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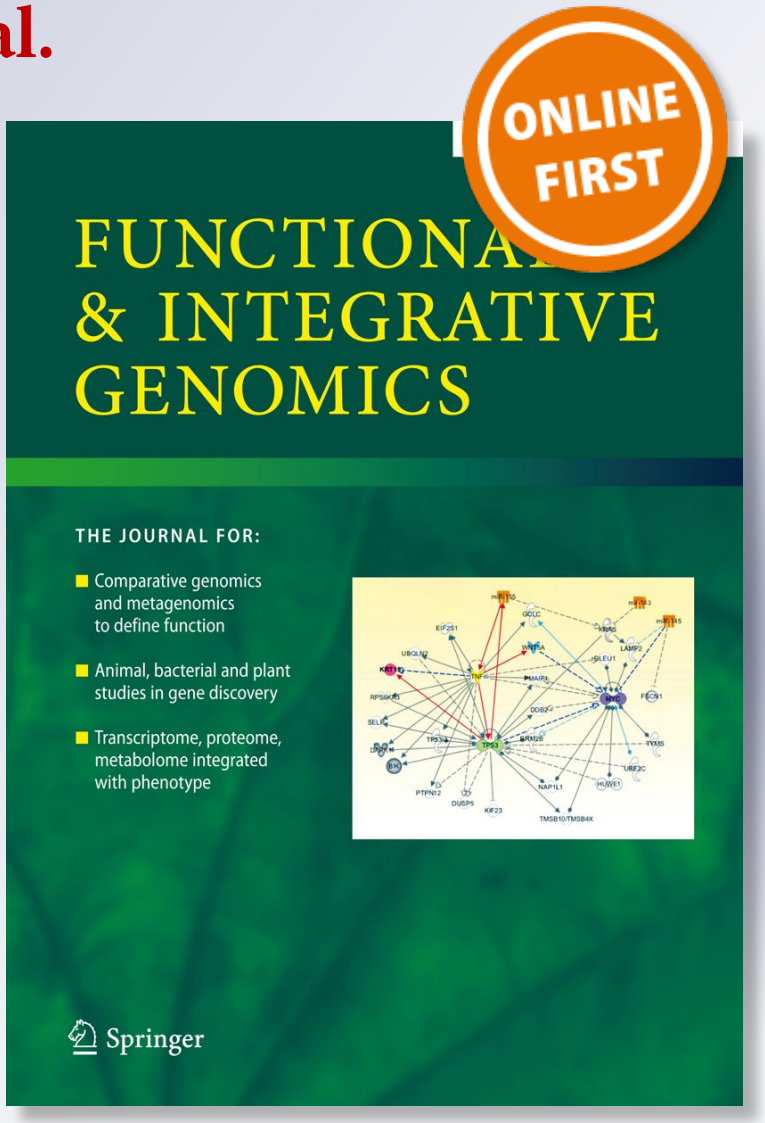

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ORIGINAL ARTICLE

Evaluation of circulating leukocyte transcriptome and its relationship with immune function and blood markers in dairy cows during the transition period

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Abstract

Dairy cows during the transition period are faced with important physiological changes which include a dysfunctional immune system and an increased inflammatory state. New data are necessary to understand the key factors involved in the immune system regulation. Six dairy cows were sampled during transition period to investigate the leukocyte transcriptome changes and its relationship with blood biomarkers. Blood samples were collected at − 20 ± 2, − 3 ± 1, 3, and 7 days from parturition (DFP). Leukocyte transcriptome was analyzed by deep sequencing technology (Hiseq1000 Illumina, USA). Plasma was analyzed for metabolic biomarkers. Differentially expressed genes (DEG) were used to run an enrichment analysis through the Dynamic Impact Approach (DIA). Considering − 20 DFP as references time, the main KEGG impacted pathways were activated before calving (− 3 DFP) and were connected to lipid metabolism, lipid transport in plasma, and phagosome. The greatest differences were found after parturition with 281 DEG (179 upregulated and 102 downregulated). The activated pathways were mainly related to immunity and endocrine aspects, while metabolic pathways related to lipid and amino acid metabolism were inhibited. Plasma BHBA had a substantial inhibitory impact on KEGG pathways related to DNA replication and cell cycle, while plasma IL-1β had an inhibitory impact on fatty acid elongation in mitochondria and an activated impact in several pathways related to cellular energy metabolism. Overall, this study confirmed that many changes in lipid metabolism and immune competence of the circulating leukocytes occurred in dairy cow around calving. Interestingly, BHBA and IL-1β connected with the transcriptome.

Keywords Immune system · Transition period · Dairy cows · Transcriptomics · Plasma metabolites · RNA-Seq

Introduction

The transition period of dairy cow encompasses physiological changes (Goff and Horst 1997; Drackley 1999; van Knegsel et al. 2014), including the reduction of immune competence (Kehrli et al. 1989a, b; Goff and Horst 1997; Mallard et al. 1998; Crookenden et al. 2016), occurrence of the negative energy balance (Drackley 1999), hypocalcemia (Goff and

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Horst 1997; DeGaris and Lean 2008), overt systemic inflammatory response (Bionaz et al. 2007; Sordillo et al. 2009), and oxidative stress status (Sordillo and Aitken 2009; Celi and Gabai 2015). When these adaptations are too intense and prolonged, the cow struggles to return to homeostasis and some adaptive mechanisms can be dysregulated, explaining the appearance of metabolic and infectious diseases, such as mastitis (Goff and Horst 1997; Drackley 1999). With the exception of some components of the immune system that act days before parturition, most of these changes are concentrated to the time immediately after the calving (Trevisi et al. 2015; Jahan et al. 2015; Trevisi and Minuti 2018).

Some functions of the immune system are depressed before calving, including neutrophil phagocytosis (Kehrli et al. 1989a; Mallard et al. 1998; Lacetera et al. 2005), the ability of lymphocytes to respond to mitogens and to produce antibodies (Kehrli et al. 1989b; Lacetera et al. 2005), and concentrations of relevant components in plasma such as immunoglobulins (Goff and Horst 1997; Mallard et al. 1998; Lacetera et al. 2005), IFNγ (Lacetera et al. 2005), complement (Trevisi et al. 2010), and lysozyme (Trevisi et al. 2012).

In some studies, the alteration of immunity has been associated with various pathologies, mainly evident after calving, which include the recurrence of existing intra-mammary infections or new mammary infections (Goff and Horst 1997) and postpartum uterine diseases (Sheldon et al. 2008). However, the susceptibility of the circulating leukocytes to release pro-inflammatory cytokines after LPS stimulation increases significantly a few days before calving (Jahan et al. 2015), suggesting that LPS could quickly trigger the immune response during this period.

