INTERPRETIVE SUMMARY

Effect of non-esterified fatty acid levels on global transcriptomic profiles in circulating peripheral blood mononuclear cells in early lactation dairy cows. Cheng. Excessive production of non-esterified fatty acids (NEFA) was previously identified as a major risk for postpartum immunosuppression. Our results demonstrated that increased circulating NEFA concentration altered various aspects of immune system process in peripheral blood mononuclear cells (PBMC) in early lactation cows. When the NEFA concentration exceeded 750 µM, their cell-to-cell adhesion was inhibited, which would reduce the efficiency of diapedesis. This would contribute to decreased body defence/immunity and predispose animals to infection.

Effect of diet and non-esterified fatty acid levels on global transcriptomic profiles in circulating peripheral blood mononuclear cells in early lactation dairy cows

Z. Cheng[1*], A. Wylie[2], C. Ferris[2], K. L. Ingvartsen[3], D. C. Wathes[1] and GplusE consortium[#]

[1]Royal Veterinary College, Hatfield, Herts, UK

[2]Agri-Food and Biosciences Institute, Newforge Lane, Upper Malone Road, Belfast BT9 5PX, UK

[3]Department of Animal Science, Aarhus University, DK-8830 Tjele, Denmark

* Corresponding author: zcheng@rvc.ac.uk

[#]List of authors in the GplusE Consortium: Mark Crowe, Niamh McLoughlin, Alan Fahey, Elizabeth Matthews, Andreia Santoro, Colin Byrne, Pauline Rudd, Roisin O'Flaherty, Sinead Hallinan, Claire Wathes, Zhangrui Cheng, Ali Fouladi, Geoff Pollott, Dirk Werling, Beatriz Sanz Bernardo, Mazdak Salavati, Laura Buggiotti, Conrad Ferris, Alistair Wylie, Matt Bell, Mieke Vaneetvelde, Kristof Hermans, Geert Opsomer, Sander Moerman, Jenne De Koster, Hannes Bogaert, Jan Vandepitte, Leila Vandevelde, Bonny Vanranst, Leslie Foldager, Klaus Ingvartsen, Martin Sørensen, Soren Ostergaard, Torben Larsen, Mogens Krogh, Charlotte

Gaillard, Jehan Ettema, Tine Rousing, Federica Signorelli, Francesco Napolitano, Bianca Moioli, Alessandra Crisa, Luca Buttazzoni, Jennifer McClure, Daragh Matthews, Francis Kearney, Andrew Cromie, Matt McClure, Shujun Zhang, Xing Chen, Huanchun Chen, Junlong Zhao, Liguo Yang, Guohua Hua, Chen Tan, Guiqiang Wang, Michel Bonneau, Andrea Pompozzi, Armin Pearn, Arnold Evertson, Linda Kosten, Anders Fogh, Thomas Andersen, Matthew Lucy, Chris Elsik, Gavin Conant, Jerry Taylor, Nicolas Gengler, Michel Georges, Frederic Colinet, Marilou Ramos Pamplona, Hedi Hammami, Catherine Bastin, Haruko Takeda, Aurelie Laine, Anne-Sophie Van Laere, Martin Schulze, Cinzia Marchitelli and Sergio Palma Vera.

ABSTRACT

After calving, lipid mobilization caused by increased nutrient demands for lactation leads to elevated circulating concentrations of non-esterified fatty acids (NEFA). Excessive NEFA levels have previously been identified as a major risk factor for postpartum immunosuppression. The aim of this study was to investigate changes in global transcriptomic gene expression of peripheral blood mononuclear cells (PBMC) in dairy cows offered different early lactation diets (High, n = 7; Medium, n = 8 or Low, n = 9 concentrate) and with differing circulating levels of NEFA. Cows were classified as having NEFA concentrations of either <500 µM (Low, n = 6), 500 - 750 µM (Medium, n=8) or >750 µM (High, n=10) at 14 days in milk (DIM). Plasma urea concentrations were greater for cows on the High concentrate diet but β-hydroxybutyrate (BHB) and glucose concentrations did not differ significantly between either dietary treatments or NEFA groups. Cows with High NEFA weighed more at drying off and suffered greater BCS loss after calving. PBMC were isolated at 14 DIM and RNA was extracted for RNA sequencing. Differential gene expression was analysed with DESeq2 with q-value for false discovery rate control followed by Gene Ontology (GO) Enrichment. While there were no differentially expressed genes (DEG) associated with lactation diet, 304 DEG were identified between cows with High and Low circulating NEFA, with 118 upregulated and 186

downregulated. GO enrichment analysis demonstrated that biological adhesion and immune system process were foremost amongst various PBMC functions which were altered relating to body defences and immunity. High NEFA concentrations were associated with inhibited cellular adhesion function by down-regulating 20 out of 26 genes (by up to 17 fold) related to this process. Medium NEFA concentrations altered a similar set of functions as High NEFA, but with smaller enrichment scores. Localization and immune system process were most significant, with biological adhesion ranking only eleventh. Our results demonstrated that increased circulating NEFA concentrations, but not diet, were associated with immune system processes in PBMC in early lactation cows. Leukocyte cell-to-cell adhesion was inhibited when the NEFA concentration exceeded 750 μM, which would reduce the efficiency of diapedesis and so contribute to decreased body defence mechanisms and predispose animals to infection.

Key words: non-esterified fatty acids; peripheral blood mononuclear cells; RNA sequencing; cows.

INTRODUCTION

Extensive nutritional, metabolic and hormonal changes during the transition period from pregnancy to lactation in dairy cows are risk factors for both metabolic and infectious diseases (Goff and Horst, 1997; Moyes et al., 2013). These changes, together with the inflammatory responses in the reproductive tract caused by parturition, can lead to decreased appetite and thus reduce feed intake (Ingvartsen and Moyes, 2013). The changes in hormones and tissue sensitivity during the transition period promotes the nutrient supply to the mammary gland in support of lactation. When feed intake fails to meet the increasing energy demands, then the cow enters a period of negative energy balance (**NEB**) which may last for several months in severe cases (Taylor et al., 2003). The catabolism of adipose tissue results in excessive release

81 of non-esterified fatty acids (**NEFA**) into the circulation and production of β-hydroxybutyrate
82 (**BHB**) by the liver (Adewuyi et al., 2005). This may exacerbate the metabolic imbalance, as
83 hepatic oxidation of some free fatty acids may trigger a satiety signal and so depress feed intake
84 further (Ingvartsen and Andersen, 2000). Some cows are less capable than others at regulating
85 the balance between their intakes and requirements.

