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PAPER

Pro-inflammatory cytokine profile in dairy cows: consequences for new lactation

Erminio Trevisi,[1,2] Nusrat Jahan,[1,3] Giuseppe Bertoni,[1] Annarita Ferrari,[1] Andrea Minuti[1,2]

[1]Istituto di Zootecnica, Catholic University of the Sacred Heart, Piacenza, Italy
[2]PRONUTRIGEN - Centro di Ricerca sulla Proteomica e Nutrigenomica, Catholic University of the Sacred Heart Piacenza, Italy
[3]IUBAT - International University of Business Agriculture and Technology, Dhaka, Bangladesh

Abstract

To verify the potential relevance of pro-inflammatory cytokine (PIC) with periparturient health problems and performances, the changes of plasma interleukin-1beta (IL-1β) and interleukin-6 (IL-6) have been investigated in 21 Holstein-Friesian cows from 35 d before to 28 d after parturition. The overall PIC concentration was higher during late pregnancy compared to the first month of lactation, but showed a high variability among the cows. Therefore, cows were retrospectively divided in 3 groups according to the values of area under the concentration curve of IL-1β concentrations from -35 d before to the day of parturition and designated as up (UPIL1), intermediate (INIL1) and low (LOIL1) IL-1β group. The concentrations of IL-6 and to some extent the concentrations of albumin and reactive oxygen metabolites (ROMs) were well related to the grouping based on IL-1β concentrations. After calving the UPIL1 cows showed a more severe acute phase reaction (APR), based on the marked increase of haptoglobin and the lower plasma albumin concentrations during the first week of lactation, and the highest oxidative stress, based on the higher concentrations of ROMs. Moreover, the UPIL1 group showed higher number of mastitis, lower feed intake and milk yield compared with INIL1 and LOIL1. Our results demonstrated that cows with the highest PIC concentrations in the last month of pregnancy showed the worse health status in early lactation (clinical and subclinical problems) and a lower milk yield. Thus, these data support the utility of PIC measurement in late pregnancy as prognostic markers for a risky transition period.

Introduction

The transition period, 3 weeks before to 3 weeks after parturition, is important for health, reproduction, production and profitability of dairy cows (Bertoni *et al.*, 2008). In this period cows are affected by different stressors including the increase of body lipid mobilization, oxidative stress, major changes in endocrine status, and altered immune function, which together result in an increased risk of diseases (Jonsson *et al.*, 2013).

Inflammation is involved in most of these changes through several mediators, such as eicosanoids (Sordillo *et al.*, 2009) and pro-inflammatory cytokines (PIC; Kushibiki, 2011). The PIC [*e.g.* tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6), and interferon-gamma (INF-γ)] are released mainly by macrophages (Koj, 1998), but they are also produced in other tissues (*e.g.* liver, brain, adipose tissue), and exert both paracrine and systemic effects. In the liver, PIC stimulate the synthesis of positive acute phase proteins (pos-APP), such as haptoglobin, serum amyloid A and ceruloplasmin (Gruys *et al.*, 2005). Concomitantly, PIC impair the synthesis of negative acute phase proteins (neg-APP), some of them being important for normal liver metabolism (Bertoni and Trevisi; 2013). Indeed, the low neg-APP concentrations (*e.g.* albumin, lipoproteins, retinol binding protein) have been associated with decreased liver function, reproductive performance, milk yield and increased disease incidences (Bionaz *et al.*, 2007; Bertoni *et al.*, 2008; Bossaert *et al.*, 2012). Moreover, PIC have a role in worsening feed intake, possibly even anorexia (Johnson, 1997), a condition often occurring around parturition (Grummer, 1995; Drackley, 1999; Trevisi *et al.*, 2012). In accordance to these results, the role of PIC have been investigated as potential indicators of postpartum reproductive diseases (Ishikawa *et al.*, 2004; Islam *et al.*, 2013b). Recently, the plasma concentration of IL-6 in the early lactating cows has shown a positive correlation with the severity of the inflammatory response (Trevisi *et al.*, 2012). Interestingly, this study also showed a great variability of the plasma concentration of IL6 at calving time, suggesting the importance of the absolute level of the PIC in the transition period. Thus, we hypothesize that the concentration of PIC before or around calving can be related with the success or the failure (*i.e.* high occurrence of metabolic and infectious diseases, unsatisfactory performance, poor fertility, early culling) of the transition period.

In previous studies, the variations of PIC was not systematically and frequently investigated during the transition period, together a wide metabolic and inflammatory profile. Therefore, the main objective of this study was to establish the pattern of changes of IL-1β and IL-6 in the peripartum of dairy cows along with several biomarkers of the metabolism and inflammation.

Materials and methods

This study complied with Italian laws on animal experimentation and ethics. The experiment was carried out in the dairy farm of Università Cattolica del Sacro Cuore (Piacenza, Italy) from 2010 to 2012.