Thus, the better understanding of the origin of these modifications and the time of their appearance, with respect to the time of calving, seems pivotal for the discovery of the breakpoint in the homeostasis during the transition period. The aims of this study were to investigate changes of leukocyte gene expression in the peripartum and to highlight possible links between leucocyte transcriptome and metabolic conditions of dairy cows.

Materials and methods

Animals and diets

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted with the DL No. 116, 27/01/1992 and its later amendments or comparable ethical standards.

The study was carried out at the experimental dairy farm of Università Cattolica del Sacro Cuore (Piacenza, Italy) using 6 multiparous Holstein Friesian cows housed in an artificially lit and ventilated tie-stall barn. Climatic conditions were maintained under almost constant settings: environmental temperature around 20 °C, relative humidity between 60 and 70%, and photoperiod with 14 h of light (from 5:00 to 19:00 h) and 10 h of dark. Cows were fed individually, and the diet offered ad libitum: forages every 12 h and concentrates by an autofeeder every 12 or 3 h in dry and lactating phases, respectively. Details of the diets (ingredients and main chemical composition) are reported in supplementary materials (Table S1). On average, cows received 9 to 12 kg of grass hay, 8 to 10 kg of maize silage, and 1 to 2 kg of concentrate per day. After calving, the diet included 2 kg of grass hay and 3 kg of alfalfa hay, while concentrate and maize silage were gradually increased (on day 30 of lactation, the cows received on average 11 to 13 kg of concentrate and 18 to 20 kg of maize silage).

Milk yield, body condition score, and health problems

During the experimental period, the animal's health condition was checked every day by individual inspections. Rectal temperature was also measured daily in the morning. The milk yield of each cow was weighted at each milking. Body condition score (BCS) was individually evaluated fortnightly, by the same observer, according to a 4-point scale (ADAS 1986).

Experimental schedule

The experimental days for the study were − 20 ± 2 days, − 3 ± 1 days, 3 days, and 7 days from parturition (DFP). At each experimental day blood samples were collected to assess a wide range of metabolic and inflammatory plasma profiles; and total RNA was extracted from circulating leukocytes.

Blood sampling and processing

Blood samples to assess metabolic and inflammatory status were collected in the morning before feeding on all experimental days from the jugular vein into evacuated 10-mL tubes containing lithium heparin as an anticoagulant (Vacuette, Kremsmünster, Austria) and immediately cooled in ice water. A small amount of blood was used for packed cell volume determination (Centrifugette 4203, ALC International srl, Cologno Monzese, Italy); the remainder was centrifuged at 3500×*g* for 15 min at 4 °C and the plasma was frozen at − 20 °C until analysis. The plasma samples were analyzed by a clinical auto-analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA, USA) using commercial kits and methods indicated by Calamari et al. (2016). The parameters included in the metabolic profile were as follows: (i) energy and protein metabolism markers: glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA) urea, creatinine; (ii) inflammatory response markers: haptoglobin, ceruloplasmin, albumin, paraoxonase (PON),

cholesterol, zinc total bilirubin; (iii) liver markers: aspartate aminotransferase (GOT), γ-glutamil transferase (GGT), alkaline phosphatase (ALP); (iv) oxidative stress response markers: total reactive oxygen metabolites (ROMs), total nitric oxide metabolites (NO_x), nitrites (NO_2), nitrates (NO_3); (v) minerals: Ca, P, Mg, Na, K, Cl; (vi) other parameters: total proteins and globulins.

Peripheral blood cell transcriptomics

RNA was extracted from blood collected from the jugular vein into evacuated 10-mL tubes containing sodium citrate as an anticoagulant (Vacuette, Kremsmünster, Austria). The blood was immediately diluted 1:1 with RBC lysis buffer (RBC LYSIS solution, 5 PRIME, Code: 733-1035) kept in ice water for 20 min and then processed for RNA extraction. The total RNA was isolated from blood samples following the procedure adapted from Chomczynski and Sacchi (2006). Briefly, RBC lysis buffer was used to lyse red blood cells followed by several centrifugation steps until obtaining the cell pellet and immediately treating with Trizol (Ambion RNA, Life Technologies, USA). Total RNA was extracted using a phenol:chloroform solution. The total RNA was then recovered by precipitation with isopropanol and washed with 75% ethanol. The pellet was then dried and RNA eluted in RNA storage solution (Ambion Inc., Austin, TX). The samples were stored at − 80 °C until analyses.

RNA sequencing

A total of 1 μg RNA was evaluated for integrity using the Agilent Bioanalyzer assay (Agilent 2100 Bioanalyzer system, Agilent Technologies). Only samples that had RNA integrity number (RIN) higher than 7 and a 28s/18s greater than 1.6 were considered. At this point, 2 out of 24 samples were rejected and consequently 22 samples were used for library construction and sequencing. Total RNA was used for the library construction following the TruSeq® RNA protocol. The libraries were then sequenced on a Hiseq1000 (Illumina, USA) with a 2× 100PE protocol. The barcoding technique was used to sequence 11 samples/lane with 300 million reads/lane.

Data analysis and bioinformatics

Raw sequence reads were quality controlled using FastQC v 0.11.6 and subsequently trimmed with Trimmomatic v 0.33 accounting for adapter sequences and eliminating start-end low quality bases (under a Phred score of 3). Trimmed reads were aligned to the cow reference genome (UMD3.1; Zimin et al. 2009) with TopHat2 v 2.1.1 (Trapnell et al. 2009) using the default parameters except for the *−b* parameter that accounts for fragment bias due to library construction.

Cufflinks was launched with default parameters and setting *−u* that corrects for the multiple hits, using the reference annotation (Ensembl Genes 89). This step produced a list of transcripts that were used for the downstream analyses. The aligned data were analyzed using HTSeq-Count (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) to measure their relative abundances with respect to the generated transcripts.