86 Parturition in dairy cows is also characterised by suppressed immune function, including
87 a reduction in circulating leukocyte numbers (Mallard et al., 1998; Wathes et al., 2009) and
88 functional capacity (Ster et al., 2012; Ingvartsen and Moyes, 2013). After calving, phagocytosis
89 and oxidative burst activity by polymorphonuclear leukocytes (**PMNL**) are impaired (Kehrli
90 et al., 1989; Ingvartsen and Moyes, 2013). Reductions in both cellular and humoral immunity
91 have been observed, in which the responsiveness of circulating T-cells to mitogenic agents and
92 production of immunoglobulin by B-cells were reduced (Nonnecke et al., 2003; Lacetera et al.,
93 2005). Disorders of immune function predispose postpartum cows to infectious diseases such
94 as endometritis, metritis and mastitis (Vangroenweghe et al., 2005; Sheldon et al., 2006).

95 Many factors may contribute to the impairment of immune function around the time of
96 calving. Immune cells require an adequate supply of glucose, amino acids, fatty acids and
97 cholesterol/oxysterols for their own proliferation and for production of immune molecules
98 (Ingvartsen and Moyes, 2013; Loftus and Finlay, 2016; Dimeloe et al., 2017). In cows with
99 NEB, decreased nutrient availability may lead to an inadequate energy supply to the immune
100 cells. Lactating cows prioritize glucose supply to the mammary gland for lactose synthesis, and
101 this limits glucose availability to the immune cell population (Habel and Sundrum, 2020). The
102 increase in circulating NEFA concentration also plays a crucial role in impairing immune cell
103 function. A previous study compared the functions of peripheral blood mononuclear cells
104 (**PBMC**) *in vitro* when exposed to serum samples obtained at five DIM (high NEFA and BHB),
105 61 DIM (low NEFA and BHB) or 61 DIM enriched with NEFA or BHB. This provided

evidence that high NEFA concentrations inhibited PBMC proliferation, oxidative burst activity and production of interferon-γ, while supplementation of BHB alone did not alter PBMC proliferation (Ster et al., 2012). Nutritional management of cows to control the increase in NEFA in early lactation may therefore improve resistance to infection (Ster et al., 2012; Lacasse et al., 2018). However, excessive tissue mobilization in early lactation is often linked to a high body condition score (**BCS**) obtained during late lactation or the dry period prior to calving (Ingvartsen, 2006). Improving resistance to infection should therefore consider optimal feeding throughout the lactation cycle and the dry period. Few previous studies have, however, examined the impact of postpartum concentrate feed level on immune function *in vivo*.

Peak NEFA concentrations after calving vary considerably between cows, usually in the range 250 to 1,200 μM (Wathes et al., 2007; Roche et al., 2015; Liang et al., 2020). Previous reports have demonstrated that concentrations above 500 μM inhibited leukocyte function (Ster et al., 2012) while values ≥1,000 μM are associated with an increased risk of developing a displaced abomasum (Chapinal et al., 2011; Lyons et al., 2014). Our hypothesis was that PBMC functionality in response to differing NEFA concentrations would result in major changes in their gene transcriptome. In the present study we investigated the associations between diets, circulating NEFA levels and PBMC function in early lactation at the global transcriptomic level using next generation sequencing and bioinformatics approaches.

MATERIALS AND METHODS

Animals

The study was performed under the Home Office Animals (Scientific Procedures) Act 1986 and was approved by both the Agri-Food and Biosciences Institute (**AFBI**) and the Royal Veterinary College's Ethics and Welfare Committees. The primary outcome was the changes

in immune pathways. Our previous global transcriptomic study showed that 6 cows in each group were sufficient to detect the changes in many immune-related pathways caused by metabolic disorders (Wathes et al., 2009). Twenty-four multiparous Holstein-Friesian cows weighing 638 ± 21 Kg were recruited from AFBI (Hillsborough, Northern Ireland, UK). All cows were milked twice daily and daily yields were recorded. These animals were a subset of animals used in a previous study which had investigated immune responses in cows fed differing amounts of concentrate (Little et al. 2019). Following calving cows were offered one of three diets comprising concentrates and grass silage mixed in different dry matter (**DM**) ratios, namely 70 : 30, 50 : 50 and 30 : 70 (High, Medium or Low concentrate, respectively). The ingredients and chemical composition of the concentrates offered with each treatment differed, with all diets designed to be iso-nitrogenous. Cows were also offered an additional 0.5 kg concentrate at each milking (i.e. 1 kg per day) via an in-parlour feeding system. Full details of the diets offered and the feeding systems adopted have been described by Little et al. (2019). Body weights (**BW**) were recorded at the end of the previous lactation and the three dietary treatment groups were balanced for PTA for fat plus protein (kg), pre-calving BW and BCS, and previous lactation 305-day milk yield. After calving BCS was estimated according to a common protocol at 14 ± 1.8 and 35 ± 1.8 DIM (mean ± SD) using a five-point scale, with each score subdivided into quarters so that in effect it functioned as a 20-point scale (Edmonson et al., 1989). Blood samples were collected from the jugular vein into heparin coated tubes at 14 ± 2 DIM (Mean ± SD), separated via centrifugation and stored at -20 °C until analysis.

Analysis of Circulating Metabolites

Laboratory analysis of blood metabolites was performed at the Department of Animal Science, Aarhus University, Denmark according to previously published methods (Bjerre-

Harpøth et al., 2012). NEFA concentrations were determined with the ACS-ACOD method using NEFA C Kit (Wako, 41468 Neuss, Germany) following the supplied protocol. Concentration of plasma glucose was determined using an enzymatic method (ADVIA 1800 Clinical Chemistry System, Siemens Healthcare Diagnostics, Ballerup, Denmark). BHB was quantified by measuring absorbance at 340 nm due to the production of NADH at alkaline pH in the presence of BHB dehydrogenase. Urea was determined by spectrophotometry (Nielsen et al., 2005). Intra- and inter-assay CV were in all cases below three and four percent, respectively, for both low and high control samples.