Animals and feeds

Twenty-one multiparous Holstein dairy cows (parity 2.5±1.0) in transition period [from -35 d to +28 d from parturition (DFP)] were included in this study. Cows were housed in the barn of the university, equipped with air

conditioning (summer), heating (winter), artificial lighting and ventilation systems to guarantee relatively *stable* conditions in the whole year. On average the climatic conditions were: environmental temperature around 20°C, relative humidity between 60% to 70%, photoperiod with 14 h of light (from 5:00 to 19:00 h) and 10 h of dark. Cows were individually fed and, every morning, the remnants were weighed and recorded. Forages were offered *ad libitum* every 12 h, whereas concentrates were distributed by an auto-feeder every 3 h and 12 h for lactating and dry cows, respectively.

On average, dry cows received 9-12 kg of grass hay, 8-10 kg of corn silage and 1 kg of concentrate per day (soybean meal and mineral-vitamin supplementation). After calving, the daily diet included 2 kg of grass hay and 3 kg of alfalfa hay, while amount of concentrate and corn silage were gradually increased (on d 30 of lactation, on average cows received 11–13 kg of concentrates and 18-20 kg of maize-silage). The average composition of diets consumed in the last month of pregnancy and in the first month of lactation is shown in Table 1.

Sample collection

Blood samples were collected from each cow on days -35±2,-28±2, -21±2, -14±2, -7, -2, -1, 0, 1, 2, 3, 7, 14±2, 21±2, and 28±2 from the date of parturition. On d 0, the samples were collected within 3 hours before the calving. Other blood samples were collected in the morning before feeding. Samples were obtained from the jugular vein using evacuated tubes containing Li-heparin as an anticoagulant (Vacuette, Kremsmünster, Austria). Tubes were immediately placed in ice water until arrival in the laboratory. Within one hour of collection, a small amount of the blood was used for packed cell volume determination (Centrifugette 4203; ALC International Srl, Cologno Monzese, Italy). The remainder of the sample was centrifuged at 3500×g for 15 min at 4°C and the plasma was frozen in several aliquots at -20°C until analyzed.

Milk yield, dry matter intake, body condition score and health problems

All cows were treated at drying-off with a dry-cow intramammary preparation (penethamate hydroiodide, bentamin penicillin, framycetin sulphate; Mamyzin®, Boehringer Ingelheim, Reggello, Italy) and intramuscular treatments with tylosin (Eli Lilly, Sesto Fiorentino, Italy). During the trial rectal temperature was measured every morning at feeding time (08:00). All cows were routinely vaccinated against bovine viral diarrhea, infectious bovine rhinotracheitis, parainfluenza type 3 and bovine respiratory syncytial viruses by a live attenuated vaccine (CattleMaster 4; Pfizer Animal Health, Exton, PA, USA) every 6 months, except in the 4 weeks before and after calving.

Individual milk yield was weighted at each milking. Milking procedures included cleaning of the teat with a clorexidine-moistened paper towel, forestripping and milking cluster attachment. The SCC (Fossomatic 180, Foss Electric, Hillerød, Denmark), fat, protein and lactose (MilkoScan FT 120, Foss Electric, Hillerød, Denmark) of milk were measured routinely once a week. The feed intake was measured daily by weighing differences of the supplied feed and the orts. The feed DM content was routinely assessed during the trial by the sampling of hay and concentrate when lots were changed and corn silage every 4 weeks from the bunker silo. The body condition score (BCS) was individually evaluated by the same observer according to a five-point scale (Agriculture Development and Advisory Service, 1986), at the first blood sampling (about 35 d before the expected calving date) and then every 14 d until d 28 after calving.

Blood analysis

Blood samples were analyzed to assess the following metabolites: i) inflammatory response indices: pos-APP (haptoglobin, ceruloplasmin) and neg-APP (albumin, cholesterol as lipoprotein index); ii) liver indices: total bilirubin, aspartate aminotransferase (GOT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), paraoxonase (PON); iii) energy metabolism indices: glucose, NEFA (non-esterified fatty acid), β-hydroxybutyrate (BHBA); iv) protein metabolism indices: urea, creatinine; v) oxidative stress response index: total reactive oxygen metabolites (ROMs); vi) minerals (Ca, P, Mg, Na, K, Cl, Zn); vii) vitamins: retinol (index of its carrier protein), tocopherol, β-carotene; viii) other parameters (total proteins, globulins). Methods used for the determination of these parameters were previously described by Bertoni *et al.* (2008). Briefly, glucose, total protein, albumin, total cholesterol, total bilirubin, creatinine, urea, Ca, P, Mg, GOT, GGT and ALP were determined at 37°C by a clinical auto- analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA, USA) using commercial kits purchased from Instrumentation Laboratory (IL Test). Globulins were calculated as the difference between total protein and albumin. Electrolytes (Na+, K+, and Cl−) were detected by the potentiometer method (Ion Selective Electrode connected to ILAB 600). Zn and NEFA were determined by commercial kits (Wako Chemicals GmbH, Neuss, Germany). ROMs were measured using a method patented by Diacron International S.r.l. (Grosseto, Italy). BHBA was determined by commercial kit (Randox Laboratories Ltd., Crumlin, UK).