Differential expression analysis was conducted using the edgeR package, used to estimate biological variation between replicates and conduct exact tests of significance on small counts (Li et al. 2010). The input was a matrix composed by the transcripts identified by the cufflinks suite: for any sample, each cell of the matrix consisted of the number of reads that uniquely mapped to that transcript. Uniquely mapped reads, used to avoid misinterpretation of the data, were normalized using the trimmed mean of *M*-values normalization procedure. This method estimates scale factors between samples and corrects for biases introduced by RNA composition and differences in the total numbers of uniquely mapped reads in each sample (Robinson and Oshlack 2010). A general linear model was applied considering − 20 DFP as baseline; raw *P* values for the time effect were adjusted using Benjamini and Hochberg's false discovery rate (FDR). Differentially expressed genes (DEG) between − 20 DFP and − 3, 3, and 7 DFP were considered significant at an FDR-adjusted ≤ 0.05. The lists of DEG were produced and used for data mining. In order to study the relationship between peripheral blood cell transcriptome and plasma parameters, the partial correlations between read counts (standardized for each sample and expressed as read counts for 100k read counts) and plasma parameters were calculated using the GLM procedure of SAS® (SAS Institute Inc.) with the MANOVA statement. The lists of genes correlated with the blood parameters were filtered accordingly with the following criteria: *P* value of correlation lower of 0.01; FDR of correlation lower of 0.2; partial correlation coefficient lower of − 0.6 or higher of 0.6. The lists of correlated genes (CG) with blood parameters were further used for an enrichment analysis.

The data mining was performed by enrichment analysis through the Dynamic Impact Approach (DIA) with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Bionaz et al. 2012a), after uploading into the system the datasets of DEG and dataset of CG. The details and the validation of DIA have been reported previously (Bionaz et al. 2012a, b).

Statistical analysis of plasma parameters

Statistical analyses were performed with SAS software (SAS Inst. Inc., Cary, NC; release 8.0). Each parameter was tested for normal distribution using the UNIVARIATE procedure of SAS and normalized when necessary, using either natural logarithmic (cholesterol, urea, bilirubin, BHBA, NO_x, NO_2, and

NO_3) or cubic (glucose) transformation. Data were subjected to ANOVA using the REPEATED statement in the MIXED procedure of SAS. The statistical model for the data of the metabolic profile used DFP as fixed factors (− 20, − 3, 3, 7) and the cow was the random effect. Each parameter was subjected to 4 covariance structures (first-order autoregressive, compound symmetry, spatial power, and Toeplitz) and the best covariance structure was retained (Littell et al. 1998). Statistical significance was designated as $P < 0.05$.

Results

Body condition score, milk yield, and health condition

All animals were in good health condition during the experimental period and no disease symptoms related to organ systems were observed during the clinical inspection. The body condition score (ADAS 1986) was 2.64 points at − 20 DFP and was quite stable until calving. Thereafter, BCS had a gradual drop and an average of 0.18 points reduction was observed in the first week of lactation (Table 1). The average rectal temperatures of all cows were within physiological values and had a significant increase as the time of calving approached. The temperature at − 3 to 7 DFP ($P < 0.05$) was higher compared with − 20 DFP (Table 1). No fever episodes were observed during the experiment. Milk yield increased progressively during the 1st month of lactation and the average milk production was 38.4 kg/day.

Biochemical chemistry

Changes of blood parameters around calving are summarized in Table 1 and Fig. 1. The glucose concentration had a typical post calving drop from 3 to 7 DFP ($P < 0.01$), whereas NEFA and BHBA increased markedly ($P < 0.01$) at 3 and 7 DFP

Table 1 Body condition score (BCS) rectal temperature and plasma concentration of blood metabolites during the transition period of dairy cows

	− 20	− 3	3	7	SEM	*P*
Body condition score and rectal temperature						
BCS	2.64	2.62	2.53*	2.46**	0.06	0.02
Rectal temperature (°C)	38.5	38.8*	38.9*	38.8*	0.13	0.07
Energy and protein metabolism						
Urea (mmol/L)	3.25	3.42	4.26	3.69	0.74	0.3
Creatinine (μmol/L)	103	112	107	106	6.89	0.013
Minerals						
Ca (mmol/L)	2.65	2.67	2.44*	2.58	0.09	< 0.01
Mg (mmol/L)	1.04	0.96	0.86*	0.94	0.06	0.0139
P (mM)	1.79	2.30	1.59	1.37	0.57	0.047
K (mM)	4.23	4.32	4.22	4.01	0.20	0.2136
Na (mmol/L)	147	148*	146	144	1.38	< 0.01
Cl (mmol/L)	109	110	107	105	1.48	< 0.01
Zn (μmol/L)	13.47	11.78*	10.80	12.30	1.75	< 0.01
Enzymes						
ALP (U/L)	53.55	47.92	40.45**	37.92**	7.44	< 0.01
GOT (U/L)	89	88	133	160	19.37	< 0.01
GGT (U/L)	23.30	19.50*	21.10	22.09	1.71	< 0.01
Oxidative stress						
ROMs (mg H_2O_2/100 mL)	13.03	14.26	16.11*	15.52	1.46	0.2279
NO_x (μmol/L)	25.94	36.27**	18.47**	16.78**	2.29	< 0.01
NO_2 (μmol/L)	3.43	3.29	2.41*	2.23*	0.35	< 0.01
NO_3 (μmol/L)	22.51	32.98**	16.05*	14.54**	2.22	< 0.01
Other parameters						
PCV (v/v)	0.33	0.32	0.32	0.31	0.01	< 0.01
Globulin (g/L)	39.45	35.30*	36.03	39.55	2.67	< 0.01
Total protein (g/L)	76.1	70.5**	71.0*	76.2	2.18	< 0.01

ALP, alkaline phosphatase; *GGT*, γ-glutamyl transpeptidase; *AST/GOT*, aspartate aminotransferase; *ROMs*, reactive oxygen metabolites; *NO_x*, total nitric oxide metabolites; *NO_2*, nitrites; *NO_3*, nitrates; *PCV*, packed cell volume

Statistically significant differences were calculated among concentration at − 3, 3, and 7 compared with concentration at − 20 DFP, and are marked as *$P < 0.05$ and **$P < 0.01$

