Global transcriptomic profiles of circulating leucocytes in early lactation cows with clinical or subclinical mastitis

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Abstract

Background. Bovine mastitis, an inflammatory disease of the mammary gland, is classified as subclinical or clinical. Circulating neutrophils are recruited to the udder to combat infection. We compared the transcriptomic profiles in circulating leukocytes between healthy cows and those with naturally occurring subclinical or clinical mastitis.

Methods and Results. Holstein Friesian dairy cows from six farms in EU countries were recruited. Based on milk somatic cell count and clinical records, cows were classified as healthy (n = 147), subclinically (n = 45) or clinically mastitic (n = 22). Circulating leukocyte RNA was sequenced with Illumina NextSeq single end reads (30 M). Differentially expressed genes (DEGs) between the groups were identified using CLC Genomics Workbench V21, followed by GO enrichment analysis. Both subclinical and clinical mastitis caused significant changes in the leukocyte transcriptome, with more intensive changes attributed to clinical mastitis. We detected 769 DEGs between clinical and healthy groups, 258 DEGs between subclinical and healthy groups and 193 DEGs between clinical and subclinical groups. Most DEGs were associated with cell killing and immune processes. Many upregulated DEGs in clinical mastitis encoded antimicrobial peptides (*AZU1*, *BCL3*, *CAMP*, *CATHL1*, *CATHL2*, *CATHL4*,*CATHL5*, *CATHL6*, *CCL1*, *CXCL2*, *CXCL13*, *DEFB1*, *DEFB10*, *DEFB4A*, *DEFB7*, *LCN2*, *PGLYRP1*, *PRTN3*, *PTX3*, *S100A8*, *S100A9*, *S100A12*, *SLC11A1*, *TF* and *LTF*) which were not upregulated in subclinical mastitis.

Conclusion. The use of transcriptomic profiles has identified a much greater up-regulation of genes encoding antimicrobial peptides in circulating leukocytes of cows with naturally occurring clinical compared with subclinical mastitis. These could play a key role in combatting disease organisms.

Key words bovine mastitis • inflammation • next generation sequence • transcriptome

List of abbreviations AMP antimicrobial peptide • **BH** Benjamini Hochberg • **DEG** differentially expressed genes • **DIM** days in milk • **GO** gene ontology • **LDH** lactate dehydrogenase • **NAGase** N-

59 acetyl-β-D-glucosaminidase • **PAMP** pathogen - associated molecular patterns • **PMNL**

60 polymorphonuclear leukocytes • **SC** somatic cells • SCC: somatic cell count

61

Introduction

Bovine mastitis is an inflammatory disease of the mammary gland. Clinical mastitis has easily recognisable symptoms. The most obvious is udder inflammation, with redness, swelling and heat around the affected area. There are visible changes in milk appearance and quality associated with a high milk somatic cell count (SCC). Mastitis can be caused by metabolic disorders, by tissue trauma and, most commonly, by environmental or contagious pathogenic microorganisms [1,2]. Causative agents are mainly bacteria but may also include fungi, yeasts and viruses. The annual costs to the dairy industry of dealing with mastitis have been estimated at over $2 billion per annum in both Europe and the USA [3,4]. There have been huge investments over many years into the development of new strategies for the prevention, diagnosis and management of mastitis, but it nevertheless remains the most economically significant bacterial disease of dairy cattle worldwide [2]. Continued advances in mastitis control are therefore necessary to ensure sustainability of dairy farming.

The most common pathogenic organisms causing bovine mastitis include *Escherichia coli*, *Streptococcus uberis* and *Staphylococcus aureus* [5-7]. In recent years the bacteriological aetiology has changed from primarily contagious forms (such as *S. aureus*) to environmental pathogens (such as *E. coli* and *S. uberis*) [8]. These bacteria enter the mammary gland and are recognised by the interaction of their pathogen - associated molecular patterns (PAMP) with Toll-like receptors (TLR2 and TLR4) expressed in resident macrophages and epithelial cells. This leads to a cascade of inflammatory responses, including the recruitment of inflammatory cells (predominantly circulating leukocytes) and upregulation of inflammatory mediators [9,10]. The resulting inflammation causes tissue damage within the udder and the associated shedding of somatic cells (SC) into the milk. Milk SC consist of many cell types, including epithelial cells, macrophages, polymorphonuclear leukocytes and lymphocytes. Both the amount of SC and changes in their gene expression are associated with physiological and pathological processes in the mammary gland. Numerous studies have found that an increase in SCC is associated with the presence of bacterial infection and this has been widely used in the diagnosis of bovine mastitis [11]. Cows with a healthy udder have a SCC less than 100,000 cells/ml milk [2]. When

88 the SCC is greater than this, mastitis is possible. A SCC greater than 400,000 cells/ml of milk is deemed
89 to be unfit for human consumption (EEC directive 92/46).

90 The development of intra-mammary inflammation activates signalling pathways involving common
91 and hepatic-specific transcription factors and pro-inflammatory mediators, which in turn leads to
92 differential expression of acute phase proteins, complement components, chemokines, antimicrobial
93 peptides (AMPs) and cell adhesion molecules [12-14]. Circulating leukocytes are then recruited to the
94 inflamed mammary gland via a series of mechanisms, such as chemokine ligand-mediated cell
95 migration and adhesion [15]. An assessment of the common genes upregulated in response to
96 experimental infections with either *E. coli* or *S. uberis* concluded that the main signalling pathways
97 activated were: 1) granulocyte adhesion and diapedesis, 2) ephrin receptor signalling, 3) RhoA
98 signalling and 4) LPS/IL1 mediated inhibition of RXR function [16]. In order to achieve an appropriate
99 balance between pathogen elimination and excessive tissue damage, then it is important that the
100 movement of leukocytes into the mammary gland occurs in a timely fashion and is properly controlled
101 [17].