Haptoglobin and ceruloplasmin concentrations were measured using methods proposed by Bertoni *et al.* (2008) adapted to ILAB 600 conditions. Plasma PON activity was assessed by adapting the method of Ferré *et al.* (2002) to the ILAB 600. Plasma retinol, α-tocopherol and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using Spherisorb ODS-2.3 μm, in a 150×4.6 mm column (Alltech, Deerfield, IL, USA); a UV detector set at 325 nm (for retinol) or 290 nm (for α-tocopherol); 460

Table 1. Ingredients and main chemical and nutritional traits of the diets fed to dry (last month of pregnancy) and lactating (first month of pregnancy) dairy cows.

	Dry cows	Lactating cows
Components, % DM		
Grass hay	75.0	11.9
Alfalfa hay	-	11.3
Corn silage	15.3	23.0
Soybean meal	8.8	-
Minerals and vitamins supplementation°	0.9	0.8
Commercial concentrate	-	53.0
Chemical composition of total diet		
NE$_L$, Mcal/kg DM	1.26	1.57
CP, % DM	11.9	17.6
NDF, % DM	48.4	34.6
Starch + sugar, % DM	10.6	29.4
EE, % DM	1.6	4.70

DM, dry matter; NEL, net energy lactation; CP, crude protein; NDF, neutral detergent fibre; EE, ether extract. °Mineral and vitamin supplement provided at each cow every day: vitamin A, 150,000 U; vitamin D, 20,000 U; vitamin K, 100 mg; vitamin E, 750 U; vitamin B₁₂, 0.5 mg; 500 mg; vitamin PP, 2 mg Co; I, 20 mg; Mn, 350 mg; Cu, 80 mg; Se, 3 mg; Zn, 800 mg; yeast, 15 g (Yea-Sacc$^{102.6}$; Alltech Italia S.r l., Bologna, Italy).

nm (for β-carotene) and 80:20 methanol:tetrahydrofurane as the mobile phase.

Cytokine assay

The concentrations of IL-1β and IL-6 were analyzed using commercially available ELISA kits specific for the bovine species (Pierce, Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction. All the samples were analyzed in duplicate. The intra- and inter-assay coefficients of variation were 4 and 17% and 3 and 13% for IL-1β and IL-6, respectively.

Data handling and groups

To investigate the association of plasma concentration of cytokines and other blood parameters and productive performances, the 21 cows were retrospectively divided into 3 groups, each consisting of 7 cows, according to the value of area under the concentration curve (AUC) of IL-1β concentrations during dry period (from -35 to 0 DFP), and were designated as high IL-1β (UPIL1), intermediate IL-1β (INIL1) and low IL-1β (LOIL1) groups. We have chosen IL-1β for grouping as it is one of the most important PIC. Data of plasma parameters during calving were also divided into 4 periods, *i.e.* from -35 to -14 (DRY1), from -7 to -1 (DRY2), from +1 to +7 (LACT1) and from +14 to +28 DFP (LACT2). Data of plasma measured over time within the period of interest were aggregated according to these periods.

Statistical analyses

Statistical analyses were performed with SAS software (SAS Inst. Inc., Cary, NC, USA; release 8.0). These data were subjected to ANOVA using the REPEATED statement in the MIXED procedure of SAS. The statistical model for plasma parameters included the fixed effect of groups (UPIL1, INIL1, LOIL1), DFP (-35, -28, -21, -14, -7, -2, -1, 0, 1, 2, 3, 7, 14, 21 and 28) or periods (DRY1, DRY2, LACT1 and LACT2) and groups by DFP or period interaction. The statistical model for DMI, BCS, milk yield and milk parameters included the fixed effect of groups (UPIL1, INIL1, LOIL1), and DFP with group by DFP interaction. For all the parameters the random variable was cow within the groups. Each parameter was tested for normal distribution using UNIVARIATE procedure of SAS and normalized when necessary, using either natural logarithmic (IL-1β, IL-6, glucose, cholesterol, urea, magnesium, GOT, GGT, bilirubin, haptoglobin, tocopherol, somatic cell count) or cubic (calcium) conversion. Each parameter was subjected to 4 covariance structures: first order autoregressive, compound symmetry, spatial power, toeplitz, and the best covariance structure was retained (Littell *et al.*, 1998). Partial correlations were computed among plasma parameters using the GLM procedure of SAS with the MANOVA statement.

Prevalence of health problems recorded during the study was evaluated by χ^2 analysis (PROC FREQ of SAS) to determine whether prevalence differed among groups.

Statistical significance was declared at P≤0.05, with trends noted at P<0.10.

Results

Pro-inflammatory cytokines

The patterns of changes of the concentration of IL-1β and IL-6 from -35 DFP to 28 DFP are shown in Figure 1. The plasma concentrations of both cytokines remained almost stable in the last month of pregnancy, but showed a great variability among the cows. Concentrations of both the cytokines were higher before calving, showed increase variability at calving time and then decreased gradually after parturition. To better describe the pattern of changes of cytokines, the values observed in the transition period were compared with the value detected at -35 DFP (beginning level). The concentration of IL-1β decreased significantly after calving for the entire 1st month of lactation. The IL-6 showed a similar pattern of change of IL-1β, but the significant reduction (P<0.01) was observed from the 7 DFP in comparison to the value observed at -35 DFP.

