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Discovery and Validation of Immunological Biomarkers in Milk for Health Monitoring of Dairy Cows - Results from a Multiomics Approach

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Abstract

At onset of milk production and in early lactation highly producing dairy cows are most susceptible for inflammatory diseases due to functional suppression of immune cells. Intensive supervision of the animals is essential and implementation of new technologies to on-farm routines will be the next step to provide automation and improvement of herd health monitoring programs. Objective of our study was to identify and validate immunological biomarkers in milk that indicate extra-mammary inflammatory diseases to characterize the general health status of highly-producing dairy cows.

In total 89 healthy and 75 diseased animals (German Holstein cows) were included. Diseases were distinguished by either systemic (extra-mammary) occurrence or those affecting the mammary gland (mastitis) and further classified by their severity. For protein biomarker discovery we used a top-down approach to narrow down a broad range of secreted gene products of the milk cell transcriptome (microarray) and proteome to a few promising candidates which were validated using real-time PCR and ELISA. The most promising biomarker candidates were statistically evaluated. Receiver operating characteristic analysis revealed haptoglobin, secretory component, lactoferrin and vascular endothelial growth factor showing the highest discriminatory capability for diseased vs. healthy cows. Values for sensitivity at a specificity of 94% were 82% for haptoglobin, 59% for secretory component, 55% for lactoferrin and 67% for vascular endothelial growth factor. Statistical evaluation by multinomial logistic regression and k-nearest neighbor method confirmed haptoglobin as the best single-use biomarker. In combination with secretory component or lactoferrin an increase in overall sensitivity or specificity, depending on the classification method, could be achieved.

The application of the validated health biomarkers in combination with an easy high-throughput detection system would offer a solution to adapt dairy herd management to changing requirements on animal welfare, farming efficiency, milk supply and food safety in modern agriculture.

Keywords: Dairy cow; Health monitoring; Milk biomarkers; Acute phase proteins

Abbreviations APP: Acute Phase Proteins; aRNA: Amplified RNA; AUC: Area Under the Curve; BL: Peripheral Blood Leukocytes; CCL25: C-C motif Chemokine 25; CFD: Complement Factor D; CFU: Colony-Forming Units; CV: Cross-Validation; CXCL10/11: C-X-C motif Chemokines 10 and 11; DEG: Differentially Expressed Gene; DPP: Days Post-Partum; FC: Fold Change; FG: Fibrinogen; HEOM: Heterogeneous Euclidean-overlap Metric; HP: Haptoglobin; Ig: Immunoglobulins; IL: Interleukin; K-NN: K-Nearest Neighbour method; LTF: Lactoferrin; MC: Milk Cells; MLR: Multinomial Logistic Regression; MS: Mass Spectroscopy; PIGR: Polymeric Immunoglobulin Receptor; PMN: Polymorph-nuclear Neutrophilic Granulocytes; PTX3: Pentraxin 3; RIN: RNA Integrity Number: RMA: Robust Multiarray Average; ROC: Receiver Operating Characteristic; RT: Room Temperature; RT-PCR: Reverse-Transcription PCR; S100A8/9: S100 Calcium Binding Protein A8/9; SAA: Serum Amyloid A; SC: Secretory Component; SCC: Somatic Cell Count; sCD25: Soluble Cluster of Differentiation 25; TNF-α: Tumor Necrosis Factor α; VEGF: Vascular Endothelial Growth Factor.

Introduction

Due to the growing population, the demand for animal-derived food products increased tremendously. Also the dairy industry was forced to face this development by continuous enhancement of the milk production yield. Dairy farmers increased their livestock which resulted in the need for automation of routine processes. Breeding programs focused on high milk yield adversely affect post-partum metabolism, fertility and health. During the last years health and well-being of dairy cows became a major economical and ethical issue. Mastitis or lameness are the major health problems and dairy farmers are affected by evolving treatment costs and production losses [1]. The consumers changing view regarding the farm animals' sentience is also contributing [2-4]. Especially after calving and in early lactation highly producing dairy cows are susceptible for inflammatory diseases of the udder or uterus [5-7]. The onset of lactation leads to metabolic distress. The negative energy balance causes metabolic and hormonal disorders [8]. As a consequence functional suppression of immune cells e.g. macrophages and polymorph-nuclear neutrophilic granulocytes (PMN) can occur [8-10]. Therefore, the early lactation period is highly critical concerning the well-being and economic consequences of diseased cows. Intensive and ambitious health monitoring programs are essential in this time and innovative methods are required to overcome current problems regarding shortage of farm staff and lack of automation in the health monitoring. Also in dairy cow breeding, functional traits such as longevity, fertility or the stability of health become more important, aiming for healthy and long-lived cows [1].

Immunological biomarkers indicating inflammatory diseases would offer a solution for the reliable and objective evaluation of the general health status of dairy cows. Routine biomarker screenings throughout the herd would facilitate the detection and subsequent treatment of diseased animals in an early state. An early diagnosis could reduce disease severity and maximize the success of the therapy. Thus, expenses for prolonged therapies, antibiotic treatments, reduction of the milk yield, or the loss of animals could be avoided. Reliable data on long-term health could support breeding programs.

Mainly acute phase proteins (APP) are generally considered as suitable biomarkers. Elevated serum concentrations of serum amyloid A (SAA), fibrinogen (FG) and haptoglobin (HP) can indicate acute or chronic inflammation [11-13]. In the milk HP, SAA or lactoferrin (LTF) are well-known indicators for clinical or subclinical mastitis [14-16] and more recently discussed as indicators for extra-mammary diseases [17,18].

In our study we intended to identify and validate immunological biomarkers for inflammatory diseases of highly-producing dairy cows in detail using a top-down approach. To facilitate fast, simple and automatic sampling and detection, we focused on dissolved and highly regulated proteins in milk. Furthermore, the milk biomarkers should not only indicate mammary gland inflammation, but especially also extra-mammary, inflammatory processes to facilitate the characterization of the udder as well as the systemic health status.

Materials and Methods

Blood and milk sampling

Animal grouping and diagnoses are summarized in Table 1. Diseases were distinguished in either systemic (extra-mammary) or those affecting the mammary gland (mastitis) to account for influences on the milk composition from local inflammatory processes. From these animals (German Holstein cows), milk and blood samples were collected in cooperation with six local, conventional dairy farms, the Large Animal Clinic for Internal Medicine and the Clinic for Ruminants and Swine of the University of Leipzig (Faculty of Veterinary Medicine), Germany. Diseases were grouped by their occurrence in the mammary gland or extra-mammary. Furthermore, the diseases were classified by their severity. All cows were examined thoroughly by the dairy herd manager, trained staff, or a veterinarian. Control animals (2-4 years old, 1st to 3rd lactation, one animal 4th and one 8th lactation) showed no clinical signs of disease and had no abnormalities in the udder or milk. Their somatic cell count (SCC) was less than 100,000 cells/ml milk. Most of the control samples were taken during early lactation (10-100 days post-partum, dpp). Thirty-six control samples were taken between 100 and 300 dpp (Table 1). Diseased cows were in their 1st to 8th lactation period within 10-220 dpp. Extra-mammary diseases or disorders that were recognized within several hours up to some days after appearance of first clinical signs were considered as early state and therefore as mild extra-mammary diseases. These animals were detected on-farm during the regular health-screening routines. Their health condition was specified by the dairy herd managers. Cows admitted to the animal clinics had a longer anamnesis of disease (several days to some weeks) and were considered as severe extra-mammary diseases or disorders. The majority of these animals had a combination of severe uterine inflammatory diseases, abomasal displacement, metabolic disorders and some additional minor diagnoses (skin abnormalities, claw diseases). All diseases were diagnosed by veterinarians. The farm or owner was anonymized if the sample was provided by the animal clinic. Thus it was not possible to consider the influence of different farming conditions.

Most samples from cows with acute, clinical mammary gland inflammations were taken on-farm. Mastitis was caused by different pathogens as being indicated in Table 1.