102 A high proportion of dairy cows undergo various infections of their reproductive system, such as
103 endometritis/metritis and mastitis [18,19] due to suppressed immune function during early lactation.
104 This immunosuppression includes a reduction in the number of circulating leukocytes [20,21] and their
105 functional capacity [22,23]. Moreover, polymorphonuclear leukocytes (PMNL) exhibit impaired
106 phagocytic and oxidative activity [22,24] and a reduction of both cellular and humoral immunities was
107 observed, in which the responsiveness of circulating T cells to mitogenic agents and production of
108 immunoglobulin by B cells were decreased [25,26].

109 Circulating leukocytes therefore play crucial roles in the initiation, development and resolution of
110 mastitis as they are the major source of immune cells attracted to the mammary gland during an
111 infection. Most previous studies of the leukocyte inflammatory responses during mastitis have used
112 experimentally developed models of clinical mastitis [3,27,28]. Less information is available
113 concerning subclinical mastitis or naturally occurring disease. The present study investigated cows with
114 naturally occurring subclinical or clinical mastitis in early lactation identified on six unrelated farms.

Changes in global transcriptomic gene expression were determined using next-generation RNA sequencing and bioinformatics approaches. This has enabled us to compare the differing systemic responses associated with the two forms of this important disease.

Materials and methods

Animals and Sample Collection

Holstein Friesian cows for circulating leukocyte RNA sequencing were sampled as part of GplusE, a multinational research consortium FP7 project (http://www.gpluse.eu/). All sampling and diagnostic methods were performed according to standard operation procedures agreed within the consortium [29,30]. Cows were recruited from six dairy farms located in the UK (Agri-Food and Biosciences Institute Hillsborough, Northern Ireland), Denmark (Aarhus University), Ireland (University College Dublin), Germany (Leibniz Institute for Farm Animal Biology), Belgium (Walloon Agricultural Centre) and Italy (Consiglio per la Ricerca in Agricoltura e l'Analsi dell'Economia Agraria). More details are given in Supplementary file 1(1.1). All procedures had local ethical approval and complied with the relevant national and EU legislation under European Union Directive 2010/63/EU. Details of the nutritional management of each herd and the milk yields by herd over the first 50 DIM were provided previously [30]. For all cows in the study, milk yield over the initial 7-week period averaged 33.3 ± 9.3 kg/day. A summary of the milk composition data by herd is provided in Supplementary file 2.

All cows were milked twice daily, and milk yields were recorded from approximately three days in milk (DIM) onwards. Milk samples were collected from consecutive morning and evening milkings twice weekly between seven to 49 DIM, stored at 4°C and subsequently analysed for composition of protein, fat and lactose and for SCC through milk quality testing. Clinical mastitis was diagnosed using standard methods based on daily observations for abnormal changes in milk appearance (e.g. flakes, clots), quality, milk yield and mammary inflammatory responses (redness, swelling, heat, or pain). Additional morning milk samples (two × 8 ml) were collected twice weekly and stored at -18°C. The enzymes lactate dehydrogenase (LDH) (EC. 1.1.1.27) and *N*-acetyl-β-D-glucosaminidase (NAGase) (EC

3.2.1.30) were analysed by fluorometric assays [31]. Raised concentrations of both enzymes are indicators of mastitis [31].

Blood samples were collected by jugular venepuncture from a total of 214 multiparous cows from the six herds at 14 ± 4 DIM into Tempus™ blood RNA tubes (Thermo Fischer Scientific, Loughborough, UK) using a standard protocol. The Tempus tubes were shaken vigorously for 15-20 sec immediately upon collection, then frozen and stored at -80ºC for RNA extraction. The milk SCC readings obtained in week 2 of lactation from the day nearest to the blood sample collection (± 2 days) and the clinical diagnoses provided by the farms were subsequently used to categorize the cows into three groups at the time when the RNAseq analysis was performed. Healthy cows were defined as having a SCC < 100,000 cells/ml milk and no clinical symptoms (n = 147). Sub-clinically mastitic cows were defined as having a SCC between 100,000 and 400,000 cells/ml milk and no apparent clinical symptoms (n = 45). Cows diagnosed as having clinical mastitis had a SCC > 400,000 cells/ml milk and showed some of the above clinical symptoms (n = 22).

RNA Extraction

Total RNA from whole blood samples collected in Tempus tubes was extracted using Tempus spin isolation kits (Thermo Fisher Scientific) following the supplied protocol. RNA quantity and integrity were assessed using an Agilent BioAnalyzer 2000 (Agilent, Cheshire, UK) and Agilent RNA 6000 Nano Kit. RNA quantity and purity were also validated using a DeNovix DS-11 spectrophotometer (Cambridge Bioscience, UK). All selected RNA sample had a reasonable integrity (RIN number >7) and purity (260/280 between 1.8 and 2.3). Quality data are summarised in Supplementary file 3. The RNA was kept at -80°C for subsequent RNA-Sequencing.

RNA-sequencing, Mapping and Quantification

RNA-seq libraries were prepared from 750 ng of whole blood total RNA with the Illumina TruSeq Stranded Total RNA Library Prep Ribo-Zero Gold kit (Illumina, San Diego, California, USA) using the epMotion liquid handling workstation (Eppendorf, Hamburg, Germany). Pooled cDNA libraries were sequenced on the Illumina NextSeq 500 sequencer at 75 nucleotide length single end reads to reach an average of 30 million reads per sample. FASTQ files were deposited to the European Nucleotide Archive (ERP019874).

A CLC Genomics Workbench V21 (Qiagen Digital Insights, Redwood City, CA 94063, UAS) was used for sequencing analysis based on its built-in workflows, including trimming the poor quality reads, quality control and mapping the reads to a reference genome of *Bos taurus* assembly (ARS-UCD1.2) and quantifying reads per gene.