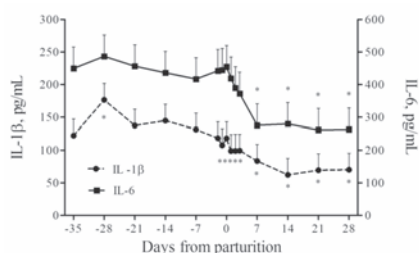Grouping the animals, the average concentrations of IL-1β during the last month of preg-

Figure 1. Mean (±SEM) plasma concentrations of IL-1β (diamond, ♦) and IL-6 (square, ■) during the transition period of 21 dairy cows. Statistically significant differences between each day from parturition (DFP) and -35 DFP are marked with * (P<0.05).
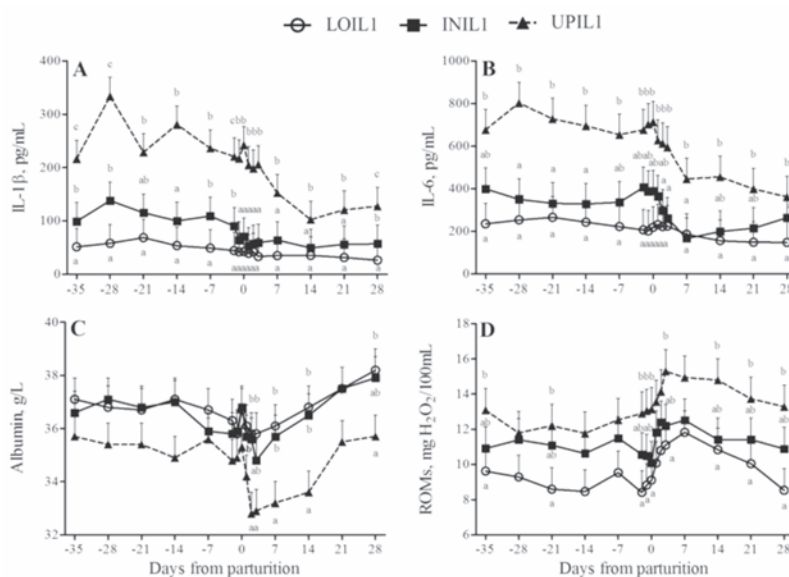
Figure 2. Changes of plasma IL-1β (A), IL-6 (B), albumin (C), and reactive oxygen metabolites (D) concentrations (mean±SEM) in the last month of pregnancy and in the first month of lactation in cows with the upper (UPIL1; 7 cows) (triangle, ▲), intermediate (INIL1; 7 cows) (square, ■) and lower (LOIL1; 7 cows) (circle, ○) groups. The groups were defined in accordance with the values of the area under the curve of IL-1β (interleukin 1β) concentrations during the dry period (from -35 to 0 d from parturition). For each blood parameter, a significant (P<0.05) difference between groups at the same day from parturition is shown by different letters (a,b,c).

nancy were 51.1, 98.2 and 246.9; SEM 35.09 pg/mL for LOIL1, INIL1 and UPIL1, respectively (P<0.01; Figure 2A). During the first month after calving a significant difference (P<0.01) was observed only between UPIL1 and LOIL1 groups. Concentrations of IL-1β were significantly correlated with those of IL-6 (r=0.46, P<0.01), but no significant correlations were found with other blood parameters. Statistical differences among the 3 groups were also found for IL-6 concentration (Figure 2B). The cows of UPIL1 group showed higher concentration of IL-6 (P<0.05) compared to LOIL1 group during the whole study period and to INIL1 for -28, -21, -14 DFP (P<0.05).

Inflammometabolic profiles

The changes of some plasma metabolic and immune parameters during the study period are shown in Figures 2C and 2D and Table 2. Albumin concentrations (Figure 2C) remained quite stable in the dry period and showed a reduction after calving. The more marked decrease was observed in UPIL1 group compared to INIL1and LOIL1 groups. Differences between UPIL1 and other groups were significant (P<0.05) for the entire first month of lactation. The ROMs concentrations (Figure 2D) rose after calving in all groups (P<0.05). The difference of ROMs between LOIL1 and UPIL1 groups were significant (P<0.05) for almost the whole period (*i.e.* -35, -21, -2, -1, 0, 1, 3, 14,

21, 28 DFP). The calcium concentration (Table 2) of UPIL1 group was lower compared to other groups in the whole period (P<0.05). Moreover, the calcium concentration of UPIL1 group was lower (P<0.05) compared to INIL1 group at DRY1 (from -35 to -14 DFP), and to LOIL1 group at LACT1 (from +1 to +7 DFP). Zn also showed a significant difference (P<0.05) between INIL1 and LOIL1 groups at LACT1. Globulin concentrations showed a significant effect on the period (P<0.01) and the interaction between IL1β group and period (P<0.01). The UPIL1 group showed a marked increase after calving and reached the highest concentration at LACT2 (P<0.05 *vs* INIL1 and NS *vs* UPIL1).

