way, the data generated is specific to the particular spectrometer used. Differences between predictions originated from characteristics and modes specific to each spectrometer model, different uses, replacements, and maintenance operations (Grelet et al., 2017). The noise due to differences between the instruments reduced the prediction accuracies (Tiplady et al., 2019). For a large-scale usage of FTIR milk KET indicator data i.e., in breeding value estimations, standardization of infrared spectral data is mandatory. Several studies and projects (e.g., OptiMIR) were implemented to harmonize FTIR data from different analyzers to create comparable result (Grelet et al., 2017; Tiplady et al., 2019; Gruber, 2021).

Grelet et al. (2017) evaluated if a spectral standardization method would enable the use of multiple equations within a network of different spectrometers. By comparing the spectral variability between 66 instruments from three different brands and 26 laboratories in Austria, Belgium, Canada, France, Germany, Luxembourg, Switzerland, and the United Kingdom the standardization was assessed. Standardization procedure based on the piecewise direct

standardization (**PDS**) method which relates milk spectra on a standard primary instrument to spectra on other instruments using identical milk based reference samples was considered. The standardization resulted in a reduction of variability between spectrometers, more precise, fine milk predictions (i.e., PUFA predictions) and an increase in the accuracy of the predictions. The PDS method has been shown to be valuable for the transfer of spectra from one instrument to another. Grelet et al. (2017) assumed that a standardization process will have positive effects on breeding studies which have to rely on many comparable records. In breeding evaluations nonstandardized data would inflate residual variance, reduce heritability and genetic progress. Additionally, genetic correlations between FTIR traits and direct traits would be lower with nonstandardized FTIR data from different instruments (Grelet et al., 2017). According to that, Tiplady et al. (2019) compared different standardization methods for FTIR spectra regarding classical milk components such as protein, fat, and lactose predictions including PDS and retroactive percentile standardization (**RPS**) to reduce between-instrument variability. The RPS method used percentiles of the observed spectra from routine milk test samples to map and exploit relationships between standard primary instrument and the other instruments. Tiplady et al. (2019) demonstrated that the PDS approach lead to the most consistent reduction in prediction errors across time, is less sensitive to shifts in milk composition and non-instrument errors and concluded that this was the optimal standardization approach.

Most of the equation models were developed in research contexts and not practicable in a routine usage. Hence, Grelet et al. (2021) recommended a development of international guidelines and collaborations to generate large robust milk spectra data sets and consistent routine model use.

Conclusions and recommendations

This thesis specifies relationships of metabolic disease KET, innovative KET milk indicators, production traits measured via FTIR technic on the first test-day and fertility interval trait ICF. Trait relations were phenotypically, genetically and genomically analyzed in consideration of a cow reference group. Metabolic disease KET significantly influenced the first test-day FPR, acetone, BHB, UFA, MUFA, and C18:0 milk concentration. Thus, there is great potential for the milk indicators measured via FTIR with regard to reliable KET detection in early lactation. Genetic correlations between KET, FTIR ketone body, UFA, MUFA, and C18:0 concentrations were strong positive indicating genetic improvement in KET due to an inclusion of the assessed indicator traits in health indices of breeding programs. Low to moderate positive genetic correlations were estimated for KET, UFA, MUFA, C18:0 with ICF. Hence, an enhancement

in metabolic health might simultaneously lead to an earlier resumption of the oestrus. The GWAS results provide new insights in the genomic architecture of complex disease trait KET and corresponding innovative milk measurements. The results of the GWAS lay the basis for more profound investigations regarding the genetic expression that underlies KET progression, innovative milk indicator traits and ICF. In future, this might offer the opportunity to select for healthier cows regarding early lactation disease KET.

The major results of this work and recommendations are:

- Strong phenotypic relations between KET and milk indicator traits i.e., the FPR, acetone, BHB and UFA, MUFA, C18:0 concentrations on first test-day exist and could be used to derive management decisions. According to our results we suggest the usage of lower threshold values for ketone body concentrations of 0.15 m*M* and 0.10 m*M* for acetone and BHB, respectively, compared to commonly used thresholds in KET prevention and monitoring. Additionally, the inclusion of FA, especially C18:0 (threshold value of 0.47 g/100 g of milk), will have practical implications and may also allow the identification of cows suffering from NEB but not showing the typical increased BHB concentration. Moreover, we recommend a more frequently milk sampling regarding the assessed indicator traits in the first weeks after calving.
- Phenotypic and genetic relations between KET and ketone body concentrations with first test-day milk production traits were favorable suggesting positive influence of selecting for KET healthier cows on milk production traits in the first weeks after calving.
- Genetic parameter estimates of KET, indicator traits and ICF revealed low to moderate heritabilities. Genetic correlations between KET diagnosis and novel milk indicator traits were large positive. We recommend their implementation in metabolic health breeding goals e.g., in KET index included in the RZmetabol. Low to moderate positive genetic correlations were detected for KET, UFA, MUFA, C18:0, and ICF. Accordingly, selection strategies for metabolic health improvement might have positive effects on the fertility interval trait. For large scale integration of FTIR measurements in breeding programs uniform screening and standardization of FTIR data should be used.
- On the basis of innovative FTIR milk indicators and direct KET diagnosis, genomic regions of interest and potential candidate genes for KET were identified. Future functional analyses might improve the biological understanding of KET.

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Further Publications

2019	Klein, S.-L., Scheper, C., Swalve, H. H., König, S. (2019): Genomische und quantitativ-genetische Assoziationen für Fettsäureprofile in der Milch in Bezug zu Ketoseerkrankungen in der Frühlaktation. Jahrestagung/Vortragstagung der DGfZ und GfT, 11. - 12. September 2019, Gießen Klein, S.-L., Scheper, C., Swalve, H. H., König, S. (2019): Association analyses for ketosis indicators – ketone bodies and fatty acid profiles in Holstein cows. 70th Annual Meeting of the European Federation of Animal Science, 26. - 30. August 2019, Ghent, Belgien
2018	König, S. und Klein, S.-L. (2018): Probleme wegzüchten. Elite, Magazin für Milcherzeuger, 1, 56-57 König, S., Klein, S.-L., Brügemann, K. (2018): Genomische Selektion: Internationale Aktivitäten, Wettbewerb und Perspektiven. DGfZ-Schriftenreihe 75, 132-142 König, S., Klein, S.-L., Brügemann, K. (2018): DNA-basierte Züchtung: Umsetzung in Zuchtprogrammen – internationaler Wettbewerb. 8. Rinder-Workshop, 6. - 7. März 2018, Uelzen Klein, S.-L., Scheper, C., May, K., König, S. (2018): Phänotypische und genomweite Assoziationen für ß-Hydroxybutyrat und Aceton in der Milch als potentielle Indikatoren für Ketose in der Frühlaktation. Jahrestagung/Vortragstagung der DGfZ und GfT, 12. - 13. September 2018, Bonn
2017	König, S. and Klein, S.-L. (2017): The modern Holstein cow and practical milk production: Prospects for improving animal health by using breeding strategies. Elite Herd Management-Conference, 12. January 2017, Wageningen

2017	Klein, S.-L., Brügemann, K., Naderi, S., König, S. (2017): Genetic parameter and SNP marker effects for metabolic diseases and stress indicators. 68th Annual meeting of the European Association for Animal Production, Tallinn, Estland, 28. August - 1. September 2017
	König, S. und Klein, S.-L. (2017): Sind unsere heutigen HF-Kühe noch praxistauglich? Züchterische Möglichkeiten zur Verbesserung der Tiergesundheit. Elite Herdenmanagement-Konferenz, 13. Januar 2017, Osnabrück
	Klein, S.-L., Scheper, C., Brügemann, K., Naderi, S., König, S. (2017): Fett-Eiweiß-Quotient: Ein Indikator für Ketose in der Frühlaktation? Kuh-L Abschlussworkshop, 10. Oktober 2017, Kassel
	Klein, S.-L., Scheper, C., Brügemann, K., Naderi, S., König, S. (2017): Genetische Parameter und genomweite Assoziationen für Stoffwechselerkrankungen und metabolische Stressindikatoren bei HF-Kühen. Jahrestagung/Vortragstagung der DGfZ und GfT, 20. - 21. September 2017, Stuttgart

Curriculum Vitae

Der Inhalt wurde aus Datenschutzgründen entfernt.

Formal Declaration

Erklärung gemäß der Promotionsordnung des Fachbereichs 09 vom 07. Juli 2004 § 17 (2)

„Ich erkläre: Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen, den _____

_____

Sarah-Luisa Klein