Statistical Analysis

The differentially expressed genes (DEG) between the groups were identified with a toolbox of Differential Expression for RNA-Seq built in CLC Genomics Workbench V21. This included trimmed mean and Z-score normalizations across all samples and a statistics based on a negative binomial generalized linear model, in which mastitic group was set as test variable and herd as confounding variable to control the differences of gene expression arising from herds. The genes with an absolute fold change ≥ 1.25 in pairwise comparisons between the three groups (Healthy, clinical mastitis and subclinical mastitis) were selected for subsequent analysis. P-values for the genes were adjusted using the Benjamini-Hochberg (BH) procedure and significance was considered at $P < 0.05$. The DEG identified as significant in blood leucocytes were uploaded into Partek Genomics Suite (Partek Incorporation, Missouri, USA) for GO enrichment analysis focussing on Biological Processes with a genome version of ARS-UCD1.2 to investigate the biological functions and interactions between genes and pathways. Fisher's exact test with BH adjustment was used and statistical significance was considered at $P < 0.05$.

Results

The cows were classified as healthy, subclinically mastitic or clinically mastitic. The corresponding SCC values were 38,000 ± 21,400 (n = 147), 194,000 ± 80,400 (n = 45) and 2,137,000 ± 2,144,100 (n = 22) cells/ml milk respectively (mean ± SD). The values remained in the same ranges by group at weeks 3 and 5 (Supplementary file 1 (1.2)). The milk enzyme LDH was significantly higher at the time of blood sample collection (week 2) in the cows with clinical mastitis compared with the healthy cows or those with subclinical mastitis ($P<0.0001$) while NAGase showed a progressive increase healthy<subclinical<clinical ($P < 0.05$ - 0.0001, Supplementary file 4). The mean lactation numbers did not differ between groups (healthy, 2.58 ± 1.25, sub-clinical, 2.71 ± 1.75, clinical, 2.91 ± 1.69, mean ± SD, Supplementary file 1 (1.1)). Metritis was recorded in 17/147 (11.6%) healthy cows, 3/45 (6.7%) subclinical mastitis cows and 3/21 (14.3%) clinical mastitis cows. A breakdown of the mastitis classifications by herd is also given in Supplementary file 1 (1.1), showing that infected animals were distributed across all 6 herds. A Venn diagram showing the differentially expressed genes (DEGs) in each group and their overlap is given in Supplementary file 5.

Comparison between the healthy cows and those with clinical mastitis

Firstly, we compared the global transcriptomic gene expression in leukocytes between healthy cows and those with clinical mastitis. This identified 769 DEGs after BH adjustment for multiple tests (listed in Supplementary file 6), of which 377 were upregulated and 392 downregulated in the cows with clinical mastitis compared with the healthy cows. The top 20 upregulated and downregulated DEGs are given in Table 1. This demonstrated that various types of AMPs were upregulated by over three fold in the cows with clinical mastitis, including *PTX3*, *CATHL2, CATHL5*, *CATHL6, CAMP*, *AZU1*, *TF, LTF*, *PGLYRP1* and *PRIN3*. Of these, the greatest fold change of 64 related to *PTX3*. Eight out of the 20 top upregulated genes are involved in immune/inflammatory process, including *VEPH1*, *HSPA6, CRISP3, STEAP1*, *EREG, MMP8*, *CD177* and *TNFAIP6*. The top 20 downregulated genes are involved in various functions without clear themes. For example, five are involved in protein binding activity (*DES, SEMA6B, CNN1*, *TAGLN* and *AARSD1),* of which *DES* and *SEMA6B* were decreased by 31 and 6.2 fold respectively. The remainder had fold changes between 1.9 to 4.2 and included four involved in

immunity/inflammation (*TAFA4, LYNX1, LY6D* and *TMEM18*) and four involved in metabolism (*MBAOT2, ANGPTL3, GLT8D2* and *SLC22A7*).

The upregulated and downregulated DEGs were separately subjected to GO enrichment analysis to identify the functional groups and pathways. For the upregulated DEGs, over 1000 biological functions were significantly enriched. The top GO functions were: 1) interspecies interaction between organisms, with 44 DEGs involved in body defence and killing other organisms; 2) immune system process, with 46 DEGs involved in various immune processes (immune response, leukocyte migration, immune effector process and activation of immune response, *etc*.) and 3) response to stimulus, with 122 DEGs involved in antimicrobial and immune activities (Fig 1A, Table 2). For the downregulated DEGs, many fewer biological functions (218) were identified and the enrichment scores were smaller compared with those of the upregulated DEGs (Fig 1B). The top biological functions were multicellular organism process (49 DEGs), localisation (66 DEGs) and signalling (7 DEGs).

Comparison between the healthy cows and those with subclinical mastitis

The analysis identified 258 DEG between the healthy cows and those with subclinical mastitis (Supplementary file 7), most of which (198) were downregulated in subclinically mastitic cows, with only 60 DEGs upregulated. The top 20 upregulated and downregulated DEGs are presented in Table 3. GO enrichment analysis showed that the upregulated DEGs play significant roles in 352 biological functions (Fig 1C). The top 5 enriched functions were: 1) locomotion (*CCL26, ALOX5, EDNRB, ADTRP, GFRA3* and *ADAMTS12*); 2) immune system process (*CCL26, FGA, AICDA, ALOX5, EDNRB, MX2* and *RSAD2*; 3) multicellular organismal process (*MYH2, TBX3, ACTA1, CHRM3, NMUR2, ALOX5, SLC7A11, ADGRG1, EDNRB, TGM3* and *CCDC151*); 4) response to stimulus (17 DEGs) and 5) interspecies interaction between organisms (*FGA, AICDA, MX2, RSAD2* and *GZMA*). The downregulated DEGs were involved in 257 biological functions and the GO enrichment summary is demonstrated in Fig 1D. The top five functions were: 1) localization (43 DEGs); 2) detoxification (*HBA, HBM* and *HBB*); 3) signalling (4 DEGs); 4) biological adhesion (7 DEGs) and 5) developmental process (34 DEGs). Among the 43 downregulated DEGs in the "localization" function, 12 were solute carriers (*SLC16A2, SLC20A2, SLC22A7, SLC23A1, SLC24A1, SLC25A21, SLC38A11, SLC4A1, SLC4A3,*

SLC5A7, *SLC9A3* and *SLC9A5*). The "detoxification" function contained genes encoding three haemoglobin subunits (*HBA*, *HBB* and *HBM*).