The pos-APP (haptoglobin and ceruloplasmin) did not differ among the three groups before calving, and increased after calving in all cows (LACT1 *vs* DRY2; P<0.05). Haptoglobin concentration was higher (P<0.05) in INIL1 and UPIL1 groups than in LOIL1 group in LACT1 (Table 2), while ceruloplasmin resulted only numerically higher in UPIL1 group compared with LOIL1 in LACT1 (3.65 *vs* 3.30 µmol/L). Concomitantly, plasma concentrations of creatinine were numerically lower in UPIL1 group cows in comparison to the INIL1 and LOIL1 groups during the entire peripartum, but statistical difference between UPIL1 and LOIL1 was observed only during the first week of lactation (P<0.05). The NEFA

(Table 2) and BHBA (data not shown) concentrations rose gradually from DRY1 to LACT1 (P<0.01) in all cows, but differences among groups were not significant.

Health problems and body condition score

Major disease frequency in the first month of lactation for each group and criteria for the diagnosis of the diseases are reported in Table 3. No clinical diseases were detected until parturition. In lactation, LOIL1 group showed a lower number of cows with clinical diseases (28%) in comparison to INIL1 (57%) and UPIL1 (71%). Of the 7 cows in each group used in the study, 4 cows of UPIL1 group and 2 cows of INIL1 group were diagnosed with clinical mastitis in the first month of lactation, while LOIL1 group cows did not show any mastitis (P<0.10 compared UPIL1 *vs* LOIL1). Cows with clinical mastitis were treated timely with intramammary preparation (rifaximine and cefacetrile). The SCC were controlled routinely once a week and as a whole tended to be higher (P<0.1) in UPIL1 groups (242.59±84.86 n/µL) in comparison with LOIL1 (104.04±84.86 n/µL) and INIL1 (182.54±84.25 n/µL) groups. The average rectal temperature measured at the morning did not differ among groups. All groups showed a tendency to have a similar pattern of BCS changes from 35 d before calving to 28 d in milk, with numerically

Table 2. Mean values of plasma concentrations of calcium, zinc, globulin, haptoglobin and creatinine in different groups of transition cows (35 d before to 28 d after calving).

	Group	Phases		SEM	P		
	DRY1	DRY2	LACT1	LACT2	Group	Period	Group×Period
Calcium, mmol/L	LOIL1	2.65[ab]	2.66	2.36[b]	2.61	0.07	*	**
	INIL1	2.72b	2.69	2.32[ab]	2.61			
	UPIL1	2.60a	2.60	2.20[a]	2.53			
Zinc, µmol/L	LOIL1	13.84	12.18	11.18[b]	13.57	1.03	**	
	INIL1	12.91	10.88	8.99[a]	12.13			
	UPIL1	13.73	10.94	9.30[ab]	11.88			
Globulin, g/L	LOIL1	43.43	35.08	35.85	40.93[ab]	2.64	**	**
	INIL1	42.51	36.88	36.93	40.34[a]			
	UPIL1	44.20	37.06	36.80	45.94[b]			
Haptoglobin, g/L	LOIL1	0.233	0.146	0.531[a]	0.339	0.092	**	
	INIL1	0.220	0.182	0.716[b]	0.313			
	UPIL1	0.214	0.246	0.729[b]	0.423			
Creatinine, µmol/L	LOIL1	102.2	111.5	110.9[a]	98.4	3.1	**	
	INIL1	104.2	111.4	108.1[ab]	96.3			
	UPIL1	98.6	108.5	103.5[b]	91.3			
	LOIL1	0.134	0.255	0.891	0.484			
NEFA, mmol/L	INIL1	0.141	0.305	0.874	0.453	0.098	**	
	UPIL1	0.106	0.319	0.842	0.542			

DRY1, from 35 to 14 d from parturition; DRY2, from 7 to 1 d from parturition; LACT1, from 1 to 7 d from parturition; LACT2, from 14 to 28 d from parturition; LOIL1, low IL-1β group; IL-1β, interleukin 1β; INIL1, intermediate IL-1 β group; UPIL1, upper IL-1β group; NEFA, non−esterified fatty acids. Groups are formed according to values of area under the curve of IL-1β concentrations during dry period (from -35 to 0 d from parturition). [a,b]Means with different superscript within the same column differ significantly at P<0.05. *P<0.05; **P<0.01.

higher body losses in UPIL1 (-0.45 points) and INIL1 (-0.45 points) in comparison to LOIL1(-0.33 points).

Dry matter intake and milk yield

In the dry period, the average DMI (Figure 3a) was 13.9, 12.2 and 12.3 kg/d (SEM=0.4680) in LOIL1, INIL1 and UPIL1 groups, respectively while during lactation period DMI was 19.6, 18.1 and 17.4 kg/d (SEM=0.4680) in LOIL1, INIL1 and UPIL1 groups, respectively. The DMI of UPIL1 group was lower ($P<0.01$) during the postpartum period in comparison with the other two groups. Milk yield (Figure 3b) was higher in LOIL1 than INIL1 ($P<0.05$) and UPIL1 ($P<0.01$) groups. The average milk yield in the first 30 d of lactation was 39.7, 36.1 and 34.0 kg/d (SEM=1.71) in LOIL1, INIL1 and UPIL1 groups, respectively. Milk content of protein, fat and lactose did not show any differences among the groups.