Diagnosis / surgical treatment	n	dpp, mean (SD)
Control		103.8 (61.3)
Healthy	89	
Mild extra-mammary diseases (detected on-farm)		16.4 (3.4)
claw infection, fever	1	
complications after calving, reduced milk yield	1	

Peritonitis	1	
Tonsitis	1	
uterus infection, abnormal lochia (amount, odor)	6	
uterus infection, metabolic disorder, joint inflammation	1	
Abomasal displacement (+ metabolic disorders, diagnosed in large animal clinic)		38.8 (34.8)
abomasal displacement	9	
abomasal displacement, ketosis	8	
abomasal displacement, ketosis, claw diseases	3	
abomasal displacement, ketosis, omentopexy	1	
Severe extra-mammary disease or disorder (diagnosed in large animal clinic)		34.5 (22.5)
endometritis, sepsis	1	
Enteritis	1	
inflamed eye injury, claw infections, scabies	1	
Metritis	1	
non-specific	1	
peritarsitis, ketosis, sepsis	1	
post-operative wound complication	1	
Severe extra-mammary disease or disorder + abomasal displacement (diagnosed in large animal clinic)		22.2 (11.9)
abomasal displacement, ketosis (severe course after surgical treatment), absence of defecation, pericarditis	1	
abomastitis, abomasal displacement, uterus surgery, claw diseases	1	
endometritis, abomasal displacement, gastric ulcer	1	
endometritis, abomasal displacement, ketosis	1	
endometritis, abomasal displacement, ketosis, chronic claw inflammation	1	
endometritis, abomasal displacement, omentopexy	1	
endometritis, sepsis, abomasal displacement, ketosis	1	
enteritis, abomasal displacement	1	
metritis, abomasal displacement	3	
metritis, abomasal displacement, ketosis, udder eczema	1	
peritarsitis, sepsis, abomasal displacement, udder eczema	1	
peritonitis, endometritis, sepsis, abomasal displacement, ketosis, udder eczema	1	
pyometra, abomasal displacement, ketosis	1	
retained placenta, metritis, abomasal displacement, pelvical phlegmone, udder edema	1	
sepsis, abomasal displacement, ketosis	1	
Acute, clinical mammary gland inflammation (detected on-farm or in large animal clinic)		89.8 (66.1)
blocked teat canal, stenosis	1	
Gram-negative	6	

not specified	5	
Yeast	1	
Acute, clinical mammary gland inflammation + severe extra-mammary disease or disorder (diagnosed in large animal clinic)		24.0 (4.9)
bronchitis, abomasal displacement, mastitis	1	
diarrhea, mastitis	1	
endometritis, abomasal displacement, mastitis	1	
endometritis, sepsis, abomasal displacement, ketosis, mastitis	1	
enteritis, sepsis, mastitis	1	
retained placenta, sepsis, mastitis	1	

Table 1: Diagnoses and animal grouping. (dpp: days post-partum).

In this study, 26.6% of the diseased cows included were locally or systemically pre-treated with antibiotics or received an anti-inflammatory medication before sampling.

Samples from a subset of clinically well-characterized animals shown in Table 2 were used for transcriptome and proteome analysis.

Sample ID	Diagnosis	Age	Lactation	dpp	Microarray	Proteome analysis
Control (dairy farm 1)						
69	no clinical signs of disease, SCC<100,000 cells/ml (at last monthly milk analysis)	2	1	60	x	-
70		3	2	66	x	x
74		2	1	66	x	x
165		3	2	93	x	-
175		3	2	28	x	x
176		2	1	44	x	x
Mild extra-mammary diseases / uterus infection (detected on-farm, dairy farm 2)						
156	postpartum uterus infection, vaginal discharge aqueous and mucous, uterus enlarged postpartum uterus infection, accumulated lochia purulent and fetid, uterus highly enlarged	2	1	16	x	x
157		2	1	13	x	-
158		2	1	18	x	x
169		2	1	19	x	x
108		2	1	11	x	-
177		2	1	12	x	x
Severe extra-mammary diseases (+AD, diagnosed in large animal clinic)						
83	endometritis, abomasal displacement, ketosis	5	4	30	-	x
109	catarrhal enteritis	2	1	50	-	x
117	peritonitis, abomasal displacement, ketosis	2	1	25	-	x
125	endometritis, sepsis, abomasal displacement	5	4	14	-	x
Acute, clinical mammary gland inflammation (detected on-farm, dairy farms 3 and 4)						
82	not specified	3	2	76	x	x

87	*Streptococcus uberis*	3	2	61	x	x
139		2	1	37	x	x
144		3	2	90	x	-
88	coagulase-negative *Staphylococci*	2	1	39	x	x
91		3	2	92	x	-

Table 2: Animals included in transcriptome and proteome analysis.

Blood samples were taken from jugular or tail vein and collected in 9 ml EDTA monovettes (Sarstedt, Nümbrecht, Germany). Prior to milk sampling the udder and teats were wiped clean and disinfected. First jets of milk were discarded. Milk of control animals or cows with extra-mammary diseases was collected as sample from just one quarter or a composite milk sample (equal volumes from all 4 quarters mixed). Mammary gland secretions of cows with mastitis were obtained as a mixture from the infected quarters. Depending on the SCC 1-2 liters of milk were collected from control animals, 50-500 ml from cows having extra-mammary diseases and 50 ml from cows suffering from mastitis, to obtain a sufficient cell amount for further analysis. Sterile plastic tubes or autoclaved glass bottles were used for milk collection and transport. For bacteriological evaluation, milk samples were plated on Columbia agar plates and incubated overnight. The count of colony-forming units (CFU) was assessed. The mean±SD was $4.5 \times 10^5 \pm 10^6$ CFU/ml for mastitic milk and $3.9 \times 10^3 \pm 5 \times 10^3$ CFU/ml for control samples. Mastitic samples were additionally characterized by Gram's stain (Merck, Darmstadt, Germany). For selected samples, information about the disease-causing agent/pathogen could be obtained from the records of the dairy farmers.

Preparation of samples and cells

Milk samples were centrifuged (20 min, 700 × g at 4°C). Cream was removed with a spatula and skim milk was aliquoted and frozen. For preparation of whey skim milk was centrifuged 60 min, 20,000 × g at 4°C. The crude whey fraction was aliquoted and frozen. Skim milk and whey samples were stored at -80°C until usage.

Samples of anti-coagulated blood were centrifuged (30 min, 1000 × g at 4°C). Plasma was removed carefully, aliquoted, frozen and stored at -80°C until use. Buffy coat and the upper layer of the red cells were aspirated and resuspended in haemolysis buffer (0.15 M NH_4Cl; 10 mM $KHCO_3$; 0.13 mM EDTA) and incubated for 5 to 10 min at room temperature (RT). After haemolysis, phosphate-buffered saline (PBS, 0.15 M NaCl; 2.7 mM KCl; 6.5 mM $Na_2HPO_4 \times 2H_2O$; 0.15 mM KH_2PO_4) was added and preparations were centrifuged for 10 min, 700 × g at 4°C.

Pellets of peripheral blood leukocytes (BL) and milk cells (MC) were washed twice, resuspended in PBS and cells were counted using a haemocytometer. Trypan blue exclusion was applied to assess viability (>90% for BL and >60% for MC).

Isolation of total RNA

Total RNA from 5×10^6 to 1×10^7 BL or MC was extracted using TRI® Reagent (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. To remove residual genomic DNA, RNA was incubated with RNase-free DNase I (VWR International, Darmstadt, Germany) as instructed in the manual. If required, RNA cleanup was performed with RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA purity and integrity was checked with the Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using Agilent RNA 6000 Nano Kit. Only samples with an RNA integrity number (RIN)>6 were used for further analysis.

Analysis of the milk cell transcriptome

The milk cell transcriptome of animals in three different conditions (healthy control (1), mild extra-mammary diseases/uterus infection (2), mammary gland inflammation (3)) was analyzed including six animals per group (Table 2) by means of the Affymetrix GeneChip® Bovine Genome Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Preparation of samples for expression analysis was done by means of the GeneChip®3′ IVT Express Kit (Affymetrix). RNA amplification, cDNA synthesis, labelling and array hybridization were performed according to manufacturer's instructions using 100 ng total RNA. In brief, *in-vitro* transcription reaction was incubated for 16 hours, then, amplified RNA (aRNA) was quantified and checked using the NanoDrop ND-1000 UV-VIS Spectrophotometer. It has to be noted that samples 165 and 176 yielded an aRNA concentration below 12 µg. For these samples RNA amplification, cDNA synthesis and labelling was repeated and pooled with the corresponding probe of the first set. All samples were concentrated in a lyophilizer for 2 hours. Thereafter, 12 µg labelled aRNA was used for fragmentation followed by hybridization on Affymetrix GeneChip® Bovine Genome Array using 10 µg of labelled and fragmented aRNA. Hybridization was performed for 16 hours in a GeneChip® Hybridization Oven 645 (Affymetrix). Afterwards, arrays were washed and stained using the Fluidics Station FS450 (Affymetrix) and the GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix) according to the manufacturer's instructions. Finally, the arrays were scanned with the GeneChip® Scanner 3000 7G (Affymetrix).

Bioinformatic analysis of microarray results

Affymetrix microarrays have been analysed by using the software programs R [19] and Bioconductor [20]. Background adjustment and inter-array normalization was performed by Robust Multiarray Average (RMA) with estimation of non-specific probe binding [21]. Only samples delivering values above the background signal and non-specific variance above the pre-defined cut-off were retained for differential expression analysis. In detail, only samples with values ≥ $\log_2(30)$ in at least 4 out of 18 arrays and interquartile ranges of IQR ≥ 0.5 passed the non-specific filter (R package genefilter), [22]. Linear models were fitted using the R package limma [23] and reliable

variance estimates were obtained by Empirical Bayes moderated t-statistics. False discovery rate was controlled by Benjamini-Hochberg adjustment [24].

GO term enrichment analysis and functional annotation were performed with the software program PANTHER [25]. The proteins which could be mapped were clustered according to biological processes (GO_BP_FAT) and to cellular compartment (GO_CC_FAT).