Comparison of cows with clinical and subclinical mastitis

The analysis identified 193 DEGs between the cows with subclinical and clinical mastitis, among which 166 were upregulated and 27 downregulated when fold changes were calculated as clinical/subclinical mastitis (Supplementary file 8). In the top 20 upregulated DEGs (Table 4), there were 11 genes encoding various AMPs (*PTX3*, *CATHL1*, *CATHL2*, *CATHL4*, *CATHL6*, *AZU1*, *CAMP*, *LTF*, *PGLYRP1*, *PRTN3* and *DEFB10*) and 8 DEGs associated with other immune/inflammatory processes (*CRISP3*, *MMP8*, *NGP*, *CD177*, *VEPH1*, *HSPA6*, *STEAP3* and *EREG*). Of these, *PTX3* was again the most differentially expressed (FC 61.7). In the top 20 downregulated DEGs (Table 4), seven are associated with immune/inflammatory processes (*ATP6V0C*, *BDKRB2*, *KIR3DS1*, *CXCL2*, *CD209*, *PID1* and *WNT9A*) and many others are associated with cellular homeostasis, such as protein binding (*DES*, *MYH2*, *ACTA1*, *TAC3* and *STMN3*) and cellular development (*THEG*, *RNF212B* and *ZFYVE28*). Over 700 biological functions were identified for the upregulated DEGs using GO enrichment analysis. Among them, the top functions were 1) interspecies interaction between organisms with 24 DEGs associated with response to and killing of other organisms; 2) response to stimulus (57 DEGs); 3) immune system process (21 DEGs); 4) detoxification (6 DEGs) and 5) developmental process (42 DEGs) (Table 2, Supplementary file 9A). GO enrichment analysis detected 100 altered biological functions for the downregulated DEGs in which only developmental process (*THEG*, *ACTA1*, *PIDA1*, *WDT74* and *WNT9A*) had an enrichment score over 1 (Supplementary file 9B).

Comparison of the common DEGs between healthy cows and those with subclinical or clinical mastitis

A Venn diagram (Supplementary file 5) for the three groups of cows showed that there were 100 DEGs in the comparisons of both the clinical and subclinical mastitis groups with the healthy group. Among

these common DEGs, most of them (79) were downregulated in the mastitic cows and only 21 were upregulated (Supplementary file 10). There were 169 DEGs in the comparisons of both the subclinical mastitis and healthy groups with the clinical mastitis group, in which most of them were upregulated (153) and only 16 were downregulated in the cows with clinical mastitis. The upregulated DEGs included a large proportion of genes encoding various AMPs (such as *AZU1, CAMP, CATHL1, CATHL2, CATHL4, CATHL5, CATHL6, CXCL13, DEFB1, DEFB10, DEFB4A, DEFB7, LCN2, LTF, PGLYRP1, PRTN3, PTX3, S100A8, S100A9* and *S100A12*) and molecules associated with immune/inflammatory processes (such as *CD14, CD34, CD163, CD177, CFB, CRISP3, ERG1, MMP8, MMP9, VEPH1)* (Supplementary file 11). Of these, *CD14* and *CD163* are commonly used markers for monocytes/macrophages, and *MMP8, MMP9* and *CD177* are markers for neutrophils. There were only two common DEGs (*CATHL4* and *GCA*) shown in all three comparison pairs.

Discussion

Bovine mastitis is a significant problem for the dairy industry, resulting in both reduced milk quality and yield. This multifactorial disease is complex in origin, as many factors contribute to the development of mastitis, including different microbial species, and key aspects of the management and environment, particularly with relation to dry cow therapy, hygiene and housing [32]. Individual cows also exhibit varying degrees of susceptibility and resistance [24,33]. In response to mastitis, the SCC increases due to the influx of immune cells, along with an inflammatory process. Previous studies have investigated circulating leukocyte gene expression in cows with mastitis induced by experimental infection with *E. coli* [34,27] or *S. aureus* [35], but information on leukocyte gene expression profiles in cows with naturally occurring subclinical mastitis is lacking. Subclinical mastitis is, however, considered as the most economically important type of mastitis due to its higher prevalence and longer term effects [2,36]. Whole peripheral blood has previously been widely used in gene expression studies used to investigate disease due to its initial ease of collection and because it can be processed directly without the requirement to separate out specific cell types. The transcriptional changes measured between different cows in the study therefore represent changes in gene expression within particular cell types (which will include T and B lymphocytes, natural killer cells, platelets, PBMC and

298 granulocytes (neutrophils, eosinophils and, basophils)) combined with alterations in their relative
299 proportions [37]. Despite this limitation, transcriptional signatures of whole blood can reliably
300 differentiate individuals with a variety of infections (e.g. human tuberculosis [38]). The present study
301 demonstrated both the shared and different gene expression profiles in circulating leukocytes between
302 cows with naturally occurring clinical or subclinical mastitis using next generation sequencing and
303 bioinformatics approaches.

304 **Inflammation and immune defence mechanisms**

305 Invasion of pathogenic microorganisms into the mammary gland triggers inflammation and leads to the
306 development of subclinical or clinical mastitis. These two types are interdependent. The initial stage of
307 bovine mastitis may be subclinical which can subside, persist as a chronic inflammation or develop into
308 a clinical inflammation [2]. Circulating leukocytes, as a major source of immune/inflammatory cells
309 and body defence mechanisms, play crucial roles in initiation, maintenance and resolution of all types
310 of mastitis [3,39]. In the present study, we identified 258 DEGs in circulating leukocytes isolated from
311 cows with subclinical mastitis and 769 DEGs from cows with clinical mastitis compared with the
312 healthy cows. This suggests that the number of DEGs by circulating leukocytes was associated with the
313 severity and development of the inflammatory process in the udder. The differences may also reflect
314 the particular disease causing organism involved, but this was not possible to evaluate within the present
315 experiment.