Discussion

Immune alterations and inflammatory conditions are signalled in the peripartum period, and often without clinical symptoms. The understanding of this phenomenon is very relevant to improve the health and welfare of dairy cows. The changes of PIC concentrations, as IL-1b and IL-6, during the transition period are not well known and until now it was erroneously assumed that their changes in plasma were very quick and difficult to detect. In this research we observed that concentrations of IL-1b and IL-6 remained almost stable in late pregnancy, but surprisingly, animals showed a wide variability. Therefore, cows were retrospectively divided in tertiles, on the basis of the concentrations of IL-1b in the last month of pregnancy, to study their relationship with the occurrence of periparturient health problems, dry matter intake, milk yield and plasma inflammometabolic parameters.

Effect of transition period on plasma concentration of pro-inflammatory cytokines

The concentration of PIC showed a short-lasting increase in the sample collected after the calving (within 24 hours following). This increase was not statistically different in comparison to previous weeks, but it is in agreement with the results of Hebisch et al. (2004) who measured IL-1b and IL-6 in women during pregnancy, delivery and early puerperium. Moreover, our data also partially agree with

Jonsson et al. (2013), who observed an increase of the IL-1β expression in the leukocytes collected from heifers within 24 h after calving. Our data suggest that a proper evaluation of the calving effects on PIC production in dairy cows require more frequent samplings around parturition.

In this study we observed a reduction of PIC concentration after parturition in UPIL1 and INIL1, but barely in LOIL1. The reduction of IL-6 agrees with the study of Ishikawa et al. (2004) and Trevisi et al. (2012) in clinically healthy dairy cows, while the reduction of IL-1b has also been reported by Islam et al. (2013a). Similar results were also observed in murine model by Orsi et al. (2006), who found a raise of PIC (e.g. TNF-α, IL-1β and IL-6) in serum during mid and late gestation. The T

helper cells (Th) play an important role in the immunoregulation and immunostimulation and can be classified in Th1, Th2 and Th17 cells, which produce different pattern of cytokines, also called lymphokines (Saito et al., 2010). Th1 and Th2 produce cytokines with opposite effects, pro-inflammatory and anti-inflammatory respectively, but their production is suppressed by immunoregulatory cytokines, such as transforming growth factor (TGF)-β and IL10 or by cell-to-cell interaction (Saito et al., 2010). In our research, the higher concentration of PIC in late pregnancy relative to those measured after parturition partly disagrees with the opinion with respect to a low ratio of Th1 to Th2 cells for the maintenance of pregnancy. Thus, Raghupathy et al. (2000) suggested that a lower ratio of Th1 and Th2

Table 3. Prevalence of clinical events in the lower, intermediate and upper group cows during the first month of lactation.

	Group		
	LOIL1	INIL1	UPIL1
	7	7	7
Retained placenta°	0	1	0
Mastitis#	0[a]	2[ab]	4[b]
Fever§	2	3	2
Other^	1	2	2
Number of cows with clinical problems (1 or more)	2	4	5

LOIL1, low IL-1β group; IL-1β, interleukin 1β; INIL1, intermediate IL-1β group; UPIL1, upper IL-1β group. Groups are formed according to values of area under the curve of IL-1β concentrations during dry period (from -35 to 0 d in milk). °Diagnosed when foetal membranes were retained after 12 h from parturition; #diagnosed trough typical clinical symptoms (udder with oedema and hardness, clots in milk, fever, above 1000 Somatic Cells Number per μL); §rectal temperature higher of 39.5°C; ^abomasal dislocation, Partial retained placenta (diagnosed when only part of foetal membranes were retained after 12 h from parturition). [a,b]Means with different superscript within the same column differ significantly at $P<0.10$.
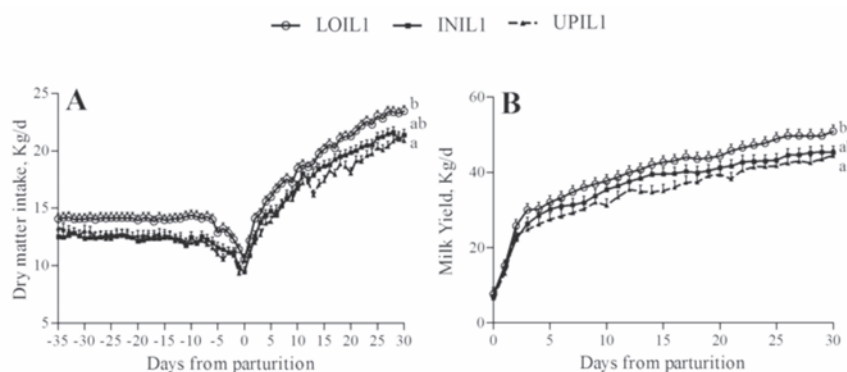