Interferon-γ production

Additional blood samples were collected in a lithium heparin tube at both 6 to 8 and 13 to 15 DIM for the measurement of IFN-γ production from stimulated lymphocytes by ELISA as described in detail by Little et al. (2017). Briefly, whole blood samples were incubated for 24 h at 37°C and 5% carbon dioxide with phosphate buffered saline as a negative control to look at the inherent IFN-γ level, and also with pokeweed mitogen (**PWM**) used as a positive stimulant of peripheral blood mononuclear cells to produce IFN-γ.

Animal Grouping

The twenty-four cows were grouped for statistical analysis based: (1) on their lactation diets (High, Medium or Low concentrates) and (2) on their circulating NEFA concentrations measured at around 14 DIM as: Low, < 500 µM; Medium, 500 - 750 µM or High, > 750 µM. The number of cows per diet and NEFA group are shown in Table 1.

Isolation of Circulating Peripheral Blood Mononuclear Cells

Equal volumes of 15 ml blood and 15 ml PBS were added to a 50 ml Falcon tube and then underlain with 15 ml Histopaque (d = 1,083, SigmaAldrich, Welwyn Garden City, UK). This was centrifuged at room temperature and 1,200 × *g* for 30 min. The PBMC suspension was collected to a new tube and 45 ml PBS added. It was centrifuged at 600 × *g* and 4°C for 10 min and the supernatant was removed. This washing procedure was repeated another two times, but with the centrifugation time reduced to 8 min. After removing all supernatant, 1.5 ml of 1 × PBS was added to re-suspend the cells. The suspension was collected into a 2 ml centrifuge vial and centrifuged at 500 × *g* and 4°C for 10 min. After removing all supernatant, the cell pellet was frozen at -80°C until extraction.

RNA Extraction

Total RNA from the isolated PBMC was extracted with a Qiagen RNeasy mini kit (Qiagen, Manchester, UK) following the supplier's protocol. The RNA concentration and purity were analyzed with a DeNovix DS-11 spectrophotometer (Cambridge Bioscience, Cambridge, UK). RNA quantity and integrity were assessed using an Agilent BioAnalyzer 2000 (Agilent, Cheshire, UK) and Agilent RNA 6000 Nano Kit. All RNA samples had RIN number >7 and 260/280 between 1.8 and 2.2. The purified RNA was stored at -80°C for next generation sequencing (**RNA-Seq**).

RNA-sequencing, Mapping and Quantification

RNA extracted from each PBMC sample was subjected to RNA-Seq by Novogene Company Ltd. (Hong Kong, China). RNA-Seq libraries were prepared from 400 ng of the total

RNA with 250-300 bp insert strand specific library after rRNA removal using a Globin-zero Gold rRNA removal kit (Illumina, San Diego, California, USA). Pooled cDNA libraries were sequenced on Illumina NovaSeq platforms with paired-end 150 bp sequencing (PE150) to reach over 30 million reads per sample.

The sequences with poor quality reads were trimmed or removed with Trimmomatic v. 0.36 (Bolger et al., 2014) and the quality of raw and trimmed FASTQ files was assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/). A splice aware aligner HISAT2 (Kim et al., 2015) was used to map the reads to a reference genome of Bos taurus assembly (ARS_UCD1.2). Conversion of the SAM files to BAM files was conducted with SAMtools (Li et al., 2009). Reads per gene were counted with StringTie (Pertea et al., 2015).

Identification of Differently Expressed Genes between Groups

DESeq2 (Love et al., 2014) on R-package 4.0 platform was used to identify the differentially expressed genes (**DEG**) between the groups using reads per gene as quantified above. This included a DESeq normalization and negative binomial generalized linear model. Q - values were used to adjust the multiple tests and significance was considered at $P < 0.05$. This analysis was performed: (1) with samples grouped by diet and (2) with samples grouped by postpartum NEFA concentration.

Gene Ontology (GO) Enrichment Analysis

The significant genes identified with DESeq2 were uploaded into Partek Genomics Suite 7.1 (Partek Incorporation, Missouri, USA) for GO enrichment analysis with a genome version of *Bos taurus* 4 to investigate the biological functions and interactions between genes and

pathways using Fisher's exact test with BH adjustment. Statistical significance was considered at $P < 0.05$.

Statistical analysis

Statistical data analysis was carried out using SPSS V26 software package (Chicago, IL, USA). The differences of blood metabolites, lactation number, daily milk yield, BW and BCS between NEFA and dietary groups were compared with two-way analysis of variance (ANOVA). The data of IFN- γ were analyzed using ANOVA with a linear mixed effect model, in which diets and NEFA group were taken as fixed effect, time as repeated measure and cow as subject. Before ANOVA, Levene's statistics were used to confirm the normal distribution of the parameters between the dietary groups or between the NEFA groups. Significance was considered where $P < 0.05$. Where ANOVA showed significance, multiple comparisons with Fisher's LSD method was carried out to identify the source of differences.

RESULTS

Circulating Metabolites and BCS Changes

Cows receiving the different lactation diets were balanced by lactation number, which were 3.14 ± 0.26, 3.50 ± 0.38 and 3.33 ± 0.29 on the High, Medium and Low concentrate diets, respectively (mean ± SE). The daily milk yields were 32.5 ± 0.96 and 35.4 ± 1.20 kg/d at 7 and 14 DIM respectively, and were not affected by diet over this time period. The effect of different lactation diet on circulating metabolites at around 14 DIM is shown in Table 2. Cows offered the Low concentrate diet had significantly lower circulating urea levels than those receiving the Medium or High concentrate diets ($P < 0.01$), but there were no differences in NEFA, BHB

or glucose levels between groups. There was no effect of diet on BCS loss between 14 and 35 DIM (-0.18 ± 0.07, -0.03 ± 0.09 and -0.08 ± 0.04 for cows on High, Medium and Low diets, respectively).