316 Clinical mastitis upregulated various biological functions related to responding, inhibiting and killing
317 the invaded pathogens. Both the top 20 upregulated genes and top activated biological processes
318 concentrated on the genes encoding various AMPs and immune/inflammatory molecules, with a clear
319 theme of body defence against pathogen invasion. The GO enrichment scores of biological functions
320 related to this theme were relatively high (31 - 22 for the top three functions). The function of
321 interspecies interaction between organisms (mainly involved in responding to and killing invaded
322 pathogens) had an enrichment score of 31 and comprised 44 upregulated genes. In contrast, the top 20
323 upregulated genes in the subclinical mastitis group had diverse functions, such as signalling (*MYH2*),
324 reproductive (*TBX3*), oxidant detoxification (*HBE2*) and immune processes (*CCL26*, *PGA*, *PRG3_1*,

PRG3_2, *GZMB_2*, *RNASE2*, *BDKRB2*, *SERPINB10* and *AIDA*). The enrichment scores were also relatively low, in which the top five functions related to body defence scored at between 4 and 5. The function of interspecies interaction between organisms and immune system process also had a relatively low enrichment score (4) and fewer players (5 and 7 DEGs, respectively). This indicates that the immune defence mechanisms activated in circulating leukocytes in the cows with clinical mastitis were more intensive than in those with subclinical mastitis.

Comparisons of the gene expression between the cows with clinical and subclinical mastitis identified 193 DEGs, in which 166 were upregulated in the clinically mastitic cows. Some of the genes might already be altered/upregulated in cows with subclinical mastitis and this may explain why fewer DEGs were detected in this comparison. The top 20 upregulated DEGs showed a clear theme of body immune defence against the invaded pathogens, as a large proportion of the listed genes were associated with AMPs and immune/inflammatory responses (see Tables 2 and 4). GO enrichment analysis demonstrated that the pathways related to responding and killing microorganisms (interspecies interaction between organisms), leukocyte development and locomotion and regulation, and immune process were the top activated pathways, with enrichment scores of 11 – 22.

Antimicrobial peptides

The common genes in the comparisons clinical mastitis vs healthy cows and clinical vs subclinical mastitic cows contained at least 16 antimicrobial peptides, which all contributed to the top pathway "interspecies interaction between organisms" (enrichment score 22). These were *AZU1*, *CAMP*, *CATHL1*, *CATHL2*, *CATHL4*, *CATHL5*, *CATHL6*, *CXCL13*, *DEFB1*, *DEFB10*, *DEFB4A*, *DEFB7*, *LCN2*, *PGLYRP1*, *PRTN3*, *PTX3*, *S100A8*, *S100A9*, *S100A12* and *LTF*. This suggests that upregulation of production of a variety of AMPs was related to the severity of the mammary inflammatory process and was one of main distinguishing differences in the way that circulating leukocytes responded to clinical mastitis compared with subclinical mastitis. This difference is likely to relate to the pathogen involved. *E. coli* infections have global effects which are generally of short duration and induce a rapid rise in the pro-inflammatory cytokines TNFA, IL1B and IL6 in mammary tissue via TLR4-dependent induced signalling [39,14]. This results in a fast influx of neutrophils to inhibit bacterial growth. In

contrast, gram-positive bacteria such as *S. uberis* cause a slower and less dramatic response [40] whereas *S. aureus* is associated with local and more persistent infections. In these cases TLR signalling increases *IL*6 expression but does not up-regulate TNFA and IL1B and so this pathogen is better able to evade the host immune response [39].

AMPs are key components of the innate immune system [41] in which leukocyte AMPs are multifunctional effector molecules [42]. They act as endogenous antibiotics to kill various pathogens directly by forming pores in their membranes via toroidal, carpet or barrel stave mechanisms. These pores allow cytoplasmic leakage that ultimately leads to cell death [43]. These antimicrobial activities were originally regarded as the primary functions of these peptides. It is now clear that, in addition to the direct activities, AMPs play important roles in regulating multiple aspects of innate and adaptive immunity, including inflammation and wound repair, and they are also involved in maintaining homeostasis [44,45]. Over 2,000 natural AMPs have been identified, of which cathelicidins and β-defensins are the most studied [45]. Members of both these families contribute to the first line of defence against many pathogens, including Gram-positive and Gram-negative bacteria, viruses, fungi and some unicellular parasites [46,47]. They both belong to a large group of cationic peptides with amphipathic properties, which enables them to permeate pathogen membranes [48]. At least seven cathelicidins and nine β-defensins have so far been identified in cattle [49,47], and of these six cathelicidins and four β-defensins were identified in this study as being upregulated in leukocytes of cows with clinical mastitis. AMPs thus possess dual capacity to control infection directly and to regulate host defences to help clearance of the invaded pathogens. Conventional mastitis control strategies include antibiotic therapy but this raises major concerns over both antibiotic residues in milk and the increase in antimicrobial resistance [50]. The use of AMPs has, therefore, been proposed as an alternative to antibiotics and immunomodulators for treatment of several bacterial infections [43]. Synthetic cathelicidins with enhanced antimicrobial activity have now been engineered and may in future provide a novel treatment option for bovine mastitis [51].

Metabolic effects

In the present study, the downregulated genes in cows with both subclinical and clinical mastitis were associated with the biological functions related to homeostasis, such as localization, biological adhesion, developmental process and signalling. The leukocyte samples were taken in early lactation, around 14 days after calving. In peripartum cows, decreased feed intakes and increased energy demands to support lactation often result in negative energy balance [22]. Immune cells re-programme their cellular metabolism in response to bacterial and viral infections to provide energy and molecules for immune processes [52]. In cows developing an infection while also experiencing negative energy balance there is competition for limited nutrients between the demands of milk synthesis and mounting an immune response [53]. This leads to a decreased number and functionality of circulating immune cells [54,21], which is likely to predispose cows to infections and inflammatory diseases, such as mastitis and endometritis [55]. In addition, metabolic hepatic pathways including those involving lipid metabolism are affected by mammary gland challenge with *E. coli* or *S. aureus*, demonstrating that the liver restricts metabolic tasks during a mammary infection [12]. The timing of the present study meant that the cows with subclinical or clinical mastitis were also likely to be experiencing a metabolic deficit, which may well have affected their immune responses.