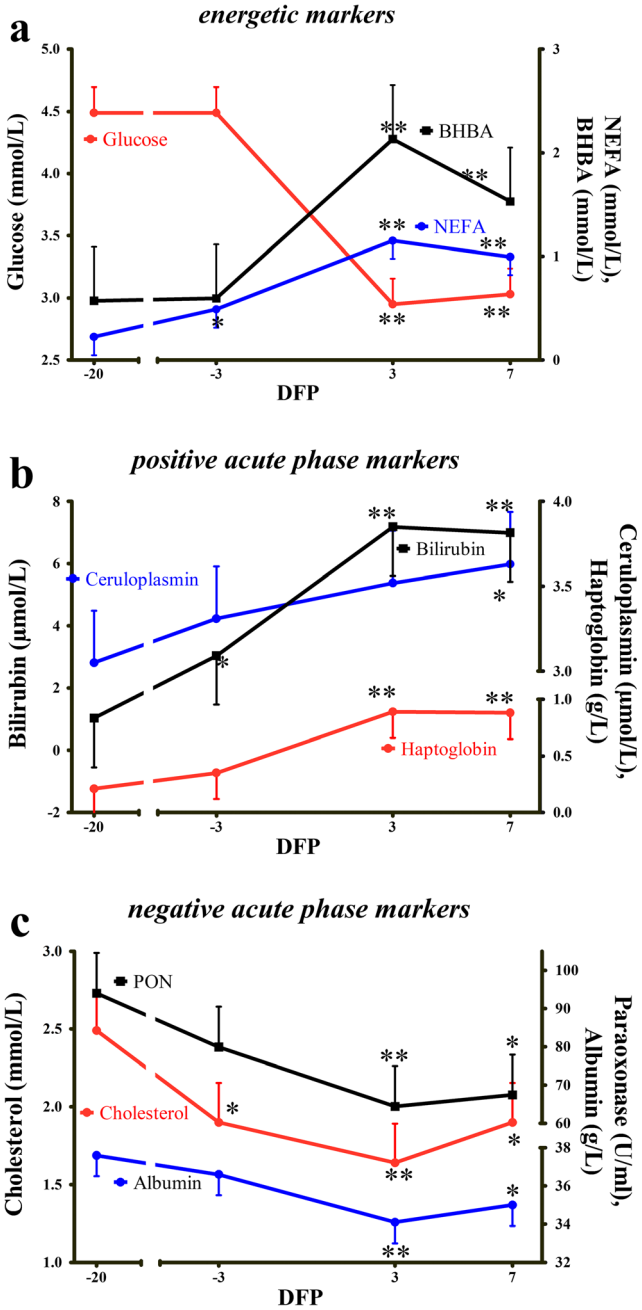

Fig. 1 Plasma concentration of **a** energetic markers (glucose, β-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA)), **b** positive acute phase markers (ceruloplasmin, haptoglobin, and bilirubin), and **c** negative acute phase markers (paraoxonase, cholesterol, and albumin) through the transition period of dairy cows. Statistically significant differences were calculated among concentration at − 3, 3, and 7 compared with concentration at − 20 DFP, and are marked as $*P < 0.05$; $**P < 0.01$

compared with − 20 DFP (Fig. 1). Creatinine concentration started to increase at − 3 DFP ($P < 0.05$) and remained elevated until the 1st week of lactation, whereas urea concentrations increased ($P < 0.05$) at 3 DFP and decreased thereafter. Haptoglobin and ceruloplasmin concentrations markedly increased after calving reaching statistical differences in comparison with − 20 DFC levels, but haptoglobin remained almost constant ($P < 0.01$ at 3 and 7 vs. − 20 DFC), while ceruloplasmin progressively increased ($P < 0.05$ at 7 vs. − 20 DFC) whereas bilirubin concentration started to increase at − 3 DFP ($P < 0.05$) and had a sharp increase after calving ($P < 0.01$; Fig. 1). Albumin, cholesterol, and PON (Fig. 1) had a decrease already before calving (− 3 DFP), reached a lower concentration at 3 DFP ($P < 0.01$), and had a raise thereafter ($P < 0.05$). The concentrations of ROMs increased after calving at 3 DFP ($P < 0.05$). The NO_x and NO_3 had a peak at − 3 DFP ($P < 0.01$) then decreased after parturition ($P < 0.01$), while NO_2 concentration progressively decreased after − 20 DFC, but reached statistical differences only after calving ($P < 0.05$).

Leukocyte gene expression during the transition period

Changes of gene expression in the leukocytes during the transition period were calculated comparing the gene expression at − 3, 3, and 7 DFP relative to − 20. The counting of the number of transcripts and differentially expressed genes (DEG), divided by upregulated and downregulated, resulting from the RNA-Seq analysis, is displayed in Table 2. A total of 562 transcripts were differentially expressed in at least one comparison with a false discovery rate (FDR) < 0.01. For 258 transcripts, it was not possible to retrieve a gene name or an annotation since they are new transcripts identified by the cufflinks suite. The transcriptome of circulating leucocytes undergoes several changes during the transition period. Comparing the gene expression observed at − 20 DFP with − 3 DFP, a total number of 41 DEG (40 upregulated DEG and 1 downregulated) were found. The greatest differences were found after parturition with 231 DEG (142 upregulated DEG and 89 downregulated DEG) comparing − 20 with 3 DFP and 164 DEG (98 upregulated DEG and 66 downregulated DEG) comparing − 20 with 7 DFP. A total of 114 DEG were in common from comparison at 3 vs. − 20 DFP and at 7 vs. − 20 DFP; moreover, considering the comparison between leucocyte gene expression at − 20 DFP and those in the two post calving checks (+ 3 and + 7 DFP), 281 DEG (179 upregulated and 102 downregulated) were detected.

Figure 2 displays a summary of the enrichment analysis carried out with the DIA approach. All the main KEGG categories (endocrine system, immune system, transport and catabolism, signaling molecules and interaction, signaling transport and catabolism) changed as parturition approached (comparison − 3 vs. − 20 DFP) and were activated. In addition, all the most impacted pathways (i.e., fat digestion and absorption, PPAR signaling pathway, adipocytokine signaling pathway, hematopoietic cell lineage, ECM-receptor interaction, phagosome) were activated already before parturition (Fig. 3).

Table 2 Number of differentially expressed genes (DEG; filtered by FDR < 0.01) in circulating leucocytes of dairy cow across different time points of transition period. The comparisons were made between expression at − 3, 3, and 7 days from parturition (DFP) versus expression at − 20 DFP

	− 3 vs. − 20 DFP	+ 3 vs. − 20 DFP	+ 7 vs. − 20 DFP	+ 3 and + 7 vs. − 20 DFP
DEG, *n*	41	231	164	281
Upregulated, *n*	40	142	98	179
Downregulated, *n*	1	89	66	102

After calving the changes were more intense and there was a substantial overlap of results at 3 and 7 DFP. Considering the results from these time points together (Fig. 2), the main KEGG categories inhibited were membrane transport, lipid metabolism, and excretory system, while categories as metabolism of cofactors and vitamins, endocrine system, signaling molecules and interaction, digestive system, and immune system were activated. Consequently, as shown in Fig. 3, many pathways were inhibited (Arachidonic acid metabolism, glycine, serine and threonine metabolism, porphyrin and chlorophyll metabolism, ABC transporters, pentose phosphate pathway) and many others activated (leukocyte transendothelial migration, phagosome, ErbB signaling pathway, RIG-I-like receptor signaling pathway, vitamin B_6 metabolism, ECM-receptor interaction, hematopoietic cell lineage, fatty acid metabolism, folate biosynthesis, adipocytokine signaling pathway, PPAR signaling pathway).