Figure 3. Dry matter intake (A) and milk yield (B) (average and SEM; kg/d) during the first month of lactation in the upper (UPIL1; 7 cows) (triangle, ▲), intermediate (INIL1; 7 cows) (square, ■) and lower (LOIL1; 7 cows) (circle, ○) group. The letters a and b in the figures indicate the overall significant differences between the groups ($P<0.05$).

during pregnancy is useful for a successful peripartum because it optimizes the feto-maternal immune interaction and vascularisation (Reinhard *et al.*, 1998). In this condition, PIC concentrations in plasma before parturition should be low, because they are mainly released by Th1 cells. At parturition, the decrease of the Th2/Th1 ratio should occur rapidly in the uterus, switching from a condition that provide tolerance for the foetus (Th2) to a condition that offers protection against infecting agents. Orsi *et al.* (2006) indicated that the Th1-Th2 dichotomy poorly describes the complex immunological process like pregnancy in mice. In fact, they observed very high concentrations of TNF-α during pregnancy, which means unbalance of Th1 and Th2 ratio. Despite, the high concentrations of PIC in late pregnancy has been suggested as risky, Orsi *et al.* (2006) did not observe complications in pregnancy. Similar results were also reported in dairy cows by Ishikawa *et al.* (2004), Trevisi *et al.* (2012) and Jahan *et al.* (2015). The results of our present study and of Ishikawa *et al.* (2004) displayed relatively high and stable values of plasma PIC in late pregnancy in a relevant number of dairy cows. This was seen, although it is documented that cows show an immunosuppression condition that start about three weeks before calving (Mallard *et al.*, 1998). This does not mean, as stated by Ishikawa *et al.* (2004), that immune function during pregnancy is inactive. However, they did not consider that PIC can also be released by several other cells besides leukocytes (Kruse *et al.*, 2000) and that the contribution of leukocytes become remarkable only when the immune system is active and challenged by pathogens and/or antigens.

After calving the PIC concentrations were remarkably reduced. The reasons of this decline was not clear, but some authors attributed the reduction of PIC due to the strong increase of 17β-estradiol (Rogers and Eastell, 2001) and cortisol (Oppert *et al.*, 2005), both markedly increase at calving time (Goff and Horst, 1997). Moreover, Vitoratos *et al.* (2008) suggested a role of placenta in the synthesis of some cytokines (*e.g.* IL-1β, adiponectin) and explained the reduced concentration of PIC in plasma after parturition due to the removal of placenta. Nevertheless, our results suggest some contradictions during early lactation. In fact when the PIC concentration was lower the pos-APP increased (*e.g.* haptoglobin), suggesting an acute phase response. From our data the short-lived peaks of IL-1β and IL-6 observed around calving seem not to be related to the raise of pos-APP few days after it. More likely, the raise of pos-APP concentration after

calving could be influenced by a different susceptibility of cows to inflammation, which could be associated to the ratio among cytokines (and thus Th1 and Th2) and the concentration of PIC in plasma (Orsi *et al.*, 2006; Ishikawa *et al.*, 2004).

Relationship between pro-inflammatory cytokines and acute phase reactants in transition period

The association between PIC and acute phase response has been investigated for a long time (Elsasser *et al.*, 1997; Gruys *et al.*, 2005). Nevertheless, few experiments investigated this association in the periparturient period, characterized by several and pronounced phenomena, such as negative energy balance, oxidative stress, immunosuppression as well as inflammatory like condition (Sordillo and Aitken, 2009; Trevisi *et al.*, 2012; van Knegsel *et al.*, 2014).

In our experiment, metabolic and inflammatory reactants, showed the classic pattern of changes of the transition dairy cows, with an increase of NEFA and pos-APP after calving and a slight reduction of neg-APP around calving (Bionaz *et al.*, 2007; Bertoni *et al.*, 2008; Trevisi *et al.*, 2012). In this context, the concentrations of PIC showed a marked reduction after calving in all the cows. Interestingly, the concentrations of PIC in late pregnancy seem related to the severity of inflammation observed in early lactation. Indeed, when cows were classified by the AUC of IL-1β during the dry period, the concentration of haptoglobin after parturition (marker of the severity of inflammation (Bionaz *et al.*, 2007; Bertoni *et al.*, 2008) was higher in the groups of higher and medium concentration of IL-1β in late pregnancy compared with the group of lower IL-1β content.

Moreover, grouping cows based on PIC showed some differences in parameters affected by inflammation (*e.g.* decrease of albumin, increase of ROMs) in late pregnancy, but based on this ranking of cows, differences between inflammatory and metabolic parameters were mainly observed after calving. The marked reduction of albumin concentrations was previously observed either before (Abdul-Aziz, 2008; Trevisi *et al.*, 2010) or after (Bionaz *et al.*, 2007; Bertoni *et al.*, 2008; Abdul-Aziz, 2008) calving in cows with severe inflammation. The synthesis of liver proteins change after a few hours after the initiation of an infection, resulting in an increase of the pos-APP concentration while at the same time the neg-APP concentrations start to decrease (Gruys *et al.*, 2005). The decrease of neg-APP concentration is in part the consequence of

the modified vascular permeability, which changes the protein distribution between plasma and other fluids (Fleck, 1989), and, perhaps, in part the consequence of the increases of the renal clearance. These phenomena explain the marked reduction of albumin concentration immediately after parturition especially in UPIL1. The delayed reduction of plasma albumin is mainly explained by the reduction of their liver synthesis (Powanda, 1980), probably competed by the higher synthesis of pos-APP.