Study limitations

This study was based on naturally occurring cases of mastitis in six herds of cows, in different countries and with differing genetics and management. This is both a strength of, and limitation to, the study. On the one hand, the variability between animals reduced the power of the analyses performed. On the other hand, the transnational approach to cow recruitment meant that the significant differences in gene expression which were detected are likely to be of more widespread relevance. We were, however, unable to measure protein expression in the leukocytes to confirm that the mRNA changes detected were reflected in protein production. It was also not possible within the study design to perform diagnostic tests to identify the pathogens involved and it is well known that different bacterial species cause different host responses [56,39]. Despite this, similarities in response do still exist across bacterial species, and the upregulation of bacterial killing by AMPs and the downregulation of the biological functions related to homeostasis for the leukocytes are consistent with previous work. Another issue is that a number of major cytokines (IFNG, IL1B, IL6, IL8, IL10, IL12 and TNFA) are upregulated during

406 mammary infections in a time dependent manner [40], but our study did not find their differential
407 expression in the leukocytes. While their expression patterns can be well detected in cases of
408 experimentally developed mastitis, we might have missed the peak expression values of these cytokines,
409 as it was not possible to obtain samples at precise time points during the course of infection in naturally
410 occurring cases.
411
412 **Conclusions**

413 The present study described the leukocyte transcriptome from cows with naturally occurring subclinical
414 and clinical mastitis in early lactation using next generation sequencing and bioinformatics technology.
415 To our knowledge, this is the first time that the transcriptomic profiles in cows with subclinical mastitis
416 have been compared with those in both healthy cows and those with clinical mastitis. Both conditions
417 were associated with significant changes in gene expression in circulating leukocytes in accordance
418 with the severity of mammary inflammation. Cows with clinical mastitis had a greater number of
419 upregulated genes involved in various immune processes including body defence, leucocyte migration
420 and antigen presentation. These results using RNA-seq have validated previous work by showing
421 greater upregulation of AMPs in cows with clinical compared with subclinical mastitis. This is
422 consistent with the greater influx of activated neutrophils to the mammary gland experienced during
423 clinical mastitis and is likely to increase their ability to kill invading pathogens. In the cases of
424 subclinical mastitis many immune genes were also differentially expressed but to a lesser extent and
425 there was a greater emphasis on metabolic pathways.
426
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431 necessarily reflect the views of the European Commission.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

All procedures had local ethical approval and complied with the relevant national and EU legislation under European Union Directive 2010/63/EU.

Data availability

The sequencing data were deposited to the European Nucleotide Archive (ERP019874).

Table 1. Top 20 upregulated and downregulated circulating leukocyte genes between the healthy (control) cows (n = 147) and those with clinical mastitis (n = 22)

Upregulated genes			Downregulated genes		
Gene Symbol	**Fold change**	**P***	**Gene Symbol**	**Fold change**	**P***
PTX3	63.82	4.42E-42	*DES*	-30.91	5.63E-06
VEPH1	22.13	2.12E-21	*SEMA6B*	-6.23	2.14E-03
HSPA6	8.47	7.23E-34	*CNN1*	-4.17	1.20E-02
CATHL6	8.26	3.10E-07	*TAFA4*	-3.67	2.15E-03
AZU1	6.57	5.78E-06	*LYNX1*	-3.52	5.87E-03
CATHL2	6.12	1.36E-06	*TAGLN*	-2.88	7.86E-03
LTF	5.03	3.46E-08	*MBOAT2*	-2.62	1.40E-03
CRISP3	5.02	2.72E-05	*GIMD1*	-2.41	5.86E-03
STEAP1	4.81	8.45E-07	*CST8*	-2.34	2.93E-02
EREG	4.26	5.85E-06	*WDR74*	-2.32	2.54E-04
MMP8	4.16	9.55E-07	*ANGPTL3*	-2.10	1.26E-02
CAMP	3.91	8.66E-05	*GLT8D2*	-1.98	1.21E-03
PRTN3	3.83	7.43E-06	*SLC25A21*	-1.97	2.07E-02
ALB	3.81	2.83E-02	*C12H13orf46*	-1.96	1.12E-02
PGLYRP1	3.52	1.02E-06	*ADAM32*	-1.96	1.64E-03
TF	3.34	3.48E-02	*SMIM18*	-1.95	3.24E-03
CATHL5	3.17	9.28E-04	*AARSD1*	-1.92	2.11E-02
MYRFL	3.15	1.49E-06	*LY6D*	-1.89	9.44E-03
CD177	3.12	3.56E-05	*TMEM232*	-1.88	9.23E-04
TNFAIP6	3.11	1.96E-07	*SLC22A7*	-1.87	3.52E-03

*P values were adjusted using the Benjamini-Hochberg method for false discovery rate control.

446 **Table 2**. Summary of GO enrichment main functions of DEGs upregulated in the cows with clinical
447 mastitis or subclinical mastitis compared with the healthy cows.