The number of genes correlated with parameters of the hematochemical profile are reported in Table 3. Considering a strong restriction (*P* value of correlation < 0.01; FDR < 0.2; correlation coefficient lower < − 0.6 or > 0.6), only 6 hematochemical variables had correlations with transcriptomic genes. Namely, glucose, ceruloplasmin, bilirubin, and haptoglobin had a correlation just with a low number of genes, while BHBA and IL-1β showed numerous correlations. BHBA was correlated with 271 genes (28 positively and 243 negatively) and IL-1β was correlated with 385 genes (297 positively and 88 negatively). An enrichment analysis using DIA was conducted using these two lists of genes (Fig. 4). The BHBA had a substantial inhibitory impact on several KEGG pathways and the most affected were DNA replication; glycosaminoglycan biosynthesis—keratan sulfate; homologous recombination; base excision repair; valine, leucine, and isoleucine biosynthesis;

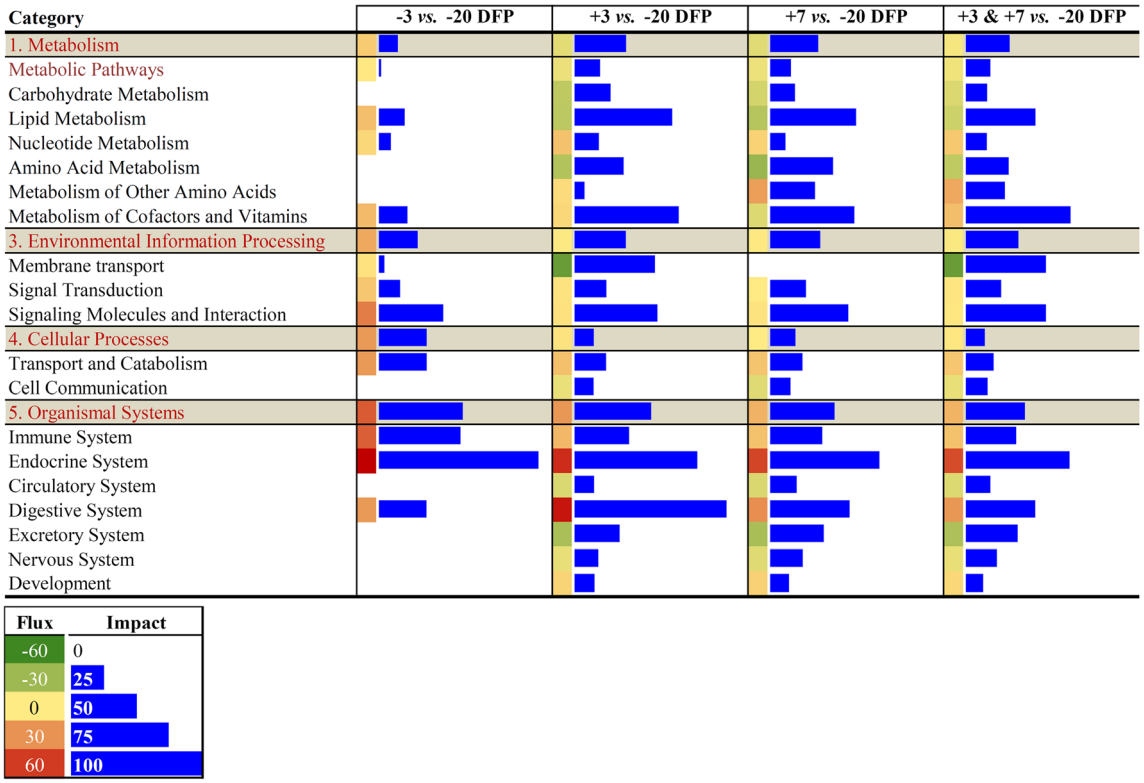

Fig. 2 KEGG category changes in transition dairy cow. Changes of circulating leucocyte gene expression in dairy cows from pre-calving (− 3 DFP) and early lactation (3 and 7 DFP) compared with − 20 DFP. The data were analyzed using the Dynamic Impact Approach (DIA). The DIA results of KEGG analysis are reported. Shown are the impact (blue bars) and the direction of the impact (red = activated; green = inhibited) of selected KEGG category from the Dynamic Impact Approach analysis

