SERUM HEALTH BIOMARKERS SIGNIFICANTLY CORRELATED WITH GENE EXPRESSION IN TRANSITION DAIRY COWS

A. Kudriashova[1,2], A.J. Chamberlain[1,3], J.E. Newton[1], C.R. Bath[1], C.J. Vander Jagt[1], C.M. Reich[1], B.A. Mason[1], J.E. Hemsworth[1] and M.E. Goddard[1,2]

[1] Agriculture Victoria, AgriBio, Centre for AgriBioscience, Bundoora, VIC 3083 Australia
[2] Faculty of Veterinary and Agricultural Sciences, University of Melbourne, VIC 3647 Australia
[3] School of Applied Systems Biology, La Trobe University, Bundoora, VIC, 3083 Australia

SUMMARY

The transition to lactation often results in health issues that impact on longevity of a dairy cow in the herd. Physiological processes involved in energy metabolism and immune response during this period can be measured by blood health biomarkers. These processes are partly genetically driven. In this study, we aim to determine gene expression patterns in circulating leukocytes and investigate associations with serum health biomarkers during the transition period. A single blood sample was collected within 21 days of calving from 110 commercial dairy cows, located on 5 farms in south-eastern Australia. Samples were used for RNA sequencing and serum analysis for glucose, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), urea, albumin, globulin, albumin to globulin ratio (A:G), triglycerides, bilirubin, cholesterol and total protein content. Of the 12470 expressed genes, 2641 were significantly correlated with serum biomarkers. Immune response pathways associated with serum health biomarkers included the chemokine signalling pathway being significantly correlated with total protein and albumin and the NOD-like receptor signalling pathway being significantly correlated with triglycerides. Urea was enriched with the most pathways. We also identified genes previously associated with negative energy balance (NEB) and several genes correlated with multiple biomarkers. This study adds to our understanding of the pathways that are contributing to transition cow health. Future work will identify which of these gene expression changes are under genetic control and the associated variants that can be used in a genomic prediction for transition health.

INTRODUCTION

The transition period from late pregnancy to early lactation for dairy cows coincides with considerable metabolic stress and impacts longevity of a dairy cow in the herd. During this period, the energy requirements of lactating dairy cows cannot be met from feed. This leads to nutrient shortages and NEB when body reserves are mobilised. A prolonged period of NEB is associated with dairy production diseases like mastitis, metritis, retained fetal membranes, abomasum displacement, milk fever and ketosis (LeBlanc *et al*. 2006). The transition period is also often accompanied by overt inflammation response that occurs shortly after parturition and normally is resolved within 3-4 days. However, the immune and inflammatory response is often dysregulated during early lactation. The failure to adapt to metabolic changes and resolve inflammatory reactions may reduce future lactation and reproduction performance (Bradford *et al*. 2015).

Improved animal health and resilience during early lactation could be achieved through genetic selection. Blood is widely used readily accessible multi-organ biofluid. Gold standard blood serum metabolic profile tests include biomarkers associated with energy balance (glucose, BHB and NEFA), immune status (A:G, globulins), protein nutritional status (urea, albumin) (Overton *et al*. 2017), lipid metabolism (triglycerides, cholesterol) and liver function (bilirubin). These biomarker levels are heritable traits and could be used for genomic prediction to improve animal health during the transition period (Luke *et al*. 2019). They also can provide accurate data for both clinical and subclinical health disorders. The mainly positive genetic correlation between the traits suggests that

selective breeding can improve the overall health of dairy cows during the transition period (Pryce *et al*. 2016).

Gene expression in blood leucocytes can help to identify biological processes underlying metabolic changes during the transition period. The gene expression pathways can help to identify candidate genes of biological significance for further genome-wide association studies (Pryce *et al*. 2020). The present study was performed to increase the understanding of metabolic adaptation of the dairy cow during the transition period. The aim was to determine gene expression patterns in circulating leukocytes and investigate their associations with serum health biomarkers during the transition period.

MATERIALS AND METHODS

Blood samples were collected within 21 days after calving from 110 multiparous cows in 5 dairy herds in south-eastern Australia. All farms had pasture-based feeding systems with supplementary forages and concentrates fed during milking time. A single blood sample (approx. 8 mL) was collected from the coccygeal vein. Whole blood (0.5 mL) was immediately subsampled into RNAprotect Animal Blood Tubes (QIAGEN) containing RNA protectant. The remaining sample was incubated for 30-60 min at 22^0 C in the dark to optimise clotting, then centrifuged at 1,500 g for 10 minutes and serum retained. Quantification of serum biomarkers was performed in either Regional Laboratory Services (Benalla, Victoria, Australia) or AgriBio (Melbourne, Victoria, Australia). RNA was isolated using the RNeasy Protect Animal Blood Kit (Qiagen), libraries prepared using Nextflex Rapid Directional RNA-Seq Kit 2.0 (Perkin Elmer) and sequenced in a 150 cycle paired end run on the NovaSeq6000 (Illumina Inc).