Function	**Enrichment score**	**DEGs in the function**
Clinical mastitis vs healthy cows		
Interspecies interaction between organisms	31	*PTX3, CATHL6, AZU1, LTF, PGLYRP1, TF, CXCL13, LRG1, CATHL1, CATHL4, LCN2, S100A9, APOA2, HP, DEFB1, CD14, S100A8, CCL8, CFB, GZMA, S100A12, FCGR1A, RSAD2, NECTIN2, ARG2, MX2, IL18, IL12B, FKBP5, SLC11A1, BCL3, RGS1, HMGB3, CSF1, CEBPE, TMEM229B, CFP, FAM20A, SCARB1, STOM, CEBPB, PYCARD, ANXA1, MX1*
Immune system process	22	*PTX3, AZU1, LTF, PRTN3, PGLYRP1, THY1, CXCL13, LCN2, VTN, DCSTAMP, S100A9, CDH26, HP, ADGRG3, CD14, S100A8, ITGA9, IL18R1, CFB, ALOX5, S100A12, EGR1, FCGR1A, IL18RAP, RSAD2, CD24, ARG2, MX2, IL18, IL2RA, IL12B, PTPRO, SLC11A1, BCL3, HMGB3, CSF1, LGALS9, MERTK, HSD3B7, CFP, PYCARD, LTBR, ANXA1, SKAP2, STAT3, MX1*
Response to stimulus	22	*PTX3, HSPA6, CATHL6, AZU1, LTF, ALB, PGLYRP1, TF, MMP9, CXCL13, LRG1, CATHL1, CATHL4, ALPL, FOLR3, CREB3L3, LCN2, VTN, GLP1R, RAB20, TBX3, TNIP3, DCSTAMP, HSPA1A, S100A9, AREG, APOA2, ORM1, CD163, HP, SLC6A2, RYR1, DEFB1, CD14, S100A8, DYSF, CFB, ALOX5, NMUR2, GZMA, S100A12, EGR1, GRPR, SOD2, SOCS1, FCGR1A, IL18RAP, RSAD2, CHI3L1, P2RX1, TREM1, CAPN3, SOCS3, ETV5, PLA2G4F, CDKN1A, SORT1, MFSD2A, GCH1, WIPI1, CD24, AK4, ROR2, ARG2, NUPR1, FOSB, MT2A, AURKB, UHRF1, GPBAR1, PAX8, MX2, IL18, MAPK13, IL12B, FKBP5, ACVR1C, MGST1, SLC11A1, BCL3, MSC, RGS1, HMGB3, CSF1, CEBPE, MANF, PRDX5, BAG3, PAM, PTAFR, TMEM229B, CFP, CADPS2, PYCR1, FAM20A, FOS, HSPA5, MYBL2, DTL, SCARB1, HK2, TFEC, SESN2, DNAJB1, METRNL, NIBAN1, PLA2G4A, CEBPB, PYCARD, LTBR, ANXA1, GADD45A, STAT3, KLF4, FAIM2, AQP9, MX1, FANCD2, PTTG1, CDC25A, MCM2, SDC4*
Clinical mastitis vs subclinical mastitis		
Interspecies interaction between organisms	22	*PTX3, CATHL6, CATHL4, AZU1, LTF, PGLYRP1, CATHL1, LCN2, LRG1, CXCL13, DEFB1, S100A9, HP, S100A8, CD14, CFB, FCGR1A, S100A12, NECTIN2, FAM20A, HSPB1, TMEM229B, RGS1, FKBP5*
Response to stimulus	13	*PTX3, CATHL6, HSPA6, CATHL4, AZU1, LTF, PGLYRP1, CATHL1, MMP9, ALPL, CREB3L3, FOLR3, LCN2, LRG1, CXCL13, DEFB1, RAB20, PLOD2, HSPA1A, TNIP3, S100A9, SLC6A2, HP, AREG, DYSF, RYR1, DCSTAMP, S100A8, CD163, EGR1, CD14, CFB, FCGR1A, S100A12, ETV5, IL18RAP, PAX8, FOSB, ROR2, MAPK13, FOS, WIPI1, GPBAR1, CHI3L1, TREM1, CDKN1A, SOD2, GCH1, FAM20A, DNAJB1, PRDX5, NAPRT, HSPB1, TMEM229B, RGS1, FKBP5, TFEC*
Immune system process	11	*PTX3, AZU1, LTF, PGLYRP1, PRTN3, THY1, LCN2, CXCL13, S100A9, HP, IL18R1, DCSTAMP, ADGRG3, S100A8, EGR1, CD14, CFB, FCGR1A, S100A12, IL18RAP, IL2RA*

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Table 3. Top 20 upregulated and downregulated circulating leukocyte genes between the healthy (control) cows (n = 147) and those with subclinical mastitis (n = 45)

Upregulated genes			Downregulated genes		
Gene Symbol	**Fold change**	**P***	**Gene Symbol**	**Fold change**	**P***
MYH2	4.62	2.52E-03	*SEMA6B*	-14.79	9.54E-09
ATP6V0C	3.46	3.63E-05	*TAFA4*	-3.50	2.03E-04
THEG	2.66	1.74E-02	*CATHL4*	-2.73	1.46E-02
CCL26	2.60	2.87E-03	*DMTN*	-2.71	7.98E-03
FGA	2.53	3.45E-02	*HBA*	-2.59	5.48E-03
TBX3	2.30	2.12E-04	*MARCO*	-2.56	1.25E-02
PRG3_1	2.30	7.89E-03	*LYNX1*	-2.18	1.77E-02
RNASE2	2.04	1.45E-02	*HBM*	-2.02	1.30E-02
ACTA1	1.92	2.06E-02	*SLC4A1*	-1.96	7.40E-03
BDKRB2	1.86	1.91E-02	*ALAS2*	-1.94	9.04E-03
PRG3_2	1.85	2.42E-02	*C17orf100*	-1.91	2.26E-02
GZMB_2	1.77	1.32E-02	*HBB*	-1.89	1.42E-02
GNG4	1.76	1.43E-02	*MSMB*	-1.87	1.06E-02
HAL	1.63	1.30E-03	*AQP1*	-1.85	2.61E-02
GPAT2	1.63	9.38E-03	*C15H11orf94*	-1.84	8.09E-03
OVOS2	1.63	9.20E-03	*ADD2*	-1.79	1.56E-02
CDHR5	1.62	9.11E-03	*DHDH*	-1.77	4.01E-03
SERPINB10	1.53	1.91E-02	*SPACA7*	-1.69	3.13E-02
AICDA	1.53	1.04E-02	*SLC22A7*	-1.67	3.42E-03
HBE2	1.52	4.96E-02	*ESRP1*	-1.62	4.87E-02