Reverse transcription and real-time PCR

For selected cytokine target genes and differentially expressed genes (DEG) from transcriptome analysis the specific mRNA expression was assessed by the real-time reverse-transcription PCR (RT-PCR). RNA samples used for quantitative real-time RT-PCR were reversely transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) and random hexamer primers using 1 µg of RNA according to the manufacturer's instructions. The reaction was performed in a Thermocycler TProfessional (Biometra GmbH, Göttingen, Germany).

The reaction mixture was prepared using Quantitect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. Gene-specific primers (Table 3) were designed using the Universal Probe Library Assay Design Center (Roche Applied Science). Bovine input sequences were listed in GeneBank Database. Primers were added to a final concentration of 0.4 µM. Five µL of 1:10 diluted cDNA were added to 15 µL of master mix. PCR was carried out in a LightCycler 480 instrument (Roche Applied Science) applying the temperature profile recommended for Quantitect SYBR Green PCR Kit (Qiagen). Normalized ratios were calculated by the LightCyler 480 Software 1.5. The target gene expression was related to the expression of the two reference genes cyclophilin B (PPIB) and ubiquitously-expressed transcript (UXT). The ratios were normalized to an external standard calibrator.

Target	Full Name (*Bos taurus*)	Primer forward (5'-3')	Primer reverse (5'-3')	NCBI Reference Sequence
ASB11	Ankyrin repeat and SOCS box containing 11	Cgcccctctttaatgcttg	actccagcagcacgttgac	NM_001034413.1
CFD	Complement factor D (adipsin)	Tacgtggcctggatcgac	gtgaccgcagcctctcag	NM_001034255.1
HP	Haptoglobin	Ggatcctgagctttgacaaga	gtcaccttcacgtacacacca	NM_001040470.1
IL10	Interleukin 10	Gcactactctgttgcctggtc	gacagggtgctcgcatct	NM_174088.1
IL1B	Interleukin 1-beta	Ctagcccatgtgtgctgaag	ccacttctcggttcatttcc	NM_174093.1
IL8	Interleukin 8	Agctggctgttgctctcttg	cagaactgcagcttcacacag	NM_173925.2
IL17A	Interleukin 17	Gctactgcttctgagtctggtg	tggactctgtgggatgatga	NM_001008412.1
IL18	Interleukin 18 (interferon-gamma-inducing factor)	cacgtttcctctcctaagaagc	ttctacttgttctgcagccatc	NM_174091.2
IL1RN	Interleukin 1 receptor antagonist	acaacccttcatcaaaagtcc	ggtcaggagaagccacattt	NM_174357.2
LTF	Lactotransferrin	Tggaagcttctcagcaagg	ctggaagctccgagacttgt	NM_180998.2
PIGR	Polymeric immunoglobulin receptor	Tgaacctggacacagtcacc	cttccttcactccacaccagt	NM_174143.1
PTX3	Pentraxin 3, long	Ccagctgtacctcagctatcg	gcatcagcgaccagtctgt	NM_001076259.1
S100A8	S100 calcium binding protein A8	Atgcggacacttggttcaa	tcaccagcacgaggaactc	NM_001113725.1
S100A9	S100 calcium binding protein A9	Cgaggagttcattatgctggt	gtgttgtgcatctcctcgtg	NM_001046328.1
SAA3	Serum amyloid A 3	Accagtttgccaacgaatg	cagcaggtctgaagtggttg	NM_181016.3
VEGFA	Vascular endothelial growth factor A	Tgctctcttgggtacattgga	accacttggcatggtgaag	NM_174216.1
PPIB	Peptidyl-prolyl cis-trans isomerase B	Ccattgccaaggaataggg	ggccacacagacagttgct	NM_174152.2
UXT	Ubiquitously expressed transcript	Ctggccaaataccttcaactg	cctgcatatataactccgagtgg	NM_001037471.1

Table 3: Gene-specific primers and according input sequences for primer design.

Analysis of the whey protein

The whey proteomes of animals in four different conditions (healthy control (1), mild extra-mammary diseases (2), severe extra-mammary diseases (inclusively abomasal displacement) (3), mammary gland inflammation (4)) were analyzed including four animals per group (Table 2). To deplete residual casein, the crude whey fraction was thawed and ultracentrifuged for 60 min, 100,000 × g, at 4°C. The upper layer was aspirated carefully and the clear supernatant was aliquoted and stored at -80°C until usage. The protein concentrations were assessed by Quickstart Bradford Protein Assay (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instructions.

1D-SDS-PAGE and tryptic digestion: Samples were boiled in SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 6% glycerol, 2% SDS, 5% mercaptoethanol, 0.05% bromophenol blue) for 5 min and subjected

onto a 1D-SDS-PAGE (4% stacking gel and 12% separation gel) according to standard laboratory procedures. The gels were cut into 10 slices per sample after staining with Coomassie Brilliant Blue G250 solution.

In-gel digestion was performed as previously described [26]. Briefly, the gel slices were destained with 50% methanol / 5% acetic acid. After reduction with 10 mM dithiothreitole, proteins were alkylated with 100 mM iodacetamide. In gel-digestion was conducted overnight at 37°C using 50 ng sequencing grade trypsin (Roche Applied Science, Mannheim, Germany) per slice. The resulting peptides were extracted two times from the gel with 5% formic acid and 50% acetonitrile. The combined extracts were evaporated, the residual peptides were dissolved in 20 µl 0.1% formic acid and the solutions were stored at -20°C until LC-MS analysis.

LC-MS/MS analysis: A nano-uHPLC system (nanoAquity, Waters, Milford, MA, USA) coupled to a LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) via chip-based nano electrospray ion source (TriVersa NanoMate, Advion, Ithaca, NY, USA) was used for LC-MS/MS analysis as previously been described [27]. Peptide elution was conducted using a 90-min gradient (2-40% acetonitrile containing 0.1% formic acid; 300 nl/min). The mass spectrometer automatically switched between full scan MS mode (positive mode, m/z 350 to 1,600, R=60,000) and MS/MS acquisition of the six most abundant peaks. Peptide ions exceeding an intensity of 3,000 counts were fragmented within the linear ion trap by collision induced dissociation (isolation with 4 amu, normalized collision energy 35, activation time 30 ms, activation Q 0.25). A dynamic precursor exclusion of 3 min for tandem MS measurements was applied.

Processing of data obtained by LC-MS/MS: LC-MS measurements were analyzed by Proteome Discoverer (Thermo Scientific, version 1.4.0.288). Proteome Discoverer was set up to perform a Mascot search against a bovine specific portion of the Uniprot database (version May 2013, only reviewed entries) assuming the digestion enzyme trypsin with a maximum of 1 proteolytical missed cleavages. As protein modifications carbamidomethylation of cysteine was specified to be fixed, whereas oxidation of methionine was specified as being variable. A minimum of two peptides considering only first ranked peptides were used for the identification of proteins. Only proteins which were quantified in at least two biological replicates based on at least three quantified peptides were considered. Proteins were quantified by a peak area based label-free quantification using the three peptides per protein given the most intense signals. Protein quantities were normalized to the original milk volume.

Quantification of protein biomarkers in milk and plasma

Selected proteins in milk and plasma were quantified using commercially available ELISA kits. In general, concentrations that were below the detection limit of the respective assay kit were recorded as zero. HP was determined using an ELISA based on polyclonal antibodies that was developed in the frame of a joint project (Sension GmbH, Augsburg, Germany). This assay was used as a prototype. Measurements of HP were either performed by Sension or in house using the same assay. All measurements were performed in undiluted samples, since this was sufficient to detect variations in the HP concentration levels related to different stages of disease. Pre-coated plates were incubated with 100 µl sample (30 min, RT). Purified HP (LeeBioSolutions, St. Louis, Missouri, USA) was used as a reference standard for preparing a calibration curve in the concentration range between 0.125 and 8 µg/ml HP. The plate was washed 3 times in assay washing buffer and then incubated with 100 µl 1:40 diluted peroxidase-conjugated anti-HP antibody (30 min, RT). After 3 washing steps 100 µl tetramethylbenzidine substrate were added (Moss, Pasadena, Maryland, USA) and incubated for 10 to 30 min at RT. The reaction was stopped using 50 µl 9.9% H_3PO_4.

Interleukin (IL) 18 was measured using a bovine IL-18 ELISA kit (USCN Life Science, Wuhan, China) according to the manufacturer's instructions. Milk was diluted 1:2 and plasma 1:5.

LTF was quantified using the bovine Lactoferrin ELISA Quantitation Set (Bethyl Laboratories, Montgomery, Texas, USA) according to the manufacturer's instructions. Milk was diluted 1:300, 1:10,000 or 1:20,000 for control samples and samples from cows with extra-mammary disease or mammary gland inflammation, respectively. Plasma was diluted 1:10.

The polymeric immunoglobulin receptor (PIGR) or more specifically declared the secretory component (SC) was quantified with a bovine PIGR ELISA kit (USCN Life Science) according to the manufacturer's instructions. Milk was diluted 1:300, 1:1,000 or 1:5,000 to 1:100,000 for control samples and samples from cows with extra-mammary disease or mammary gland inflammation, respectively. Plasma was diluted 1:100,000.