There was also no significant relationship between NEFA group and milk yield at 7 and 14 DIM. Circulating concentrations of metabolites according to the three NEFA groups are summarized in Table 2. No other metabolites measured apart from NEFA differed between groups. However, High NEFA cows tended to be slightly older, with a higher BW at drying off and a greater BCS at 14 DIM. They subsequently suffered a significantly greater BCS loss between 14 to 35 DIM ($P < 0.01$, Figure 1).

Interferon-γ production

Neither the diet nor the NEFA group had a statistically significant effect on the IFN-γ production by PWM stimulated whole blood culture in samples obtained at around 7 and 14 DIM. The production levels of IFN-γ were 19.3 ± 3.55, 11.6 ± 2.85 and 10.0 ± 2.74 ng/ml for NEFA groups <500 µM, 500-750 µM and >750 µM respectively ($P = 0.15$). The results with respect to diet were 18.5 ± 2.62, 10.4 ± 3.10 and 12.0 ± 3.45 ng/ml for the Low, Medium and High concentrate diets ($P = 0.12$). Production of IFN-γ did, however, increase from 9.8 ±1.89 to 14.8 ± 1.89 ng/ml ($P<0.001$) between 7 and 14 DIM, when the RNAseq analysis was performed.

Comparison of Global Transcriptomic Expression in Circulating PBMC According to Lactation Diet

Based on the principal component analysis (**PCA**) plot, there was substantial overlap in gene expression profiles in PBMC obtained from cows in the three dietary groups (Figure 2A). This was confirmed by the global transcriptomics analysis, which showed that there were no DEG between the dietary groups which remained significant after BH or q - value correction.

Comparison of Global Transcriptomic Expression in Circulating PBMC between Cows with High or Low Circulating NEFA levels

In contrast to the lack of effect of lactation diet, the PCA showed different patterns of the circulating PBMC transcriptomic expression profiles between the NEFA groups (Figure 2B-C). Global transcriptomic analysis identified 304 DEG in PBMC between the cows with High (> 750 µM) and Low (< 500 µM) circulating NEFA (Supplementary file 1, https://rvc-repository.worktribe.com/output/1548065). Among them, 118 genes were upregulated in the High group and 186 genes were downregulated. The top 10 most upregulated genes as ranked by the *P* values were *KLF11, CPT1A, PDK4, ALPL, CHRND, IL2RA, CAPN5, PATJ, LINGO2* and *PTH1R*, and the most downregulated were *ICAM5, STAC2, S100A14, KCNG2, IL5RA, ADGRB2, MEFV, FKBP10, KRT7* and *BHLHE40*. GO enrichment analysis of all the DEG identified over 1,000 biological functions. The top five (all with enrichment scores >10) were biological adhesion, immune system process, biological regulation, development process and locomotion (Figure 3A). The identified GO function of "Immune system process" included 37 DEG, among which 19 were downregulated and 18 upregulated in the cows with High NEFA (Table 3). Sub-analysis of these DEG showed that they were involved in various immune processes, including immune response (enrichment score 10.6), lymphocyte co-stimulation (enrichment score 6.4) and leukocyte activation (enrichment score 5.5) (Figure 3B). There were 26 DEG associated with the GO function of biological adhesion. Among these 20 out of 26

were downregulated by up to 17 fold in the cows with High NEFA compared to those with Low NEFA. Only six DEG were upregulated, but with relatively small fold changes (Table 4). Most of the DEG (n = 25) were associated with cell adhesion (cell-to-cell adhesion, cell-substrate adhesion and cell adhesion mediated by integrin).

Comparison of the Global Transcriptomic Expression in Circulating PBMC between Cows with Medium or Low Circulating NEFA Levels

PCA also illustrated different transcriptomic expression patterns in the PBMC between the cows with Medium and Low NEFA concentrations (Figure 2C). Global transcriptomic analysis identified 306 DEG in PBMC between the cows with Medium (500-750 µM) and Low (< 500 µM) circulating NEFA. Among these 188 DEG were upregulated and 118 were downregulated in the Medium NEFA cows compared with the Low NEFA cows (Supplementary file 2, https://rvc-repository.worktribe.com/output/1548065). The top 10 most upregulated genes ranked by the *P* values were *MARCO, CSTB1, HP, MEGF11, ADAMDEC1, KLRB1, PAMR1, ALPL, PPP1R16A* and *FCGR1A* and the top 10 downregulated genes were *TAFA4, MGC138914, MYO1B, NXPE4, PPM1E, SYT2, MFSD11, SP140L, KLRC1* and *SPON1*. GO analysis using all DEG again identified over 1,000 significantly altered biological functions with localization, immune system process, detoxification, locomotion and cell killing being the most significant (enrichment scores >7.3) (Figure 4A). The immune system process pathway contained 31 DEG (Table 5). Among these, 24 genes were upregulated in the cows with Medium NEFA levels with *AZU1, FCGR1A, LTF, PTX3, HP* and *CD36* all being upregulated by over two fold (2.2 - 7.9). There were only seven downregulated DEG: *TAFA4* was downregulated by 88 fold whereas the changes in other genes were below two fold. These immune genes were involved in various immune processes, with leukocyte activation, immune

response, immune effect process, production of molecular mediator of immune response and leukocyte migration being most significant (enrichment scores 4.47-11) (Figure 4B). The "Activation of biological adhesion" pathway was detected with a relatively low enrichment score (3.7, ranked eleventh). This involved six downregulated genes (*FERMT2, ICAM5, COL4A3, KIRREL1, PDGFRA* and *ITGA6*) and nine upregulated genes (*CD36, LAMA4, SLC39A8, S100A8, S100A9, CD9, ADA, MFGE8* and *KIFC3*).