All paired reads that passed trimming and quality filtering were aligned to the bovine genome ARS-UCD1.2 merged with Btau5 Y and its associated annotations using STAR v2.5.3 (Dobin *et al*. 2013) 2-pass mapping and default settings. Alignment files (.bam) with greater than 15 million read pairs and greater than 83% mapping rate were used for gene count matrix generation. A gene count matrix was generated using Subread v1.5.1 (http://subread.sourceforge.net/). Gene expression data quality was assessed by generating a multidimensional scaling plot. The gene count matrix was normalised with the Bioconductor software package edgeR in R Studio (Robinson *et al*. 2010).

Statistical analysis was performed using R version 4.2.1 (R Core Team 2022). A fixed effect model was fitted to assess the effect of parity, farm, breed, and days in milk on the gene counts and blood biomarkers.

$$y_{\text{ijklm}} = \mu + P + F + B + DIM + e_{\text{ijklm}},$$

where y is the biomarker concentration (BHB, NEFA, glucose, albumin-globulin ratio, albumin, globulin, total protein, bilirubin, cholesterol, triglycerides, urea), μ is the mean, P is parity (1 to 4 and 5+), F is the effect of farm, B is the effect of breed, DIM is days in milk (from 1 to 21) and e is the random error term. Residuals adjusted for fixed effects of parity, farm, breed and DIM indicated between cow variation in biomarkers and gene expression.

The relationship between serum biomarkers and normalised gene count was investigated by calculating the Pearson correlation between the residuals. As correlation between residuals was not normally distributed, the correlation between the raw gene counts and raw biomarkers were also investigated. Genes with significant correlation with both raw data and the residuals were identified for pathway analysis. Enrichment analyses of biological pathways (KEGG) and gene ontology terms (GO) were conducted using DAVID Bioinformatics resources (https://david.ncifcrf.gov/).

RESULTS AND DISCUSSION

In our study, 2,641 out of 12,470 genes were significantly correlated ($P<0.05$) with serum biomarkers. Ninety-eight of these genes had unknown function. The highest number of genes was significantly correlated with total protein (830), followed by albumin (458), urea (433), BHB (419),

A:G (394), globulin (359), triglycerides (252) and glucose (207). The lowest number of genes were correlated with cholesterol (109), NEFA (79) and bilirubin (74). The number of biomarkers used for all samples was unequal due to the differences in time of blood collection which might affect the power of different tests (Table 1).

Table 1. Descriptive statistics of the datasets used in this study, including number of samples, mean and standard deviation of serum health biomarkers, number of genes significantly correlated with biomarkers and significant KEGG pathways

Serum biomarkers	Number of samples	Mean (SD)	Number of genes	KEGG pathways (FDR)
BHB (mmol/L)	110	0.78 (0.36)	419	
NEFA (mmol/L)	110	0.57 (0.25)	79	
Albumin-Globulin ratio	82	1.12 (0.22)	394	
Globulin (g/L)	82	32.97 (6.11)	359	
Glucose (mmol/L)	34	2.74 (0.46)	207	
Bilirubin (mmol/L)	34	6.52 (3.12)	74	
Cholesterol (mmol/L)	34	2.14	119	
Triglycerides (mmol/L)	34	0.15 (0.08)	252	NOD-like receptor signalling pathway (<0.01)
Albumin (g/L)	82	35.76 (3.07)	458	Chemokine signalling pathway (<0.05)
Total Protein (g/L)	108	67.98 (6.52)	830	Chemokine signalling pathway (<0.01)
Urea (mmol/L)	110	5.12 (1.60)	433	Cell cycle (< 0.01) p53 signalling pathway (< 0.01) Oocyte meiosis (< 0.05) Progesterone-mediated oocyte maturation (<0.5) Cellular senescence (< 0.01) Human T-cell leukemia virus 1 infection (<0.05) Homologous recombination (<0.05)
All genes			2641	Cell cycle (< 0.01) Chemokine signalling pathway (< 0.01) Oocyte meiosis (< 0.01) Progesterone-mediated oocyte maturation (< 0.01) Osteoclast differentiation (< 0.01)