S100 calcium binding protein A9 (S100A9) was measured using a bovine S100A9 ELISA kit (USCN Life Science) according to the manufacturer's instructions. Milk was diluted 1:50, 1:200 or 1:500 for control samples and samples from cows with extra-mammary disease or mammary gland inflammation, respectively.

The tumor necrosis factor α (TNF-α) was measured using Bovine TNF-α ELISA VetSet (Kingfisher Biotech, Saint Paul, Minnesota, USA) according to the manufacturer's instructions. Milk was diluted 1:2 or 1:10 for control samples and samples from diseased cows, respectively.

Vascular endothelial growth factor (VEGF) was determined with the Bovine VEGF-A ELISA VetSet (Kingfisher Biotech) according to the manufacturer's instructions. Milk was diluted 1:10 or 1:100.

Further biomarker candidates were analyzed utilizing the following bovine ELISA kits according to the manufacturer's instructions: Cathelicidin, FG, and SAA3 ELISA kit (Shanghai BlueGene Biotech), C-C motif chemokine 25 (CCL25) ELISA kit (Wuhan EIAab Science, Wuhan, China), soluble cluster of differentiation (sCD25), C-X-C motif chemokines 10 and 11 (CXCL10, CXCL11), and S100A8 ELISA kits (USCN Life Science), complement factor D (CFD) ELISA kit (Biotang, Lexington, MA, USA), immunoglobulins (Ig) A und G ELISA kits (KomaBiotech, Seoul, Korea), IL-17A ELISA VetSet (Kingfisher Biotech), and Pentraxin 3 (PTX3) ELISA kit (Cusabio Life Science, Wuhan, China).

Statistical Analysis

Analysis of differences between groups, Spearman rank order correlations, receiver operating characteristic (ROC) analysis and graphical visualization of results were performed using the SigmaPlot 11 software program (Systat Software, Erkrath, Germany). To avoid a bias by directed sample selection, samples of animals in Table 1 were picked randomly for analysis by real-time RT-PCR or ELISA. Data sets were analyzed for normal distribution. If the Shapiro-Wilk normality test was passed, than Student's t-test was applied. If the data were not normally distributed, the non-parametric Mann-Whitney rank sum

test was applied. All included disease groups were tested against their respective control group. Data from different extra-mammary diseases groups were combined in case of low sample numbers. P values are given in two levels of significance, which are indicated as *0.05>p>0.01 and **p ≤ 0.01.

Selection and evaluation of potential biomarkers

ROC analysis was used to evaluate the discriminatory capability. An area under the curve (AUC)>0.9 was considered as highly discriminative and AUC values <0.6 as non-discriminative. Biomarkers were selected for best discrimination between mild extra-mammary diseases against healthy controls. Statistical evaluation of biomarkers and marker combinations was performed with TANAGRA 1.4.49 [28], an open-source data mining software. Since the majority of data sets were not normally distributed, multinomial logistic regression (MLR) and the k-nearest neighbour method (K-NN) were applied for classification. For K-NN the neighborhood size was 5 and the heterogeneous Euclidean-overlap metric (HEOM) applied as distance function [29]. To prevent possible over-fitting, a cross-validation (CV, 10-fold, 1 repetition) was performed. Values for sensitivity, specificity and resubstitution error rate were taken from CV. The different diseases were combined to one group. The biomarkers or their combinations were evaluated on the basis of their discriminatory capability for the diseased cows.

Results

Study design

To detect biomarkers for health monitoring of dairy cows in milk we followed a top-down approach, narrowing down a broad range of potential biomarker genes discovered by transcriptomics or proteomics to a few candidates, which were finally validated on the protein level by ELISA. In order to allow a general differentiation of disease categories in cattle and to discriminate systemically relevant biomarkers from those only derived from local inflammation we distinguished into either systemic diseases (representing all extra-mammary disease conditions) or disease conditions locally affecting the mammary gland (mastitis). The individual disease conditions which were exemplarily included in this study are listed in Table 1. In an initial approach a broad range of potential biomarker genes and proteins were screened using transcriptomics of MC and proteomics of milk (Table 2). Since cytokines are often highly regulated and are typically expressed with only low abundance, a microarray might not offer the required sensitivity for their detection. Therefore, selected cytokine target genes of interest were additionally analyzed by real-time RT-PCR to evaluate their potential to represent health-relevant biomarker candidates (Table 3). The combined list of biomarker candidates based on transcriptome data was narrowed down to a few promising candidates using key words. Results were confirmed by real-time RT-PCR. Finally, selected proteins were quantified by ELISA using commercially available assay kits. To confirm the systemic relevance of a biomarker candidate, measured in the local environment of the mammary gland, targeted genes or proteins were also quantified in BL or plasma by real-time RT-PCR or ELISA, respectively.

Comparison of the milk cell transcriptome of healthy and diseased cows

The cell populations in the respective milk samples were analyzed by flow cytometry. We hypothesized that the MC populations might be pathologically altered due to disease-induced influences and aimed to detect overall changes of expression profiles during mammary vs. extra-mammary inflammatory diseases with no regard to the mastitis-causing pathogen. To get an overview of gene products being locally expressed in the udder and secreted into the milk, the transcriptome of MC was analyzed by microarray during health and disease (Table 2).

Results were analyzed for (1) disease in general (i.e. no discrimination between uterus infection and mammary gland inflammation) vs. control, (2) uterus infection vs. control, and (3) mammary gland inflammation vs. control. Annotated DEGs were filtered using keywords related to immune response and acute-phase response. The secreted and highly regulated gene products of the remaining list (Table 4) were analyzed in detail. Complete results are provided in the gene expression omnibus database (GEO accession number GSE93082; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gxglwaiufjelhav&acc=GSE93082). In the analysis of disease vs. control we identified in total 1,891 significantly regulated DEG. Transcriptome analysis revealed activation of basic immune functions (e.g. TLR-induced or chemokine-induced signaling) and up-regulation of genes encoding for acute-phase proteins, cytokines or cytokine receptors(e.g. *IL2RA*) particularly during mastitis. *ASB11* was the only DEG in the analysis of uterus infection vs. control but out of our focus due to its intracellular expression. However, it might be an interesting new molecular target in future studies. In addition, *HP* and *SAA3*, which are discussed as biomarkers for mastitis in cattle, were detected in the analysis of mammary gland inflammation vs. control and also subjected to further analysis.

Gene Symbol	Annotation	Full Name (*Bos taurus*)	LogFC	adjusted p
Disease (uterus infection or mammary gland inflammation) vs. control				
PIGR	Bt.4695.1.S1_at	Polymeric immunoglobulin receptor	-2.28	1.14E-02
LTF	Bt.4802.1.S1_at	Lactotransferrin	-1.53	1.80E-02
CXCL11	Bt. 18368.1.S1_at	Chemokine (C-X-C motif) ligand 11	-1.33	1.49E-02
CCL25	Bt. 2539.1.S1_s_at	Chemokine (C-C motif) ligand 25	-1.27	3.94E-03
CFD	Bt.4336.1.S1_at	Complement factor D (adipsin)	1.06	1.53E-02
IL18	Bt.234.1.S1_at	Interleukin 18 (interferon-gamma-inducing factor)	1.26	4.32E-02
IL2RA	Bt.3941.1.S1_at	Interleukin 2 receptor, alpha	1.43	7.08E-04
CXCL10	Bt. 16966.1.S1_at	Chemokine (C-X-C motif) ligand 10	1.49	3.39E-02
IL1RN	Bt.4199.1.S1_at	Interleukin 1 receptor antagonist	1.56	1.01E-02
S100A8	Bt.9360.1.S1_at	S100 calcium binding protein A8	1.83	1.26E-02

VEGFA	Bt. 4138.1.S1_a_at	Vascular endothelial growth factor A	1.92	3.22E-03
PTX3	Bt. 10398.1.S1_at	Pentraxin 3, long	2.15	1.62E-02
S100A9	Bt. 16201.2.A1_at	S100 calcium binding protein A9	2.47	2.20E-03
Mammary gland inflammation vs. control				
HP	Bt. 12553.1.S1_at	Haptoglobin	1.54	4.47E-02
SAA3	Bt.278.1.S1_at	Serum amyloid A 3	1.95	5.22E-03
Uterus infection vs. control				
ASB11	Bt.7651.1.S1_at	Ankyrin repeat and SOCS box containing 11	-3.73	1.17E-03

Table 4: Selected differentially expressed genes of milk cell transcriptome. If multiple probe sets for one gene were differentially expressed, the probe set with the stronger regulation is shown. FC: fold change.

Validation of selected differentially expressed genes

The transcriptome analysis of MC was validated by determining selected DEG using real-time PCR. To confirm the systemic relevance of a potential biomarker from the local environment of the mammary gland, we also verified expression patterns of leukocyte-derived targets in BL. For selected cases indicated in Table 5 the number of animals included suffering from mild or severe extra-mammary diseases was too low. For statistical evaluation those samples were combined in one general group of extra-mammary diseases.