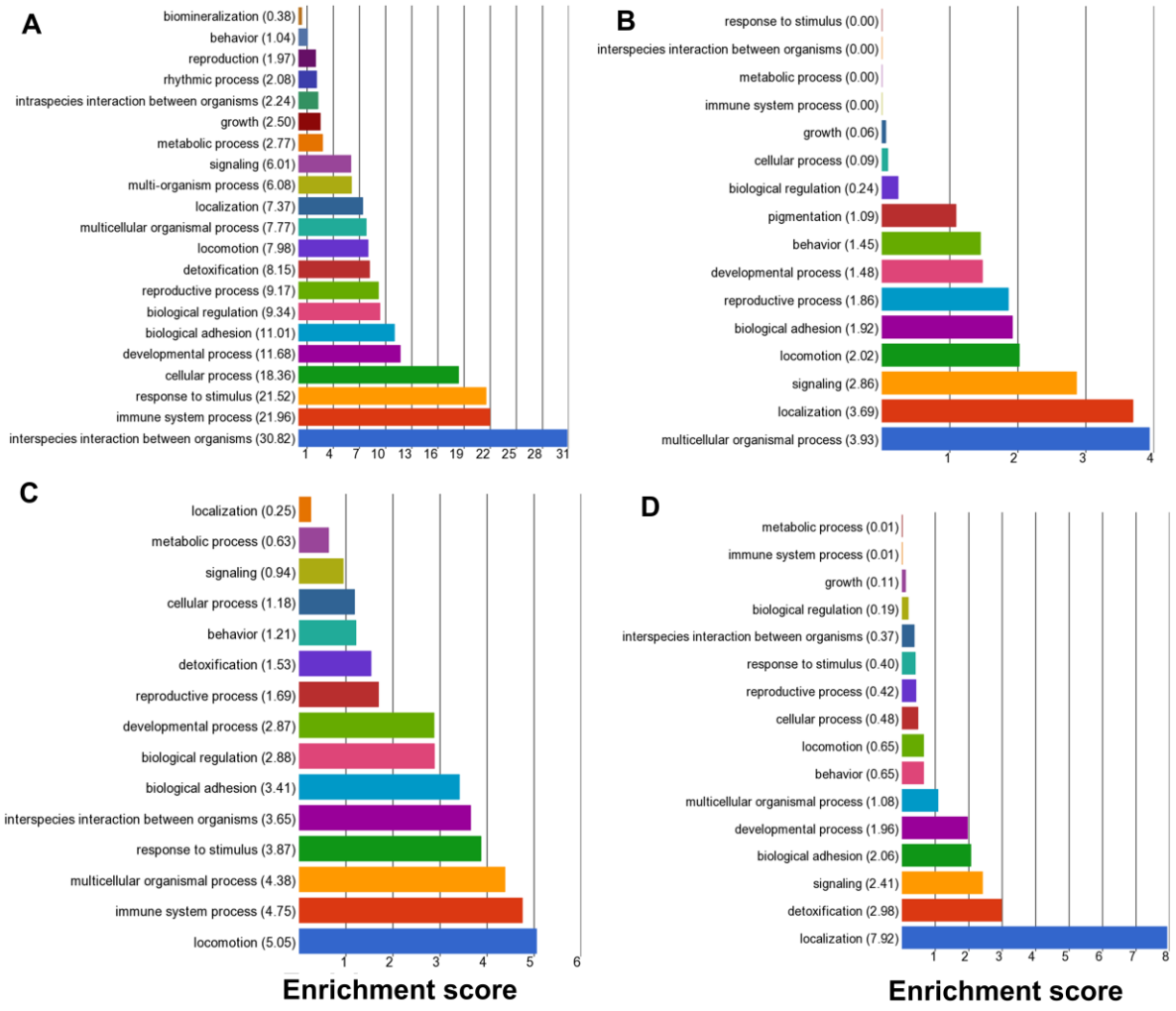
*P values were adjusted using the Benjamini-Hochberg method for false discovery rate control

457 **Table 4**. Top 20 upregulated and downregulated circulating leukocyte genes between the cows with subclinical
458 (n = 45) and clinical (n = 22) mastitis

Upregulated genes			Downregulated genes		
Gene Symbol	**Fold change**	**P***	**Gene Symbol**	**Fold change**	**P***
PTX3	61.69	8.65E-32	*DES*	-20.41	5.75E-04
VEPH1	24.32	4.13E-17	*MYH2*	-18.74	1.47E-04
CATHL6	9.86	1.79E-06	*THEG*	-4.72	2.30E-02
HSPA6	8.25	4.38E-25	*ACTA1*	-3.73	6.55E-03
CATHL2	7.14	6.60E-06	*ATP6V0C*	-2.52	4.34E-02
CATHL4	6.93	1.06E-03	*BDKRB2*	-2.45	4.17E-02
STEAP1	6.72	7.45E-07	*KIR3DS1*	-2.20	8.69E-03
AZU1	6.45	1.50E-04	*PID1*	-2.17	3.01E-02
LTF	6.20	5.36E-08	*CXCL2*	-1.98	1.79E-02
CRISP3	6.20	4.98E-05	*TAC3*	-1.93	2.07E-02
CAMP	5.36	5.62E-05	*WDR74*	-1.86	2.72E-02
MMP8	4.98	1.91E-06	*CD209*	-1.85	2.20E-02
PGLYRP1	4.84	8.19E-08	*RNF212B*	-1.84	4.01E-02
CD177	4.58	2.12E-06	*TMEM232*	-1.70	2.07E-02
NGP	4.10	5.17E-05	*NRIP3*	-1.63	2.70E-02
CCN3	4.03	2.08E-02	*ZFYVE28*	-1.62	4.74E-02
EREG	3.42	1.18E-03	*IDO2*	-1.60	3.41E-02
CATHL1	3.40	7.75E-04	*WNT9A*	-1.53	4.03E-02
PRTN3	3.39	1.14E-03	*STMN3*	-1.47	3.42E-02
DEFB10	3.36	3.28E-07	*UCHL3*	-1.44	2.14E-03

459 *P values were adjusted using the Benjamini-Hochberg method for false discovery rate control

460



461

462 Fig 1.

463

464 **Figure legend**

465 **Fig 1.** Gene Ontology (**GO**) enrichment analysis for the differentially expressed leukocyte genes
466 which were upregulated (A) or downregulated (B) between the cows with clinical mastitis (n = 22)
467 and the healthy cows (n = 147), and upregulated (C) or downregulated (D) between the cows with
468 subclinical mastitis (n = 45) and the healthy cows (n = 144).

469

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