DISCUSSION

During early lactation dairy cows mobilize adipose tissue to support milk synthesis (Bauman and Currie, 1980; Drackley et al., 2001). Excessive lipids reaching the liver during this period leading to the development of fatty liver disease in around half of high-yielding dairy cows, with the triglyceride accumulation impairing liver function and increasing inflammatory responses (Vernon, 2005; Shi et al., 2020). Bacterial infection in the postpartum period leads to around half of dairy cows being affected by uterine diseases including metritis, purulent vaginal discharge, endometritis or cervicitis (LeBlanc, 2012). The incidence of mastitis is also maximal in early lactation (Vangroenweghe et al., 2005). There is increasingly strong evidence that susceptibility to infection is associated with metabolic disorders and subsequent immunosuppression (LeBlanc, 2012; Ingvartsen and Moyes, 2013; Habel and Sundrum, 2020). Raised NEFA concentrations are an important indicator of adipose tissue mobilisation and are thought to be a crucial player contributing to immune dysfunction at this time (Moyes et al., 2009; Lacasse et al., 2018).

Circulating NEFA concentrations start to increase from about 2 to 3 weeks before calving, reach a peak during the first 1 to 2 weeks of lactation, and return to basal levels of around 100 μmol/L by about 6 to 8 weeks after calving (Pushpakumara et al., 2003; Roche et al., 2015).

340 Both the timing and height of the peak are influenced by a number of factors including parity
341 (Wathes et al., 2007), DM intake (Ingvartsen and Moyes, 2013), prepartum BCS (Liang et al.,
342 2020) and prepartum diet (Roche et al., 2015; Zhou et al., 2015). This is in accord with our
343 study, which found that differences in NEFA at around 14 DIM were associated with pre-
344 calving weight and post calving BCS loss. We did not, however, find any differences in either
345 NEFA concentration or leukocyte gene expression associated with the lactation diet, which
346 differed in concentrate proportion. This was in agreement with the previous report that these
347 diets had no impact on immune function at this time as measured by neutrophil phagocytosis
348 and oxidative burst, or on lymphocyte IFN-γ production (Little et al., 2019). Although this
349 larger study did find differences in milk yield according to diet, this did not become significant
350 until 3 weeks into the lactation, whereas the blood samples for RNAseq analysis were taken
351 earlier than this at 14 DIM. This supports previous suggestions that improved late lactation and
352 dry cow nutritional management may be a more useful strategy than lactation diet to limit
353 peripartum immunosuppression by prevention of excessive circulating NEFA production
354 (Ingvartsen, 2006; Ster et al., 2012; Ingvartsen and Moyes, 2013; Lacasse et al., 2018).

355 In the present study, samples for both PBMC isolation and NEFA quantification were
356 collected on around day 14 after calving, so at this time the cows would have been exposed to
357 elevated NEFA levels for several weeks. Using *in vitro* testing, Ster et al. (2012) showed that
358 the function of PBMC from dairy cows are directly sensitive to changes in NEFA
359 concentrations, as 13 µM inhibited their proliferation, 50 µM reduced oxidative burst activity
360 and 75 µM reduced IFNγ secretion. In our study IFNγ production by PWM stimulated
361 lymphocytes did not differ between the NEFA groups, although the values were numerically
362 higher in both groups with NEFA > 500 µM. The cows in our Low NEFA group had circulating
363 NEFA concentrations of 372 ± 119 µM compared with 1,069 ± 53 µM in the High NEFA group.
364 Our results showed clearly that increases in circulating NEFA levels were associated with a

365 greater degree of immunosuppression after calving between these two groups. It is, however,
366 likely that expression of some genes in PBMC may also have been influenced by NEFA in the
367 Low group cows, as the concentrations at around 14 DIM in these animals were much above
368 the basal values of around 100 μmol/L. This could have reduced the number of DEG detected
369 in the analysis.

370 The PBMC population includes lymphocytes (T-cells, B-cells, NK cells), monocytes and
371 dendritic cells. During an episode of mastitis or metritis such cells are attracted to the site of
372 infection and are exposed to circulating metabolites including NEFA. In the present study, gene
373 expression of many pro-immune mediators in the PBMC was significantly downregulated in
374 the cows with High circulating NEFA. The top GO enrichment scores were related to immunity,
375 including biological adhesion and immune system processes. This supports previous
376 suggestions that High circulating NEFA concentrations postpartum may be responsible for
377 inhibiting various immune functions in the PBMC and may therefore reduce resilience to
378 infectious diseases during early lactation.

379 Lymphocyte recirculation and monocyte trafficking require adhesion and transmigration
380 through blood-vessel walls towards the site of tissue damage or infection, so playing a central
381 role in immune and inflammatory responses (Ley et al., 2007). In a previous study we reviewed
382 the literature to identify candidate genes associated with responses to mastitis and found that
383 genes involved in leukocyte adhesion to the vascular endothelium and diapedesis contributed
384 to the top signaling pathway (Chen et al., 2015). This process and its regulation require the
385 involvement of selectins, integrins, immunoglobins and chemokines. In the present study,
386 alteration of biological adhesion of PBMC between cows with High and Low circulating NEFA
387 concentrations was also the top pathway identified, with a GO enrichment score of 20. Among
388 26 DEG involved in this process, 77% were inhibited in cows with High circulating NEFA,
389 suggesting a decreased ability of PBMC adhesion.

Previous studies of bovine leucocytes over the transition period have similarly noted a reduced capacity of the immune cells for extravasation (Jahan et al., 2015; Crookenden et al., 2016) and altered expression of genes involved in the extracellular matrix receptor pathway after calving (e.g. *ITGB2*, *ITGBX,* VCAM1) (Crookenden et al., 2016; Minuti et al., 2020). Amongst the DEG identified in our High NEFA cows, *CLDN4, CYP1B1, DSG2, ICAM1, ICAM5, IL1B, ITGAD, ITGA4, JAM2, KIRREL1, PCDHGA5, PDGFRA PLEK* and *TNF* were all annotated as being involved in cell-to-cell adhesion by GO terms. The most downregulated gene (by 17-fold) was *DSG2* which encodes desmoglein-2, an integral component of macula adherens junctions (Franke et al., 2006). Its' loss contributes to the pathologies of some inflammatory diseases, such as Crohn's disease in humans (Spindler et al., 2015). *ICAM1* and *ICAM5* are members of the intercellular adhesion molecule (ICAM) family, type I. These are transmembrane glycoproteins which bind to the leukocyte adhesion LFA-1 protein. They are important in inflammation, immune responses and in intracellular signaling events (Gahmberg et al., 1997). *ICAM1* encodes an endothelial- and leukocyte-associated transmembrane protein which stabilizes cell-cell interactions and facilitates leukocyte endothelial transmigration (Yang et al., 2005). The cytokines *IL1B* and *TNFα* both promote leukocyte cell-to-cell adhesion by activating ICAM1 (Myers et al., 1992). *JAM2* (junctional adhesion molecule 2) acts as an adhesive ligand for interacting with a variety of immune cell types and may play a role in lymphocyte homing to secondary lymphoid organs, also involving trans-endothelial migration (Johnson-Leger et al., 2002). *CYP1B1* expression was reduced by 8-fold in cows with by High circulating NEFA. This encodes a cytochrome P450 enzyme which was shown to regulate expression of genes involved in cell adhesion and migration in retinal astrocytes (Falero-Perez et al., 2020). *ITGAD* and *ITGA4* both encode integrins, the main receptor proteins that cells use to both bind to and respond to the extracellular matrix and which act as sensors of biological adhesion (Harburger and Calderwood, 2009; Kechagia et al., 2019). Expression