In terms of *HP, LTF, PIGR, VEGF*, and *ASB11* (Figure 1), as well as further selected target genes (Table 5) differential expression was confirmed by real-time RT-PCR. With the exception of LTF, a very similar pattern of regulation was observed in milk cells and blood leukocytes.

Screening for relevant Cytokines using real-time RT-PCR

Since the microarray did not provide the required sensitivity for the detection of low abundant cytokines in macrophages and T cells (e.g. *IFNG, IL1B, IL4, IL6, IL8, IL9, IL10, IL12A, IL12B, IL17A, IL21, IL22, IL23A, TNF*) these targets were analyzed by real-time RT-PCR to evaluate their potential as biomarkers in milk. Results are summarized in Table 5. *IL1B, IL8, IL10* and *IL17A* were found to be up-regulated in mastitic MC samples.

Milk cells				Peripheral blood leukocytes			
Group	**Mean**	**SEM**	**n**	**Group**	**Mean**	**SEM**	**n**
CFD							
control	0.08	0.02	4	Control	1.37	0.32	5
mild extra-mamm. dis.	0.16	0.11	2	extra-mamm. dis.	1.91	0.08	5
severe extra-mamm. dis. (+AD)	0.15	0.01	2				
mamm. gland inf.	0.81**	0.5	5	mamm. gland inf.	2.06	0.47	5
IL18							
control	6.78	0.95	9	Control	4.53	2.74	4
mild extra-mamm. dis.	10.54	6.64	4	extra-mamm. dis.	3.98	1.28	3
severe extra-mamm. dis. (+AD)	12.79	3.74	6				
mamm. gland inf.	81.29**	24.71	11	mamm. gland inf.	4.24	1.04	3
PTX3							
control	6.51	2.11	9	Control	6.87	3.59	8
mild extra-mamm. dis.	321.26	314.2	5	extra-mamm. dis.	22.81	20.33	6
severe extra-mamm. dis. (+AD)	148.10**	109.55	6				
mamm. gland inf. + severe extra-mamm. dis.	8773.43**	4048.34	14	mamm. gland inf.	221.87	105.1	5
S100A8							

control	4.9	13.04	9	Control	6.91	2.21	8
mild extra-mamm. dis.	6.24	4.83	4	extra-mamm. dis.	18.75*	5.33	5
severe extra-mamm. dis. (+AD)	18.78	12.01	4				
mamm. gland inf. + severe extra-mamm. dis.	174.39**	55.44	10	mamm. gland inf.	86.77**	63.97	4
S100A9							
control	6.12	27.55	8	Control	69.97	26.2	12
mild extra-mamm. dis.	12.56	9.72	4	extra-mamm. dis.	333.35*	140.59	9
severe extra-mamm. dis. (+AD)	29.36*	12.8	4				
mamm. gland inf. + severe extra-mamm. dis.	338.12**	105.71	10	mamm. gland inf. + severe extra-mamm. dis.	1124.07**	711.64	6
Exclusively quantified in Milk cells							
IL1RN				*SAA3*			
control	36.82	9.1	8	Control	2.6	0.51	7
mild extra-mamm. dis.	22.93	12.6	4	mild extra-mamm. dis.	54.75**	20.98	5
severe extra-mamm. dis. (+AD)	73.34	16.36	5	severe extra-mamm. dis. (+AD)	52.18	37.83	4
mamm. gland inf.	330.13**	56.41	11	mamm. gland inf. + severe extra-mamm. dis.	461.59**	242.94	13
IL1B				*IL8*			
control	116.44	60.46	4	Control	54.78	21.19	6
extra-mamm. dis.	67.59	19.1	5	severe extra-mamm. dis. (+AD)	141.89	38.04	5
mamm. gland inf.	304.92*	40.83	5	mamm. gland inf.	241.38*	65.12	6
IL10				*IL17A*			
control	10.7	1.28	8	Control	0.97	0.25	8
extra-mamm. dis.	29.13	21.03	6	extra-mamm. dis.	13.39	8.97	10
mamm. gland inf.	54.82*	21.79	10	mamm. gland inf.	13.39**	3.96	7

Table 5: Expression of selected genes in milk cells and blood leukocytes of cows in different disease conditions. Differential expression of genes was determined using real time RT-PCR and is shown as percent of the expression of the two reference genes PPIB and UXT (mean ± SEM). Disease groups were tested against the control group using Student's t-test or Mann-Whitney rank sum test. Detailed information on the cows in Tables 1 and 2. Bold: *0.05>p>0.01 and **p ≤ 0.01. mamm.: mammary; dis.: disease; inf. inflammation, AD: abomasal displacement.

Potential biomarkers in whey proteome

To verify the results of transcriptome analysis on protein level, the milk proteome was screened by LC-MS/MS. Since mild extra-mammary disease had a non-sufficient signal in microarray, we included severe extra-mammary disease (+AD) in the proteome analysis. Each group consisted of four animals (Table 2). Since p values were mostly non-significant, the fold-change (FC) value was the only criterion for follow-up validation. In total 291 whey proteins could be identified of which 138 could be quantified in the control group as well as in at least 2 of the 3 disease groups. Comparing mastitis vs. control 28 or 17 proteins were found to be more than 1-fold up- or down regulated, respectively. Comparing mild extra-mammary disease vs. control 48 or 15 proteins were found to be more than 1-fold up- or down regulated, respectively. Comparing severe extra-mammary disease vs. control 30 or 21 proteins were found to be more than 1-fold up- or down regulated, respectively. A selection of detected proteins is shown in Table 6. These proteins are associated with lipid metabolism, proteolysis, glycolysis and also immune functions such as antimicrobial proteins (i.e. LTF, cathelicidins), complement components or Ig chains. The secreted target gene products *CFD, HP, LTF* and *PIGR* obtained from transcriptome analysis, were also detected in the whey proteome analysis. In addition, cathelicidin-1 and FG were found to be up-regulated in mastitic milk samples, although they were not found to be regulated on transcript level. Cathelicidins

and FG have been previously shown to be elevated during inflammatory conditions of cattle [30,31] and were therefore additionally subjected to further analysis by ELISA.

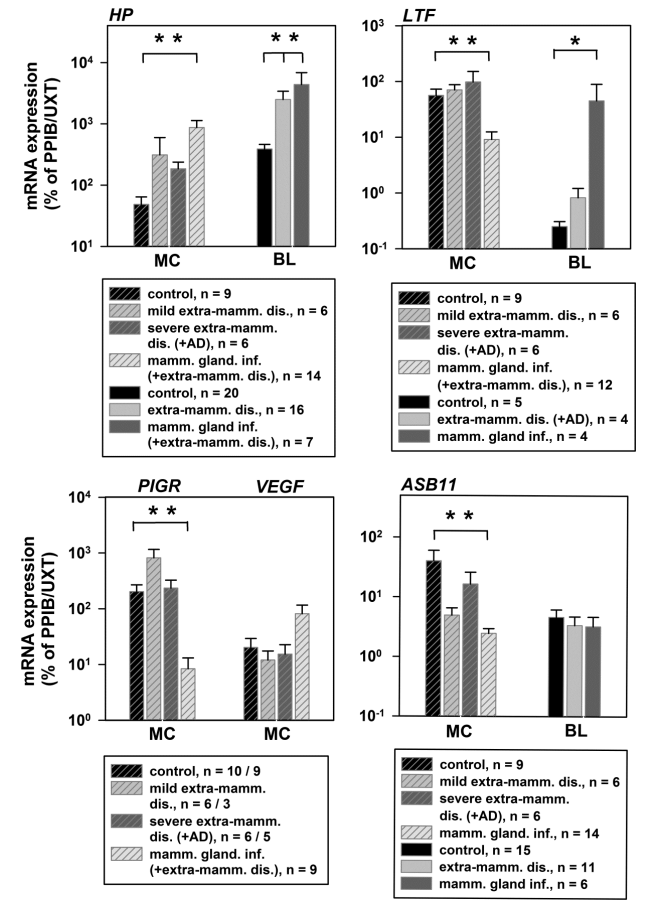

Figure 1: Expression of selected genes in milk cells (MC) and blood leukocytes (BL) of cows with different health conditions. Differential expression of genes was determined using real-time RT-PCR and is shown as percent of the expression of the two reference genes PPIB and UXT (mean ± SEM). Disease groups were tested against the control group using Student's t-test or Mann-Whitney rank sum test. Detailed informations on the cows in Tables 1 and 2. *0.05>p>0.01 and **p ≤ 0.01. mamm.: mammary; dis.: disease; inf. Inflammation.