The 2,570 genes that were correlated with biomarkers were included in an enrichment analysis of KEGG pathways and GO terms. Genes correlated with urea were enriched for cell cycle, p53 signalling pathway and cellular senescence (FDR <0.01), and for oocyte meiosis, progesterone-mediated oocyte maturation (FDR <0.05). Pro-inflammatory chemokine signalling pathway was associated with albumin and total protein and inner immune system NOD-like receptor signalling pathway was associated with triglycerides. This association may indicate the interconnection between lipid mobilisation and immune response during the transition period. In addition, 17 genes correlated with NEFA were enriched in metabolic pathways (FDR < 0.05). Moreover, we identified genes that are known to participate in several metabolic pathways and have been previously identified as important candidate genes for NEB (Soares *et al.* 2021). These genes *(AKT2, CPT1A, CPT1B, PPARA, PPARG, PPP1R3B, PPP2R3C)* are involved in insulin resistance pathway, fatty acids metabolism, PPAR signalling pathway, AMPK signalling pathway, adipocytokine signalling

pathway, and glucagon signalling pathway. Several genes in our study were associated with multiple biomarkers. For instance, 52 genes were correlated with both urea and BHB. The correlation between leukocyte gene expression and the levels of serum health biomarkers is not clearly understood and requires further investigation. Presumably, the metabolic changes in transition cow alter the gene expression in leukocytes. In our study, 178 genes negatively correlated with BHB were associated with cell cycle KEGG pathway and RNA binding molecular function which is important in the regulation of gene expression. This is in line with Minuti *et al. 2020* who identified pathways involved in genetic information processes inhibited by BHB.

The results of this study may be limited by the small sample size and unequal number of biomarkers used for all samples.

CONCLUSION

In this study, we examined the correlation between serum health biomarkers and genes expressed in leukocytes during the transition period of dairy cows. The results of this study provide evidence for the hypothesis that serum health biomarkers are significantly correlated with genes expressed in leukocytes during the transition period. This investigation identified 2641 genes significantly correlated with 11 serum health biomarkers. Some genes were correlated with several biomarkers. Significant correlations between genes that have been previously associated with the negative energy balance were found. The findings in this investigation suggest that gene expression analysis can provide a better understanding of physiological processes during NEB. The genes that are correlated with changes in metabolic health were used to identify pathways that may be associated with transition health. Further studies are needed to validate the findings and understand causation and effect of the revealed correlations.

ACKNOWLEDGEMENTS

This study was undertaken as part of the DairyBio program, which is jointly funded by Dairy Australia, the Gardiner Foundation and Agriculture Victoria and was a MAgSc research project at the University of Melbourne.

REFERENCES

Bradford B.J., Yuan K., Farney J.K., Mamedova L.K. and Carpenter AJ. (2015) *J Dairy Sci* **98:** 6631.

Dobin A., Davis C.A., Schlesinger F., Drenkow J., Zaleski C., Jha S., Batut P., Chaisson M. and Gingeras T.R. (2013) *Bioinformatics* **29:** 15.

Robinson M.D., McCarthy D.J. and Smyth G.K. (2010) *Bioinformatics* **26:** 139.

Leblanc S.J., Lissemore K.D., Kelton D.F., Duffield T.F., Leslie K.E. (2006) *J Dairy Sci* **89:** 1267.

Luke T.D.W., Nguyen T.T.T., Rochfort S., Wales W.J., Richardson C.M., Abdelsayed M. and Pryce J.E. (2019) *J Dairy Sci* **102:** 11142.

Minuti A., Jahan N., Lopreiato V., Piccioli-Cappelli F., Bomba L., Capomaccio S., Loor J.J., Ajmone-Marsan P. and Trevisi E. (2020) *Funct integr genomic* **20:** 293.

Overton T.R., Mcart J.A.A. and Nydam D.V. (2017) *J Dairy Sci* **100:** 10398.

Pryce J.E., Nguyen T., Ho P., Luke T.D.W., Rochfort S., Wales B., Moate P., Marett L., Nieuwhof G., Abdelsayed M., Axford M., Shaffer M. and Haile-Mariam M. (2020) *Proc. Assoc. Advmt. Anim. Breed. Genet* **23:** 390.

Pryce J.E., Parker Gaddis K.L., Koeck A., Bastin C., Abdelsayed M., Gengler N., Miglior F., Heringstad B., Egger-Danner C., Stock K.F., Bradley A.J. and Cole J.B. (2016) *J Dairy Sci* **99:** 6855.

Soares RAN, Vargas G, Muniz MMM, Soares MAM, Cánovas A, Schenkel F and Squires EJ. (2021) *J Dairy Sci* **104:** 602.