of *ITGA4* and *ITGB2* in polymorphonuclear neutrophil leukocytes was significantly reduced in postpartum cows fed a high fat diet pre-calving (Zhou et al., 2015). Our results therefore lend support to the importance of diapedesis and adhesion pathways in postpartum cows.

With respect to diapedesis, it is important to note that NEFA also alter the properties of the vascular endothelium as well as the leukocytes. For example, liver sinusoidal endothelial cells were less adherent and more migratory in *CYP1B1* knockout mice and expressed lower levels of the inflammatory mediators MCP1 and TNFα (Falero-Perez et al., 2018). This is in line with our present study in which *TNF* was downregulated by 2.4 fold in PBMC of the cows with High circulating NEFA. Contreras et al. (2012) tested the addition of NEFA mixtures to bovine aortic endothelial cells and reported significant alterations in mRNA expression of cytokines (*IL6*, *IL8*) and adhesion molecules (*ICAM1*, *VCAM1*) that are associated with increased inflammatory responses.

Localization and immune system process were the most significant pathways identified in PBMC from cows with Medium circulating NEFA concentrations (500-750 μM) compared with those having Low NEFA. The changes were less pronounced than in the High NEFA cows, with lower enrichment scores. Biological adhesion was only ranked eleventh in the Medium NEFA cows as opposed to being the most significant pathway affected in the High NEFA cows. In the sub-analysis of the DEG involved in the immune system process there were fewer DEG (n = 17) with smaller fold changes between 2.1 and -4.4. Nine of the top ten biological functions/pathways identified in the sub-analysis of immune system process in PBMC of cows with High and Medium circulating NEFA were, however, the same. Although the order of their GO enrichment scores was different, activations/disorders of immune system process was the top pathway for both groups. Other pathways included lymphocyte co-stimulation, leukocyte activation and migration, immune effector process and antigen presentation and processing.

This implies that most arms of the immune system were affected by both high and medium NEFA concentrations, although some aspects of the responses appeared to be dose-dependent.

The mechanisms by which increased NEFA concentrations alter immunity are not fully understood and probably include both direct and indirect effects on the PBMC. Plasma NEFA consist of many short and long chain fatty acids which are mainly transported in blood bound to albumen (Contreras et al., 2010; Ster et al., 2012). Previous studies in dairy cows demonstrated that around parturition and during early lactation the proportion of saturated fatty acids, such as palmitic acid, increased in both circulating blood and PBMC, whereas the phospholipid fraction of long-chain PUFAs in the PBMC decreased (Contreras et al., 2010). Increased cellular content of saturated fatty acids is associated with enhanced activation of human and bovine leukocytes (Scalia et al., 2006; Haversen et al., 2009). Indeed, we observed enhanced PBMC expression of some DEG involved in immune process in the cows with High circulating NEFA, such as *RARRES2* and *BOLA-DQB*. Expression of the class B scavenger receptor *CD36* was also increased, which is thought to be important for NEFA uptake (Kashyap et al., 2009; Nakamura et al., 2014). Other free fatty acid receptors (FFAR1-4, GPR84) have been identified in many cell types, including immune and epithelial cells and they play roles in the interactions between NEFA and cells, especially for the immunological responses (Alvarez-Curto and Milligan, 2016). In our study, however, *FFAR3* was down-regulated in the High NEFA cows (Supplementary Table 1).

Changes to the NEFA concentration of PBMC may modify many other key functions. For example, raised concentrations of oleic acid can inhibit prostaglandin production (Cheng et al., 2015). Prostaglandins are important signaling molecules contributing to regulation of immune cell functions (Calder, 2009). The TLR4 signaling pathway can be activated by saturated fatty acids including palmitic acid (Mamedova et al., 2013), which is a major component of circulating NEFA in the cow. Intracellular lipids also act directly as ligands for

a number of transcription factors, including PPARs and RXR (Nakamura et al., 2014), so influencing gene expression. Changes in circulating NEFA will also influence PBMC indirectly, particularly through alterations to liver function. For example, excessive triglyceride accumulation increases hepatic expression of genes encoding acute-phase proteins, so their circulating concentrations will increase, influencing leukocyte activity (Shahzad et al., 2014).

Other blood metabolites apart from NEFA have been shown to influence PBMC activity. For example, pre-incubation of bovine neutrophils with increasing concentrations of BHB decreased their bactericidal activity against *E. coli* causing mastitis (Grinberg et al., 2008), inhibited superoxide anion (SO) production in ovine neutrophils (Sartorelli et al., 2000) and reduced chemotaxis of bovine leukocytes (Suriyasathaporn et al., 1999). Previous workers have, however, concluded that excessive production of circulating NEFA is more important for immunosuppression. For example, Moyes et al. (2009; 2013) reported that circulating NEFA peripartum was a more reliable risk factor for the development of metritis, milk fever and retained placenta than plasma BHB and glucose, or calculated energy balance. Janosi et al. (2003) also observed a trend ($P = 0.08$) towards a positive correlation in Holstein cows between elevated NEFA concentrations in very early lactation (1–3 DIM) and the incidence of mastitis within the first 28 DIM. The importance of NEFA is therefore supported by our study, which has used next generation sequencing and bioinformatics approaches to demonstrate that increased circulating NEFA concentrations altered various immune processes in the PBMC, and that the other blood metabolites measured did not differ significantly between the three NEFA groups.