Quantification and selection of biomarkers

Based on the results of our screening experiments we quantified potential biomarkers with commercially available ELISA kits. Targeted proteins were determined in milk of cows under different disease conditions (Tables 1 and 2). IL-17, cathelicidins, *CCL25*, and *PTX3* were not detectable by ELISA (data not shown). Either the proteins had a concentration below the lower limits of detection or the applied test kits were not suitable for determination of target proteins in skim milk or whey. *CXCL10, CXCL11, sCD25*, and *S100A8* signals (absorption at 450 nm) were elevated in milk from cows underwent mastitis, but still very low, close to the lower detection limits (data not shown). *CFD, FG, IgA, IgG*, and *SAA3* did not show disease-dependent regulation (data not shown). We observed increased concentrations of *IL-18, LTF, PIGR (SC), TNF-α*, and *VEGF* in milk during *AD*, severe extra-mammary disease, mammary gland inflammation, and combinations of the diseases. However, elevated levels of *HP* and *S100A9* could be detected during mild extra-mammary diseases (Figure 2). The observed up-regulation of *LTF* and *PIGR* (SC) at the protein level was in contrast to the down-regulation of *LTF* and P*IGR* genes in transcriptome analysis.

To evaluate the explanatory power for the animal's systemic health status expression patterns of *HP, IL-18*, and *LTF* were also determined in plasma. The Spearman correlation coefficients (Spearman's ρ) of *HP* and *LTF* concentrations in milk vs. plasma were determined to be 0.75 and 0.33, respectively (Figure 3). The positive correlation coefficients indicate interrelations of milk and plasma protein concentrations (Table 7). In addition, the correlation of the most reliable biomarker candidates in milk was analyzed. All proteins showed a positive correlation of their concentration in milk, i.e. increased together during disease (Table 7). As most promising milk biomarker candidates, *HP, LTF, VEGF* and *PIGR* (SC) were subjected to statistical evaluation.

Protein data	MS Identification statistics			FC between Groups vs. Control		
Protein Name	Accession	Mascot Protein Score	Σ# Peptides	mild extra-mamm. dis.	severe extra-mamm. dis.	mamm. gland inf.
Complement factor B	P81187	1017.24	38	-1.7	0	-0.2
Complement component C7	F1N045	705.05	23	-1.6	0.5	n.q.
Complement C3	Q2UVX4	5075.75	134	-1.4	-0.6	-0.2
Haptoglobin	Q2TBU0	3599.52	40	-0.5	-0.2	3.7

Complement C5a anaphylatoxin	F1MY85	186.01	15	-0.3	0.2	n.q.
Cathelicidin-1	P22226	840.6	10	0	-1.4	4.7
Immunoglobulin light chain, lambda gene cluster	Q1RMN8	3835.43	16	0.4	-0.1	0.5
Complement component C6	F1MM86	359.06	19	0.6	0.4	n.q.
Immunoglobulin lambda-like polypeptide 1	F1MLW7	3681.78	17	0.6	0.3	0
Complement factor I	F1N4M7	520.8	21	0.7	1.7	1.9
Ig alpha-1 chain C region	A5D7Q2	2035.63	16	0.7	-0.7	-0.9
Fibrinogen alpha chain	A5PJE3	1630.53	36	0.8	-0.2	1.4
Lactoferrin	C7FE01	4288.07	77	1.1	-0.5	-1.3
IgG heavy chain constant region	G3N0V0	855.68	16	1.3	-0.1	0.6
Complement factor H	Q28085	461.18	31	1.3	0.6	n.q.
Complement factor D	Q3T0A3	422.25	12	1.8	2.6	n.q.
IgM heavy chain constant region	G5E513	1781.56	22	1.8	-1.2	-2.1
Fibrinogen beta chain	F1MAV0	1250.37	45	2	-0.4	1.3
IgM heavy chain constant region	G5E5T5	1637.17	16	2	-1.4	-2.5
Polymeric immunoglobulin receptor	P81265	4005.9	38	2.8	-0.6	-1.1
Immunoglobulin J chain	Q3SYR8	431.32	8	2.9	3	3.8

Table 6: Selection of proteins detected in proteome analysis of milk samples from cows with different disease conditions. Detailed informations on the cows in Table 2. MS: mascot score; FC: fold change; mamm.: mammary; dis.: disease; inf. inflammation; n.q.: protein was not quantified in this condition.

Correlation of	Spearman correlation coefficient	p	n
correlations in milk			
milk HP and milk PIGR (SC)	0.67	0.001	71
milk LTF and milk PIGR (SC)	0.61	0.001	79
milk HP and milk LTF	0.59	0.001	142
milk HP and milk VEGF	0.58	0.001	120
milk LTF and milk VEGF	0.54	0.001	132
milk VEGF and milk PIGR (SC)	0.41	0.001	79
correlations in milk and plasma			
milk HP and plasma HP	0.78	0.001	121
milk IL-18 and plasma IL-18	0.38	0.088	21
milk LTF and plasma LTF	0.33	0.005	69
correlations in plasma			
plasma HP and plasma LTF	0.59	0.001	63

Table 7: Correlations of protein biomarkers in milk and plasma.

Statistical Evaluation of potential Biomarkers in Milk

For statistical evaluation the strongly regulated and highly concentrated milk biomarkers *HP*, *PIGR* (SC), *LTF* and *VEGF* were chosen. A subset of samples, in which all four biomarker candidates had been determined, was used to test for a direct relation of the results. The biomarker candidates alone and dual combinations were evaluated. The test set contained 17 control samples and 49 samples of diseased cows. The discriminatory capability for each disease group was determined by ROC analysis (Figure 4A and Table 8). HP and PIGR (SC) showed the best discrimination of mild extra-mammary disease with AUC values of 0.69 and 0.68, respectively. All proteins were highly discriminative for severe diseases and mastitis (AUC>0.9).

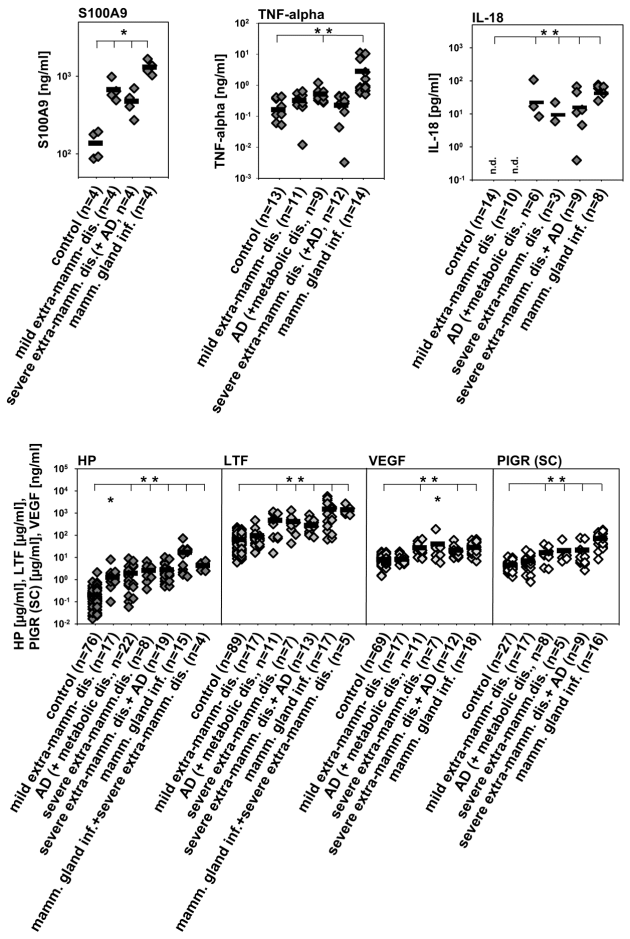

Figure 2: Concentrations of potential biomarkers in milk determined by commercial ELISA kits. Disease groups were tested against the control group using Student's t-test or Mann-Whitney rank sum test. Detailed informations on the cows in Tables 1 and 2. *0.05>p>0.01 and **p ≤ 0.01.

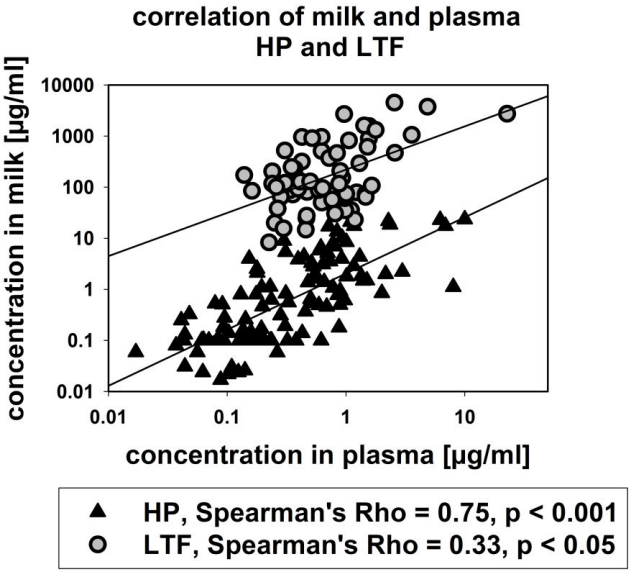

Figure 3: Concentrations of Haptoglobin (HP) and Lactoferrin (LTF) in milk and plasma determined by commercial ELISA kits. Positive correlations are indicated by the regression lines. Detailed informations on the cows in Tables 1 and 2.