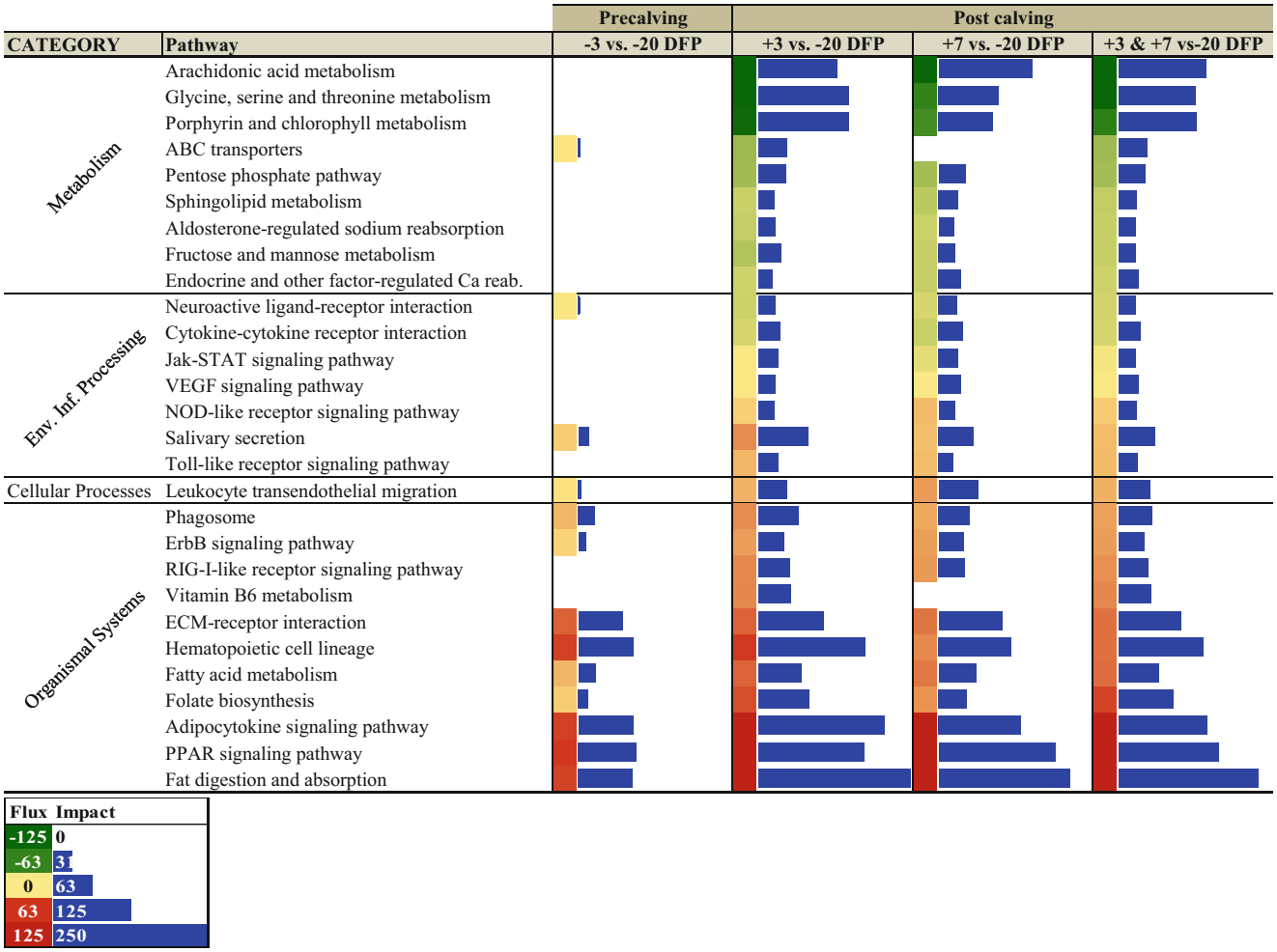

Fig. 3 KEGG pathways changes in the transition dairy cow**.** Changes of circulating leucocyte gene expression in dairy cows from pre-calving (− 3 DFP) and early lactation (3 and 7 DFP) compared with − 20 DFP. The data were analyzed using the Dynamic impact Approach (DIA). The DIA results of the KEGG pathways analysis are reported. Shown are the impact (blue bars) and the direction of the impact (red = activated; green = inhibited) of selected KEGG pathways from the Dynamic Impact Approach analysis

and cell cycle. Conversely, the IL1β inhibited only the fatty acid elongation in mitochondria while it activated many pathways such as synthesis and degradation of ketone bodies; glyoxylate and dicarboxylate metabolism; ubiquinone and other terpenoid-quinone biosynthesis; ribosome; biosynthesis of unsaturated fatty acids; and oxidative phosphorylation.

Discussion

The weeks around calving are associated with peculiar metabolic, immune, and inflammatory changes (Mallard et al. 1998; Lacetera et al. 2005; Bionaz et al. 2007; Loor et al. 2013; Trevisi et al. 2015). The objectives of this study were to investigate the changes in the peripheral blood transcriptome around

Table 3 Number of correlated genes (filtered by *P* values < 0.01 and FDR < 0.2) between circulating leucocyte transcriptome of dairy cow across different time points of transition period and some plasma parameters

	Correlated genes	Negatively correlated	Positively correlated
Glucose	15	1	14
β-Hydroxy butyric acid	271	243	28
Ceruloplasmin	6	4	2
Bilirubin	18	3	15
Haptoglobin	4	0	4
IL-1β	385	88	297

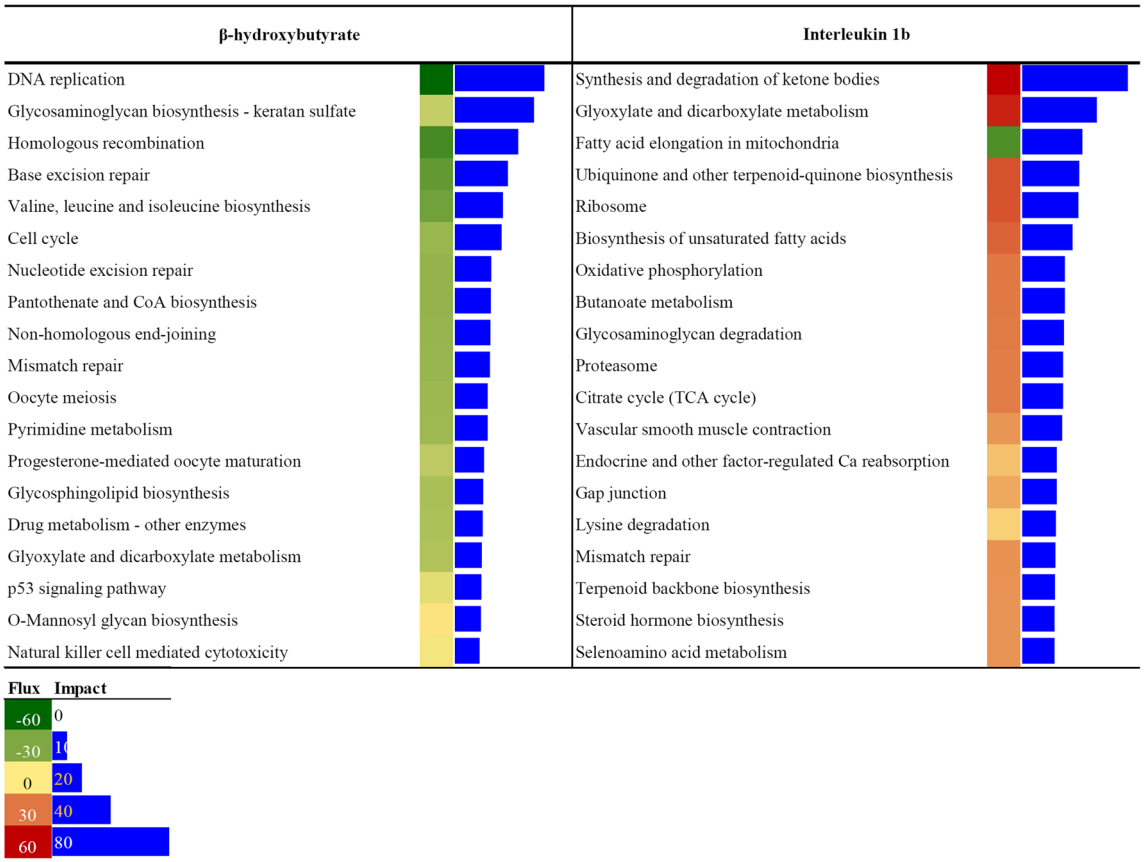

Fig. 4 Result of the enrichment analysis investigating the relationship between circulating leucocyte transcriptome and blood biomarkers. For the analysis were used the gene lists from circulating leukocytes resulted correlated with plasmatic concentrations of β-hydroxybutyrate and interleukin-1 beta in six dairy cows during transition period. The DIA results of the KEGG pathway analysis are reported. Shown are the impact (blue bars) and the direction of the impact (red = activated; green = inhibited) of selected KEGG pathways from the Dynamic Impact Approach analysis