CONCLUSION

Our results demonstrated that increased circulating NEFA concentrations after calving were associated with altered aspects of many immune system processes in PBMC, but that these were not influenced at the time point examined (14 DIM) by the post-calving diet. In particular, pathways of cell adhesion and diapedesis were inhibited by downregulated expression of many key genes. These responses were more pronounced when the NEFA concentration exceeded 750 µM, but changes were also present in cows with NEFA > 500 µM. This would lead to a decreased body defence capability and so predispose the animals to infections. Our results also supported evidence from other studies which has shown that the condition of cows pre-calving is a critical factor in altering the extent of adipose tissue mobilisation in early lactation, which is increased by calving with a high BCS. The immunosuppression should therefore be reduced by improved dry cow management.

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DECLARATION OF INTEREST

The authors have not stated any conflict of interests.

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Figure Legend

Figure 1. Lactation number, bodyweight and body condition score (BCS) parameters between cows with Low (< 500 µM, n = 6), Medium (500 - 750 µM, n = 8) or High (> 750 µM, n = 10) circulating NEFA concentrations at around 14 DIM. (a) NEFA concentrations; (b) Lactation number; (c) Body weight at drying off; (d) BCS at 14 DIM; (e) BCS at 35 DIM and (f) changes in BCS between day 14 and 35 DIM. a > b > c, $P < 0.05$ - 0.001.

Figure 2. Principal component analysis (PCA) for PBMC transcriptomic gene expression in (A) cows offered High, Medium or Low concentrate diets. This showed substantial overlap in gene expression profiles in the PBMC between the three dietary groups, indicating little effect of the diets on PBMC global gene expression. (B) PCA comparison of cows with Low NEFA (< 500 µM) or High NEFA (> 750 µM). (C) PCA comparison of cows with Low NEFA (< 500 µM) or Medium NEFA (500 – 750 µM). These plots both showed separation of the NEFA groups, so demonstrating the potential strong effect of circulating NEFA concentrations on PBMC global gene expression. Blood samples were taken at around 14 DIM and the RNA-Seq reads were normalised with DESeq.

Figure 3. Gene Ontology enrichment analysis for (A) the DEG by circulating PBMC between the cows with High (>750 µM, n = 10) and Low (<500 µM, n = 6) circulating NEFA concentrations; (B) Sub analysis of the DEG involved in immune system process.

782 **Figure 4**. Gene Ontology enrichment analysis for (A) the DEG by circulating PBMC
783 between the cows with Medium (500-750 µM, n = 10) and Low (<500 µM, n = 6) circulating
784 NEFA concentrations; (B) Sub analysis of the DEGs involved in immune system process.

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Table 1. Summary of numbers of cows from each concentrate diet within each NEFA group

Diet[1]	Low NEFA <500 µM[2]	Medium NEFA 500-750 µM[2]	High NEFA >750 µM[2]	Total
Low concentrate	2	4	3	9
Medium concentrate	4	1	3	8
High concentrate	0	3	4	7
Total	6	8	10	24

[1]Offered post calving. [2]Determined in blood samples collected at around 14 DIM

Table 2. Circulating metabolite concentrations in cows according to lactation diet or circulating NEFA, at around 14 DIM[1]

	Group	n	NEFA (µM)	BHB (mmol/L)	Glucose (mmol/L)	Urea (mmol/L)
Diet	Low	9	736 ± 109	0.49 ± 0.07	3.60 ± 0.07	2.54 ± 0.12^{b}
	Medium	8	667 ± 144	0.68 ± 0.09	3.34 ± 0.10	3.56 ± 0.31^{a}
	High	7	823 ± 85	0.58 ± 0.04	3.44 ± 0.09	3.75 ± 0.26^{a}
NEFA group	<500 µM	6	373 ± 49^{e}	0.65 ± 0.10	3.57 ± 0.11	2.93 ± 0.32
	500-750 µM	8	600 ± 27^{d}	0.47 ± 0.05	3.46 ± 0.10	3.36 ± 0.35
	>750 µM	10	1070 ± 53^{c}	0.62 ± 0.07	3.41 ± 0.08	3.32 ± 0.25

[1]Values are mean ± SE: within columns a > b, $P < 0.01$; c > d > e, $P<0.01$ - 0.001.

Table 3. Differentially expressed genes involved in immune process by circulating PBMC between the cows with circulating NEFA levels > 750 µM (n = 10) and < 500 µM (n = 6).

Gene symbol	Fold change[1]	q(P values)	Gene name
EFNB3	-6.02	0.0361	ephrin-B3
CCL2	-4.08	0.0292	chemokine (C-C motif) ligand 2
IL6	-3.79	0.026	interleukin 6
ISG15	-2.83	0.0238	ISG15 ubiquitin-like modifier
S100A14	-2.55	0.00698	S100 calcium binding protein A14
OAS2	-2.53	0.0348	2'-5'-oligoadenylate synthetase 2
TNF	-2.44	0.0270	tumor necrosis factor
IL1B	-2.20	0.0195	interleukin 1 beta
IL5RA	-2.11	0.0100	interleukin 5 receptor subunit alpha
OAS1Y	-2.02	0.04605	2',5'-oligoadenylate synthetase 1, 40/46kDa
MX2	-1.96	0.0255	MX dynamin-like GTPase 2
CLCF1	-1.80	0.0264	cardiotrophin-like cytokine factor 1
CLEC6A	-1.71	0.03059	C-type lectin domain family 6 member A
NFKBID	-1.71	0.0270	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta
EGR1	-1.53	0.0446	early growth response 1
IFNLR1	-1.33	0.0393	interferon, lambda receptor 1
CYSLTR1	-1.31	0.0302	cysteinyl leukotriene receptor 1
CMTM7	-1.27	0.0302	CKLF-like MARVEL transmembrane domain containing 7
CD40	-1.23	0.0333	CD40 molecule
RARRES2	14.26	0.0483	retinoic acid receptor responder (tazarotene induced) 2
CD36	3.41	0.0158	CD36 molecule (thrombospondin receptor)
BOLA-DQB	3.38	0.0369	major histocompatibility complex, class II, DQ beta
CD28	1.75	0.0238	CD28 molecule
IL7R	1.75	0.014	interleukin 7 receptor
HP	1.68	0.0302	haptoglobin
GZMM	1.58	0.0350	granzyme M
ENPP1	1.58	0.0328	ectonucleotide pyrophosphatase/phosphodiesterase 1
IL2RA	1.57	0.00227	interleukin 2 receptor subunit alpha
CCR6	1.52	0.0239	chemokine (C-C motif) receptor 6
CCR9	1.50	0.0159	chemokine (C-C motif) receptor 9
GATA3	1.50	0.02384	GATA binding protein 3
CD4	1.49	0.0302	CD4 molecule
S100A13	1.48	0.0255	S100 calcium binding protein A13
PDGFB	1.37	0.0430	platelet derived growth factor subunit B
GAS6	1.36	0.0302	growth arrest specific 6
TNFSF14	1.36	0.0316	tumor necrosis factor superfamily member 14
FYB1	1.30	0.0391	FYN binding protein 1