Control vs. disease group	AUC	95% confidence interval	p
HP			
mild extra-mamm- dis.	0.69	0.48-0.89	0.065
AD (+ metabolic dis.)	0.96	0.89-1.03	<0.001
severe extra-mamm.dis.	0.99	0.95-1.03	0.001
severe extra-mamm. dis.+ AD	0.99	0.95-1.02	<0.001
mamm. gland inf.	1	1.00-1.00	<0.001
PIGR (SC)			
mild extra-mamm- dis.	0.68	0.49-0.87	0.071
AD (+ metabolic dis.)	0.84	0.64-1.04	<0.05
severe extra-mamm.dis.	0.95	0.87-1.04	<0.05
severe extra-mamm. dis.+ AD	0.8	0.61-0.99	<0.05
mamm. gland inf.	0.99	0.98-1.01	<0.001
LTF			
mild extra-mamm- dis.	0.67	0.48-0.86	0.088
AD (+ metabolic dis.)	0.82	0.62-1.03	<0.05
severe extra-mamm.dis.	0.95	0.86-1.05	<0.05
severe extra-mamm. dis.+ AD	0.93	0.84-1.03	<0.001
mamm. gland inf.	0.98	0.95-1.02	<0.001
VEGF			

mild extra-mamm- dis.	0.57	0.38-0.77	0.459
AD (+ metabolic dis.)	0.99	0.96-1.02	<0.001
severe extra-mamm.dis.	0.84	0.58-1.08	<0.05
severe extra-mamm. dis.+ AD	0.96	0.90-1.03	<0.001
mamm. gland inf.	0.97	0.91-1.03	<0.001

Table 8: Discriminatory capability of milk biomarkers for different disease conditions. Data were obtained with receiver operating characteristic analysis. (control: n=17, mild extra-mamm. inf.: n 1=17, AD (+metabolic dis.): n=8, severe extra-mamm. dis.: n=5, severe extra-mamm. dis. (+AD): n=8, mamm. gland inf.: n=11) AUC: area under the curve; mamm.: mammary; dis.: disease; inf. inflammation; AD: abomasal displacement.

Marker combinations were evaluated by discrimination between diseased and control animals using two statistical classification methods (MLR and K-NN) (Table 9). A second statistical model was applied to avoid possible bias of the results. HP was found to be suitable as a single biomarker. However, using a combination of HP and either PIGR (SC) or LTF achieved an increase in overall sensitivity or specificity, depending on the classification method. In our approach these combinations revealed the best discrimination between healthy and diseased cows.

Multinomial logistic regression (cross-validation) /%				k-nearest neighbour classification (cross-validation) /%		
Marker (combination)	**Sensitivity**	**Specificity**	**Error rate**	**Sensitivity**	**Specificity**	**Error rate**
single milk biomarker						
HP	86	88	13	91	69	15
LTF	84	44	27	82	63	23
VEGF	84	38	28	73	31	38
PIGR (SC)	86	25	30	77	19	38
combinations of two milk biomarkers						
HP & VEGF	86	88	13	80	94	17
HP & PIGR (SC)	89	81	13	84	75	18
HP & LTF	89	69	17	86	81	15
VEGF & PIGR (SC)	86	63	20	82	56	25
LTF & PIGR (SC)	84	56	23	86	31	28
LTF & VEGF	82	56	25	84	44	27

Table 9: Evaluation of milk biomarkers and combinations. Classification was performed using multinomial logistic regression and k-nearest neighbor methods for control (n=17) vs. diseased (n=49). Sensitivity, specificity and resubstitution error rate were taken from cross validation (10-fold, 1 repetition).

AUC	95% confidence interval	P	Cutoff at 94% specificity	Sensitivity at 94% Specificity /%
HP				
0.88	0.80-0.96	<0.001	0.58 µg/ml	82
PIGR (SC)				
0.82	0.72-0.93	<0.001	8.20 µg/ml	59
LTF				
0.84	0.74-0.94	<0.001	120.7 µg/ml	55
VEGF				
0.82	0.72-0.92	<0.001	9.50 ng/ml	67

Table 10: Discriminatory capability of milk biomarkers for diseased animals. Data were obtained from receiver operating characteristic analysis. (control: n=17, diseased: n=49).

Practical application would require high specificity of the test in order to prevent an overestimation of the incidence of diseases in large dairy herds. Therefore, ROC analysis was performed for all disease groups vs. control to assess sensitivity (true positive), specificity (true negative), 1-sensitivity (false negative) and 1-specificity (false positive) of the biomarker determination in milk at different threshold (cut-off) concentrations. Table 10 shows values for possible cut-off concentrations at a high specificity value of 94%. Accordingly, ROC curves are shown in Figure 4B. At a specificity value of 94%, 6% of the healthy cows would be detected as sick and on the other hand, 18%, 41%, 45%, and 33% of the sick animals would be overseen when determining HP, PIGR (SC), LTF and VEGF, respectively. Attributing single biomarker candidates the determination of HP is best suitable for the disease detection in dairy cows. However, attributing the combined use of two biomarker candidates the combinations HP plus PIGR (SC) or HP plus LTF were shown to be capable of increasing overall sensitivity or specificity in ROC analysis.

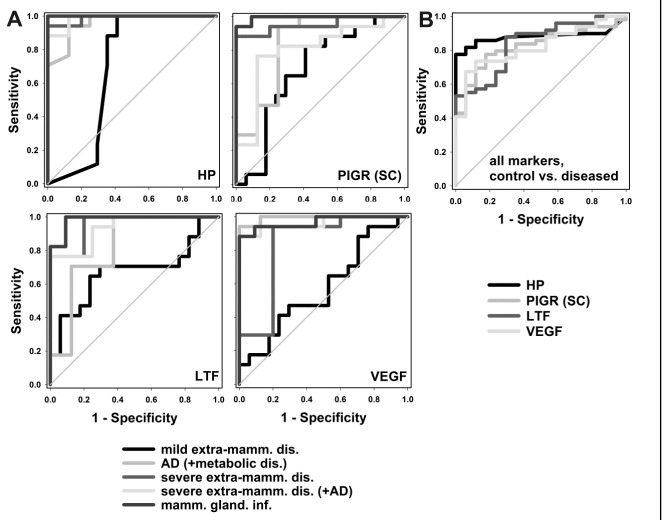

Figure 4: Receiver operating characteristic (ROC) curves of selected milk biomarkers. A: ROC analysis of the different disease conditions. B: Summarized ROC analysis for diseased animals. mamm.: mammary; dis.: disease; inf. inflammation; AD: abomasal displacement.

Discussion

The aim of this study was to identify and validate potential bovine biomarker candidates detectable in milk for the evaluation of the general health status of dairy cows.

Using a top-down approach for protein biomarker discovery, a high number of hits identified by state-of-the-art transcriptomics and proteomics was narrowed down to a few promising candidates. The central requirements for these candidates with regard to their application in practice were (i) to represent disease-dependently regulated factors and (ii) to be reliably and easily detectable in milk. In a first validation step we could confirm a small selection of leads out of the large pool identified by our multi-omics strategy at the transcript as well as the protein level by real-time RT-PCR and ELISA, respectively. Biomarker candidates confirmed in this step of validation (i.e. *HP, PIGR* (SC), *LTF* and *VEGF* - all representing proteins associated with the activation of the immune system in cattle) are currently proved for practical applicability in field studies (second validation step). As an essential prerequisite for the field validation of suggested biomarker candidates we have developed monoclonal antibodies and ELISA techniques based on these antibodies for biomarker detection (K. Zoldan, J. Lehmann, unpublished).

The up-regulation of validated biomarker candidates in milk seems to be attributed to common local as well as systemic inflammatory processes involving an acute-phase response rather than being related to special disease conditions. This is also true for the expression of CD25 (IL-2Rα) on bovine peripheral blood PMN, thus representing a cellular biomarker for the health status of dairy cows, as recently shown by our group [32].

Importantly, the concentration of all validated milk biomarker candidates in milk and plasma correlated with disease severity of post-partum mammary and extra-mammary disease conditions exemplarily investigated in this study. The quantification of *HP, PIGR* (SC), *LTF* or *VEGF* in milk was highly discriminative for diseased vs. healthy cows. Correlation of milk HP and LTF with the according plasma concentrations confirmed the representation of the systemic health status of dairy cows by measuring those biomarkers in milk. Three other secretory proteins detectable in milk (i.e. IL-18, S100A9 and TNF-α) also revealed discriminative potential between healthy and diseased cows but have to validate yet.

The rational of this study was to reflect the general health status of dairy cows in their natural environment under conventional conditions. Therefore, we decided to study milk and plasma samples derived from conventional herds or animal clinics rather than from an animal experiment specially performed for this purpose, for example utilizing an infection model. Although the latter had offered better opportunities for statistical evaluation due to well-defined disease conditions, clear identity of the causing pathogens or exact sampling time points it is impossible from the practical as well as the economic point of view to perform animal experiments using cows for all possible systemic disease conditions. Therefore, we preferred to include a variety of naturally occurring diseases and cases of different severity. In our opinion, the randomly selected diseased cows recruited for our study do more natively represent the field situation and offer a better basis for later field validation of the identified biomarker candidates which is crucial in any case.