The inflammatory condition also affects some mineral concentrations in blood (Beisel, 1977). The UPIL1 and INIL1 groups showed a greater decrease of zinc concentration after calving, although the statistical difference has been observed only between INIL1 and LOIL1. The zinc is considered as a positive acute phase reactants, in fact, its reduction in plasma is an index of higher liver synthesis of metallothionein in case of inflammation (Rink and Kirchner, 2000). The UPIL1 cows also displayed a lower concentration of calcium (table 2), particularly after calving. These data partly agree with Kimura *et al.* (2006) and Bertoni *et al.* (2008) who measured lower concentration of blood calcium in cows in more severe inflammatory like condition after calving.

A further evidence of stronger inflammations and oxidative stress in UPIL1 group compared to LOIL1 and INIL1 groups was represented by the higher concentrations of ROMs. The differences of ROMs among groups showed up in the dry period and remained during the calving period (all groups showed the same degree of increase) and persisted for the entire first month of lactation. ROMs can contribute to increased susceptibility of dairy cows to a variety of diseases (Trevisi and Bertoni, 2010; Celi, 2011). Moreover, the increased concentrations of ROMs can reiterate the inflammatory state of animals (Rimbach *et al.*, 2002; Trevisi *et al.*, 2010). Considering the high individual variability of ROMs, largely independent of the distance from calving, it is possible that genetic factors explain the differences among groups with different concentrations of PIC, in agreement with Sordillo and Aitken (2009).

Relationship among pro-inflammatory cytokines, health status and productive performance

In our study, the grouping of cows based on IL-1b concentrations in the last month of dry period seemed related to the frequency of some diseases (mainly mastitis) that occurred in the subsequent early lactation. Several studies were focused to identify inflammatory and oxidative stress markers able to predict clinical

events in periparturient dairy cows (Ishikawa *et al.*, 2004; Zebeli *et al.*, 2011; Islam *et al.*, 2013a, 2013b), but the results often do not agree. Our results on post-partum clinical problems are partly in agreement with Ishikawa *et al.* (2004), who showed the high concentrations of IL-6 in pre-partum were able to predict post-partum reproductive disorders. In contrast, Islam *et al.* (2013a) recently suggested that low concentrations of IL-1b at 15 d prior or at calving may be used to identify the cows susceptible to develop post-partum reproductive diseases. In another experiment, Islam *et al.* (2013b) claimed that increased concentration of the IL-10 (an anti-inflammatory cytokine) at 15 d pre-partum can be a good predictor of retained placenta and metritis. Unfortunately, the data of Islam *et al.* (2013a, 2013b) are difficult to compare with our results because in these studies no inflammatory or metabolic markers in plasma were investigated.

More consistent appear researches, which have investigated concentrations of PIC and the feed intake. PIC have a profound behavioural, neuroendocrine, and metabolic effect and act in the brain to reduce food intake in laboratory animal species (Plata-Salamán, 2000; Johnson, 1997). In chicks, a series of experiments demonstrated that a variety of inflammatory agents reduced feed intake and efficiency of gain, increased body temperature and plasma corticosterone (Klasing *et al.*, 1987). In cows with induced ketosis (Loor *et al.*, 2007) the increased expression of IL-6 may have been causally involved in the reduced DMI and milk production. In overall these data agree with our research, in fact cows with the higher concentration of pro-inflammatory cytokines before calving showed a lower DMI for the whole period, a higher frequency of mastitis and a lower milk yield. Surprisingly, the marked differences in DMI in the present study were not associated with increased lipid mobilization. In our experiment, the NEFA and BHBA concentrations were only numerically higher in UPIL1 in comparison to other groups, likely because of the simultaneous marked reduction of milk synthesis. Thus, our study suggests a role of plasma PIC concentrations before calving in the regulation of the DMI during the peripartum.

Conclusions

The present study demonstrates that in dairy cows the concentrations of PIC (*e.g.* IL-1b and IL-6) are almost stable during the last month of pregnancy, but shows a high variability among the cows. Although PIC concentrations were significantly higher in the dry period, only few relationships between these cytokines and other plasma parameters (*e.g.* lower concentrations of albumin and higher concentrations of ROMs) were found in late pregnancy. Importantly, none of the cows showed clinical diseases. Nevertheless, cows with the highest concentration of PIC in late pregnancy exhibited the worst condition in term of metabolic and inflammatory indices, incidences of mastitis, feed intake and milk production in early lactation, suggesting that PICs are good candidates to discover animals with an unsuccessful transition period. Future efforts should be addressed to clarify the causes of the high variability of PIC concentration among cows leading to inflammatory phenomena in the forthcoming lactation, as well as the reasons for the higher PIC concentrations in pregnancy without evidence of acute phase reaction status.

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