calving and to relate them with the main typical indices of metabolism, inflammation, and oxidative stress. Leukocytes have a relevant role in the activation of defense mechanisms of the body and their regulation can contribute in the resolution or reiteration of the diseases and can interfere with metabolism. For these reasons, the pattern of gene expression of white blood cells has been investigated around calving at specific time points (from − 20 to + 7 DFP) that have been well characterized for noticeable changes of energy and inflammatory markers in plasma (Drackley 1999; Bionaz et al. 2007; Trevisi et al. 2012).

General condition of cows

All cows used in this study were without signs of clinical disease. The metabolic and inflammatory profiles observed in this experiment are typical of peripartal dairy cattle (Bionaz et al. 2007; Trevisi et al. 2009). The decrease of BCS after parturition was in accordance with the increase of blood BHBA and NEFA concentrations, confirming that cows were in negative energy balance. The concentration of BHBA revealed that at sampling times for transcriptomic evaluation (3 and 7 DFP), cows were in a condition of subclinical ketosis (i.e., BHBA > 1.4 mmol/L; Gordon et al. 2013). Nevertheless, the ketosis condition was transient, solved few days after 7 DFP (data not shown), and without particular consequences for animal health. Similar concentrations of BHBA and without severe clinical consequences during the first week of lactation are not unusual in high-yielding dairy cows. Cows in our experiment had the characteristic inflammatory response around calving (i.e., increase of haptoglobin, ceruloplasmin, and bilirubin and decrease of cholesterol, albumin, and PON), which could be considered more severe in comparison with that previously observed in cows defined in good health conditions (Bionaz et al. 2007; Bertoni et al. 2008; Trevisi et al. 2012) even though cows used in our experiment did not show any clinical disease.

Leukocyte gene expression changes before calving

The pathways activated in leukocytes before calving were mainly connected to lipid metabolism into cells and the transport of lipid in the plasma. This was noteworthy considering that changes in lipid metabolism happen around parturition (Drackley 1999) and

that in our study an increase of plasma NEFA and the decrease of plasma cholesterol were observed also before parturition (− 3 DFP). The upregulated genes that drive the activation of these pathways before parturition (diacylglycerol O-acyltransferase 2 (DGAT2), scavenger receptor class B member 1 (SCARB1), carnitine palmitoyltransferase 1A (CPT1A), cluster of differentiation 36 (CD36)) are responsible for important metabolic functions. The cluster of differentiation 36, also known as fatty acid translocase, is a transmembrane protein that binds with number of lipid ligands including long-chain fatty acid, cholesterol crystals, and oxidized low-density lipoprotein. The CD36 has an important role in the fatty acid uptake but it is also implicated in many functions related to immune response and inflammation (Pepino et al. 2014). The SCARB1 is a membrane receptors (Valacchi et al. 2011) sharing homology with the CD36 and, in analogy with CD36 the SCARB1, is able to mediate the uptake of lipid into the cell. The function of SCARB1 is mainly related to uptake of cholesterol needed by cells for basic functions (Silver and Tall 2001), mostly using the cholesterol fraction contained in the HDL. The confirmation of changes in the lipid metabolism of circulating leukocytes was also the upregulation of the CPT1A gene, which encodes a key regulatory enzyme of b-oxidation required for the transport of long-chain fatty acids into mitochondria (Nakamura et al. 2014). Taken together these data point out that even the circulating leukocytes are influenced by the metabolic changes—mainly related to lipid metabolism—which influence the cows in the final phase of the dry period. It is therefore possible to speculate that the direct contact between cells and some circulating metabolites that change before calving (Wankhade et al. 2017) such as hormones, NEFA, and cholesterol may act to influence the gene expression of circulating leukocytes.

Leukocyte gene expression changes after calving

After calving the gene expression of circulating leukocytes had the greatest difference compared with 20 days before calving, and there was a substantial overlap of information between the two sampling times at 3 and 7 days after calving. The activated pathways were mainly related to immunity and endocrine aspects. Furthermore, we observed an inhibition of some metabolic pathways, mainly those related to lipid and amino acid metabolism. At the gene level, the strong inhibition after calving of the arachidonic acid metabolism pathway was due to downregulation of two enzymes, 5-LOX and the 12-LOX that are key for the regulation of the synthesis of leukotriene molecules (Sordillo et al. 2009). Similar results for reduction of 5-LOX expression were also found by Crookenden et al. (2016) in transition dairy cows using quantitative reverse transcriptase PCR. These results suggest a reduction of leukotriene synthesis after calving. Because these mediators have a role in inflammation, their reduction is compatible with the reduction of IL-1β and IL-6 after calving, found in the present study and in previous research from our group (Trevisi et al. 2015; Jahan et al. 2015). Clearly, the development of inflammatory processes at calving is a more complex phenomena, indeed as usually observed after calving; also cows of this experiment had a severe acute phase response which modify markedly the liver functions. It remains unclear what the driver for the inflammatory response after calving was.

Our results indicate that some important functions of the immune system were activated in the first week of lactation, including the phagocytosis pathway. Overall, this finding from transcriptomic analysis partly contradicts the literature which has emphasized a reduction of specific cell functions including phagocytosis of neutrophils (Kehrli and Goff 1989; Goff and Horst 1997). This function is very important immediately after calving and it is helpful in the uterus to allow placenta detachment and avoid the onset of endometritis (Hammon et al. 2006). Interestingly, with the exception of the myeloperoxidase gene that was downregulated, in our study, we observed an overall upregulation of genes involved in phagocytosis (supplementary figure 1). The reduction of MPO gene expression and its activity during the transition period was reported previously (Kimura et al. 2006; Zhou et al. 2015; Crookenden et al. 2016; Batistel et al. 2017). The myeloperoxidase is one of the most important enzymes in the phagocytosis process; therefore, it is possible that the reduction of its synthesis could be one of the limiting factors for the impairment of phagocytosis around calving. In general, the shortage of MPO can justify the reduction of immune capacity observed during the transition period in previous studies (Kehrli and Goff 1989; Goff and Horst 1997).