For the significance of the results the distribution of the animals throughout the farms was not considered since the farms did not fundamentally differ in animal keeping and feeding since they were all commercial German dairy farms that underlie a strict regimen of monthly milk and animal health controls.

Moreover, a critical feature of the native situation in the cow herd is the periparturient suppression of neutrophil functions which is appropriately represented in our study since the majority of cows were in early lactation. In the light of these convincing advantages we accepted some disadvantages of the chosen experimental strategy, predominantly the large variance of the results observed within the control or disease groups.

As a common post-partum, extra-mammary disease we chose the uterus infection [33] for MC transcriptome analysis and compared the results to a group of cows without any clinical signs of disease (healthy controls) and to a group of cows suffering from mastitis to account for influences of local inflammation in the udder. With this experiment we intended to record the whole spectrum of genes encoding for secreted

gene products that are expressed in the udder and secreted into milk by a mixture of cell populations e.g. different portions of PMN, macrophages, lymphocytes or epithelial cells depending on the disease condition as demonstrated by flow cytometric MC analysis by [34]. Leitner and colleagues could show that predominantly the percentages of PMN and epithelial cells were altered in milk of diseased cows [34].

ASB11 encoding for a Notch signaling molecule [35,36] was the only significant DEG during uterus infection found in our study. Finucane and colleagues found ASB11 up-regulated in mammary tissue at day 10 pp by investigating the molecular events of lactogenesis [37]. Thus, the result might be also related to the sampling time point (between days 11 and 19 pp), (Table 2). However, Notch signaling and ASB11 are involved in the development, differentiation and activation of immune cells [38]. Significant DEG from the analysis of disease (uterus infection and mastitis) vs. healthy control were dominated by genes related to the immune defense during mastitis and confirmed previous studies analyzing genome-wide expression in mammary tissue [39-41] or MC [42] in dairy cows during mastitis. This observation is of crucial importance since immune-related genes partly rule out effects of different treatment on different farms. Genes such as complement components, CD14, *CXCL10*, *HP*, *LTF*, *PTX3*, *SAA3* or *S100* calcium binding genes were found to be significantly regulated. In our study up- or down-regulation in microarray analysis of DEG such as *HP, LTF, PIGR, VEGF, IL18, S100A9* or *PTX3* in MC during disease could be validated by real-time RT-PCR. The expression patterns of the pro-inflammatory cytokine genes *IL8, IL1B* but also of *PTX3, SAA3* and *S100A9* did correlate with the results of previous studies analyzing gene expression in milk somatic cells, mammary tissue or a cell culture model during bacterial infection using real-time RT-PCR [39,43,44]. To analyze the systemic expression of biomarker candidates in parallel we used mRNA of BL. Gene expression of e.g. *HP, IL18* or *S100A9* was up-regulated during disease. However, *LTF*, which was found to be down-regulated in MC, had a contrary expression pattern in BL probably due to the presence of more, not yet terminally differentiated, band neutrophilic granulocytes actively producing LTF in blood. Furthermore, terminally differentiated segmented PMN in milk just store the LTF in their secondary granules ready for release after activation, as LTF acts as an antimicrobial protein [45] and alarmin [46]. Nevertheless, up-regulation of *LTF* mRNA expression in mammary tissue was previously shown during experimentally induced mastitis [47], which supports our observation of elevated *LTF* concentrations in milk during disease. A similar expression, release, and function pattern was shown for HP [48] in human blood PMN. However, in MC the mRNA expression was still elevated during disease in our experiments. In this case the mammary epithelial cells might be an additional source of HP mRNA [49]. In mammary tissue HP mRNA expression was also found to be increased during mastitis [50].

On the protein level we either observed similar expression patterns between the disease groups or no clear tendency of regulation. In general, the different numbers of analyzed animals with regard to the included disease conditions might be a reason for controversial results from proteome analysis and ELISA in our study. In whey proteome analysis HP, cathelicidin-1, FG, CFD and several other complement factors were detected and appeared to be up-regulated during disease which is in agreement with analyses of the mastitic whey or milk by other authors [30,43,51]. For PIGR (SC) no clear tendency of regulation was observed. In contrast to transcriptome analysis, PIGR appeared to be up-regulated during extra-mammary diseases and down-regulated during mastitis in proteome analysis. However, analysis by ELISA confirmed a significant up-regulation even during mild extra-mammary diseases. Down-regulation of *PIGR* mRNA expression in MC during disease probably occurred due to less epithelial cells in milk of sick cows and a higher portion of PMN during mastitis [34,52,53] since PIGR (SC) is expressed by epithelial cells [54]. PIGR (SC) is known as a key player in mucosal immunity. Regulation of its concentration as soluble protein might be related to the enhanced IgA transport occurring during disease [55,56]. Furthermore, it acts as a non-specific microbial scavenger [57].

The down-regulation of LTF during mastitis in proteome analysis correlates with mRNA expression results in MC. However, it does not correlate with the protein concentration in milk determined by ELISA showing a strong and significant up-regulation during severe extra-mammary diseases and mastitis.

As for LTF, the milk and plasma HP concentrations were elevated during inflammatory diseases. Even mild extra-mammary diseases could be discriminated by milk HP quantification using ELISA. Strong HP up-regulation confirmed results of mRNA expression and proteome analysis. As mentioned earlier, increased concentrations of HP and LTF in plasma and milk most likely occurred due to the release of secondary granules from PMN after activation.

As being expected MS-based proteome analysis was able to detect more than 100 of the major milk components and higher concentrated immune related proteins. Low-molecular weight or low-abundance proteins such as cytokines were not detectable, most likely due to the lower sensitivity of the method.

Measuring milk TNF-α concentrations by ELISA revealed up-regulation of this pro-inflammatory cytokine during severe extra-mammary diseases and mastitis confirming results of [58]. Furthermore, the elevated milk VEGF concentrations correspond with the higher mRNA expression in mastitic MC samples. A possible source of VEGF in milk are activated PMN [59,60] and mammary epithelial cells [61]. VEGF in milk is not only a regulator of mammary gland development and function [62] but also might influence the permeability of the intestinal epithelium in the newborn [63,64]. During inflammation VEGF serves as chemoattractant for monocytes and macrophages and stimulates angiogenesis [65,66].

Activated PMN are further able to produce S100A9 and IL-18 [67-69], what could explain the elevated mRNA expression and higher protein concentration of these factors detected in MC and milk during disease. IL-18 and S100 proteins are known as mediators of the inflammatory neutrophil response in humans [70-72].

From the spectrum of milk biomarkers in our study and the elevated number of PMN in milk during disease [34], we assume that most of the biomarker proteins are produced by activated PMNs. These cells are key players in the immune defence of the udder [73,74]. As previously stated [32], we hypothesize that the majority of milk PMNs during mastitis are not activated within the udder tissue but recruited from the blood circulation in an activated state. However, the precise mechanism causing the recruitment of PMNs into the normal, non-mastitic gland remains unidentified [74] but might explain the elevated numbers of activated PMNs in milk and thus higher milk biomarker concentrations during extra-mammary diseases.

Conclusively, our results demonstrate that extra-mammary, non-mastitic diseases influence not only the serum / plasma concentrations but also the milk composition of immunoregulatory and acute-phase proteins and that those factors can be quantified as general health

biomarkers in the milk. Thereby, the sampling can easily and quickly be carried out by farm staff and can be integrated in milking routines even if automated systems are used. Regular measurement of the suggested milk biomarkers or their combinations could facilitate the detection of not only mastitis and severe extra-mammary inflammations but also lameness and claw diseases [18,75,76] and even stress [77]. The advanced disease detection may improve health monitoring systems and animal welfare in growing dairy herds, indicate disease resistance and provide objective information about the individual, long-term health to support breeding programs.

For the application of the biomarker determination, the later intended use is critical for the definition of time points, intervals and the frequency of measurements. Due to large variations of biomarker concentrations in healthy animals, it is recommended to define periodic time points of analysis in order to detect animal-specific changes in the biomarker concentration [78]. Application of specific cut-offs at one defined sampling time point to differentiate between healthy and sick animals could not account for individual variations in healthy animals but provide information about the overall health condition of the herd. Additionally, cut-offs are specific for every method of quantification. The suggested cut-off concentrations in this study only serve as an example to explain the potential of one possible application.

Based on our findings, future studies could focus on automated and cost-effective methods for biomarker quantification. Suitable potential test formats are the lateral-flow assay (i.e. dipstick), mid-infrared measurement [16], high-throughput fluorescence polarization immunoassay [79] or the novel concept for a protein microarray on smart-phones [80].

The application of health biomarkers in combination with an easy high-throughput detection system might offer a solution to adapt dairy herd management to changing requirements on animal welfare, farming efficiency, milk supply and food safety.

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