[1]Fold change: >750 µM/<500 µM.

804 **Table 4.** Differentially expressed genes involved in biological adhesion by circulating
805 PBMC, in the cows with circulating NEFA levels between > 750 µM (n = 10) and < 500 µM
806 (n = 6).

Gene symbol	Fold change*	q(P values)	Gene name
DSG2	-16.65	0.0267	desmoglein 2
TNC	-10.77	0.0205	tenascin C
COL12A1	-10.38	0.0208	collagen type XII alpha 1
ADAMTS9	-8.80	0.0344	ADAM metallopeptidase with thrombospondin type 1 motif 9
ICAM5	-8.116	0.0022	intercellular adhesion molecule 5
CYP1B1	-7.97	0.0267	cytochrome P450, family 1, subfamily B, polypeptide 1
CLDN4	-4.87	0.0390	claudin 4
FERMT2	-4.74	0.0445	fermitin family member 2
ANGPT1	-4.71	0.0302	angiopoietin 1
EPHB4	-4.37	0.0302	EPH receptor B4
KIRREL1	-3.86	0.0238	kirre Like Nephrin Family Adhesion Molecule 1
JAM2	-2.74	0.0302	junctional adhesion molecule 2
TNF	-2.44	0.0269	tumor necrosis factor
IL1B	-2.20	0.0194	interleukin 1 beta
DDR1	-1.99	0.030	discoidin domain receptor tyrosine kinase 1
ICAM1	-1.81	0.0302	intercellular adhesion molecule 1
SSPO	-1.70	0.0315	SCO-spondin
PDGFRA	-1.70	0.0434	platelet derived growth factor receptor alpha
ITGAD	-1.46	0.0302	integrin, alpha D
PLEK	-1.44	0.0315	pleckstrin
CD36	3.41	0.0158	CD36 molecule (thrombospondin receptor)
PCDHGA5	2.11	0.0360	Protocadherin Gamma Subfamily A, 5
EPDR1	1.70	0.0302	ependymin related 1
CD4	1.49	0.0302	CD4 molecule
ITGA4	1.37	0.0305	integrin subunit alpha 4
GAS6	1.37	0.0302	growth arrest specific 6

807 [1]Fold change: >750 µM/<500 µM

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Table 5. Differentially expressed genes involved in immune process by circulating PBMC, between the cows with circulating NEFA levels 500-750 µM (n = 8) and < 500 µM (n = 6).

Gene symbol	Fold change[1]	q(P value)	Gene name
TAFA4	-87.75	4.04E-05	TAFA Chemokine Like Family Member 4
MASP2	-1.67	0.0236	mannan binding lectin serine peptidase 2
GPR174	-1.47	0.0225	G protein-coupled receptor 174
IFNLR1	-1.41	0.0225	interferon, lambda receptor 1
CYSLTR1	-1.32	0.0225	cysteinyl leukotriene receptor 1
FNIP1	-1.30	0.0370	UDENN FNIP1/2-type domain-containing protein
ITGA6	-1.30	0.03590	integrin_alpha2 domain-containing protein
AZU1	7.86	0.0190	peptidase S1 domain-containing protein
FCGR1A	3.97	0.0083	Fc Fragment Of IgG Receptor Ia
LTF	2.95	0.0247	lactotransferrin
PTX3	2.40	0.0302	pentraxin-related protein PTX3
HP	2.34	0.0015	haptoglobin
CD36	2.16	0.0391	CD36 molecule (thrombospondin receptor)
IL12B	1.99	0.0150	interleukin-12 subunit beta
GZMM	1.84	0.0131	granzyme M
IL10	1.81	0.0390	cytokine synthesis inhibitory factor
PGLYRP1	1.80	0.0226	peptidoglycan recognition protein 1
TMIGD2	1.80	0.0184	Ig-like domain-containing protein
S100A8	1.72	0.0201	S100 Calcium Binding Protein A8
S100A9	1.65	0.02256	S100 Calcium Binding Protein A9
S100A13	1.62	0.0089	S100 calcium binding protein A13
ENPP2	1.56	0.0253	ectonucleotide pyrophosphatase/phosphodiesterase 2
CD14	1.56	0.0232	monocyte differentiation antigen CD14
S100A12	1.54	0.0236	S100 calcium-binding protein A12
ENPP1	1.52	0.0324	ectonucleotide pyrophosphatase/phosphodiesterase 1
ADA	1.51	0.0201	adenosine deaminase
PDGFB	1.44	0.0236	platelet derived growth factor subunit B
RAB32	1.35	0.0229	RAB32, member RAS oncogene family
TNFSF14	1.31	0.0343	tumor necrosis factor superfamily member 14
TNFSF12	1.31	0.0276	TNF 2 domain-containing protein
KDELR1	1.23	0.0392	ER lumen protein-retaining receptor 1

[1]Fold change: 500-750 µM/<500 µM.