Other functions activated after calving from gene expression data include mechanisms of migration, adhesion, and extravasation of the immune cells such as the upregulation of transendothelial migration pathways. Crookenden et al. (2016) reported a reduction of extravasation capacity (or diapedesis) of the immune cells after calving. Migration, adhesion, and extravasation of immune cells are crucial for clearance of an infection and can affect the health status of the cow during the first month of lactation. In agreement with Crookenden et al. (2016), in our previous study, the in vivo carrageenan test revealed a reduction of response at − 3 DFP that can be interpreted as a reduction of extravasation capacity or diapedesis of the immune cells immediately close and after calving (Jahan et al. 2015).

The extracellular matrix (ECM) receptor pathway, associated with adhesion and migration of cells, also was activated after calving. The ECM consists of a complex mixture of molecules and its role is important in the maintenance of cells and tissue structure and function. Specific interaction between cells and ECM is mediated by transmembrane molecules such as proteoglycans, CD36, or other cell surface–associated components and these interactions lead to control of cellular

activity such as adhesion, migration, and apoptosis. Among the upregulated differentially expressed genes after calving were THBS1, LAMA5, FN, HSPG2, EMILIN1, and CD36 (already discussed). The gene THBS1 is strongly expressed in neutrophils and induces an intense chemotaxis response when tissues are injured (Wight et al. 1985). Hassan et al. (2006) found a dramatic upregulation of the genes involved in neutrophil chemotaxis in human uterine cervix after spontaneous parturition. Morimoto et al. (1997) and Wu et al. (1999) found a significant increase of the mRNA for THBS1 in the myometrium of women and sheep, respectively, during labor. In our experiment, the increased expression of THBS1 after calving suggests that parturition was the main cause of THBS1 expression in leukocytes.

EMILIN1 is involved in cell adhesion, migration, and trophoblast invasion (Spessotto et al. 2003, 2006). The reduction of the expression of EMILIN1 in knockout rats caused dermal and epidermal hyperproliferation and accelerated wound closure (Danussi et al. 2011). The upregulation of EMILI1 after calving agrees with the reduction of the in vivo carrageenan test response from − 3 to 7 DFP observed in our previous study (Jahan et al. 2015). Other genes such as VCAM could be implicated in this response. This gene was upregulated at 3 and 7 DFP. The VCAM is an important factor for the interaction between leukocytes and endothelial cells (Muller 2003). In particular, the synthesis of VCAM-1 (vascular cell adhesion molecular-1), well-known mediator of the adhesion of leukocytes to vascular endothelium, is induced by pro-inflammatory cytokines (Granger and Kubes 1994; Liu et al. 2004) such as IL-1β and IL-6. The increased expression of VCAM-1 gene in early lactation was previously reported by Aitken et al. (2009), who found a significant increase in mammary tissue in early lactating dairy cows. Hodgkinson et al. (2007) also reported an increase during colostrogenesis, a phase characterized by negative energy balance and oxidative stress. Therefore, the regulation of the adhesion factors between leukocytes and endothelial cells around calving seems important in dairy cows.

Relationship between leukocyte transcriptome and metabolic conditions of the transition dairy cow

The effect of BHBA on the immune system activity has been studied previously and it is well known that ketotic cows have an impairment of immune function and a consequent increase in mastitis susceptibility (Kremer et al. 1993; Zarrin et al. 2014). In vivo and in vitro studies revealed that leukocyte functions, such as chemotaxis, response to mitogenic agents, blastogenesis, phagocytosis, and superoxide anion release, are reduced by high BHBA concentration (Targowski and Klucinski 1983; Targowski et al. 1985; Suriyasathaporn et al. 1999; Sartorelli et al. 1999, 2000). To date, the mechanism for the impairment of leukocyte function is not completely understood. In our study, after calving, all cows involved in the experiment experienced subclinical ketosis (BHBA > 1.4 mmol/L; Duffield et al. 2009) and this agrees with the negative relationship between BHBA and several KEGG pathways. Our data outlined that the BHBA strongly inhibited the pathways from genetic information process, mainly related to mechanism of cell cycle and DNA replication and repair. These observation agree to previous studies of Targowski and Klucinski (1983) and Targowski et al. (1985) in which a decrease of mitogenic response of lymphocytes due to ketone bodies was observed.

On the contrary, the relationship between transcriptome data and IL-1β was in the direction of the activation of several pathways related to metabolism, in particular to aspects of cellular energy metabolism. A clear explanation of this association is challenging. In fact, the immune cells can be both the source of IL-1β production than the target of IL-1β produced elsewhere. In our previous study, we observed a high variability of plasma IL-1β concentration among cows during the transition period, and mainly in late pregnancy (Trevisi et al. 2015). Interestingly, the level of IL-1β in the dry period was related to a worse condition in terms of metabolic and inflammatory indices and clinical disease in the subsequent early lactation stage (Trevisi et al. 2015), suggesting a relationship with the immune response.

Conclusion

Although the results of this study are based on a small number of animas and need to be confirmed by further investigation, overall, the transcriptomic data confirmed changes in immune competence of the circulating leukocytes around calving indicating mainly an increase of their activity and function. These data support the idea that the dairy cow's immune system is dysfunctional but not immunosuppressed around calving. The changes of gene expression in leukocytes before the parturition have been less severe than assumed in previous experiments. These changes seem related to metabolic adaption to nutrient trafficking (mainly lipid). In opposite, genes related to immune system can start to change few days before calving but play a major role immediately after calving. Many of them suggest the reduction of extravasation capacity (or diapedesis) of the immune cells. Strong association was observed between transcriptomic profile of leucocytes during transition period and plasma concentration of BHBA and IL-1β. In this respect, the BHBA seems inhibit the gene regulation of leukocytes, while the IL-1β acts to activate the gene regulation functions.

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Availability of data and material The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. The DIA software is not publicly available but can be requested from the developer. KEGG databases are free and available online.

Compliance with ethical standards

Ethics approval and consent to participate All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted (DL No. 116, 27/01/1992)

Conflict of interest The authors declare that they have no conflict of interest.

Abbreviations ALP, alkaline phosphatase; BCS, body condition score; BHBA, β-hydroxybutyrate; DEG, differentially expressed genes; DFP, days from parturition; DIA, Dynamic Impact Approach; GGT, γ-glutamil transferase; GOT, aspartate aminotransferase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharide; NEB, negative energy balance; NEFA, non-esterified fatty acids; NO_2, nitrites; NO_3, nitrates; NO_x, total nitric oxide metabolites; PON, paraoxonase; ROMs, total reactive oxygen metabolites.

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