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EXPLORING BLOOD BIOMARKERS AS POTENTIAL INTERMEDIATE PHENOTYPES FOR TRANSITION COW DISEASES: A FOUNDATION FOR FUTURE MULTI-OMICS INTEGRATION

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SUMMARY

Transition cow health is essential for dairy cows' productivity and longevity. Current breeding methods inadequately address transition period challenges. Blood biomarkers like β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), glucose, and urea assess cows' metabolic status and provide insights into their health. This study examined the association between common blood biomarkers and transition diseases in dairy cows, identifying them as potential intermediate phenotypes. Blood samples from 200 clinically ill transition cows were compared with healthy controls. Serum biomarkers were quantified, and statistical analysis identified statistically significant differences between diseased and control groups. Linear models assessed the impact of diagnosis and other factors on biomarker concentrations. Results indicated that clinically ill cows exhibited patterns of systemic inflammation, with elevated globulins, reduced albumin, and altered lipid and liver markers. Energy metabolism biomarkers (BHB, NEFA, glucose) were affected by metabolic disorders, while inflammatory biomarkers (albumin, albumin-globulin ratio, globulin) were associated with infection-related diseases. These findings suggest that serum biomarkers related to energy metabolism and inflammation are valuable for predicting disease in transition dairy cows.

INTRODUCTION

Given the elevated occurrence of illnesses during the postpartum period, the health of transition cows is crucial for dairy cows' productivity, lifespan, and survival. Current breeding approaches do not adequately address the physiological challenges of the transition period, such as energy deficit, immune suppression, and calcium homeostasis. Genetic improvement of transition cow health could rely on records of cow disease. However, metabolic disorders generally have low heritability and are hard to collect on enough cows to generate accurate EBVs (Pryce *et al.* 2016). Intermediate phenotypes, also known as endophenotypes, might be more heritable and easier to collect than records of disease and better describe the underlying biology than the clinical symptoms of specific disorder or complex phenotypes such as overall health or productivity. Endophenotypes provide a functional link between genetic information and complex traits, potentially enhancing the accuracy of selection in breeding programs. Blood biomarkers such as BHB, NEFA, glucose, and urea are widely used to assess cows' metabolic status and provide accurate measurable insights into metabolic health. Their higher heritability compared to health data accessible from farms and veterinary records makes blood biomarkers more desirable phenotypes (Luke *et al.* 2019). Further, these biomarkers can be used to identify gene variants associated with disease susceptibility and resilience.

The present study was performed to explore associations of common blood biomarkers of health in postpartum dairy cows and to identify these biomarkers as potential intermediate phenotypes of transition cow diseases.

METHODOLOGY

Blood samples were collected from 200 clinically ill dairy cows within 70 days after calving and matched with one or two healthy control animals from the same herd with similar calving dates. The study included 15 commercial dairy farms in Gippsland, Australia, all using grazing-based feeding systems supplemented with forage and concentrates during milking. Blood samples were collected from a coccygeal vessel, incubated at ~20°C for 1.5 hours, and centrifuged at 1,500 x g for 15 minutes; serum was stored at -80°C. Serum biomarkers listed in Table 1 were quantified using a Chemwell 2910 auto-analyser (Awareness Technology, USA) at AgriBio, Melbourne, Australia. Transition cow diseases included ketosis (n =13), milk fever (n = 50), mastitis (n = 51), metritis (n = 18), endometritis (n = 38), retained foetal membrane (n = 34), subclinical hypocalcemia (n = 178), and subclinical hyperketonemia (n = 74). Several animals had multiple diseases. A total of 331 samples were included in the control group.

Statistical analyses were conducted using R version 4.4.2 (2024-10-31). Descriptive statistics (mean, standard deviation, standard error, minimum, and maximum) were calculated for each biomarker in the "Control" and "Diseased" groups (Table 1). Welch's t-test determined statistically significant differences in biomarker levels between the groups. Density plots were generated to assess normal distribution, group overlap, and outliers for each biomarker (Figure 1). BHB distribution was normalised using log-transformation.

Outliers were identified in the Diseased group for urea, magnesium, calcium, and glucose. Since these extreme values reflected true physiological states associated with disease, they were retained in the analysis but underwent proportional winsorization to reduce their influence without compromising data integrity and eliminating their relative order. For each biomarker, values exceeding a predefined upper threshold (upper normal range) were proportionally scaled down. The winsorized value was computed as:

$$X_{win} = T_{upper} + \left(X_{orig} - T_{upper}\right) \times scalefactor$$

where X_{orig} - original biomarker value, T_{upper} upper threshold, scale factor - proportion by which extreme values above the threshold are adjusted, and X_{win}new winsorized biomarker value. A scale factor of 0.3 reduced extreme values to 30% of their deviation beyond the upper threshold.

A fixed effect model was fitted to assess the effect of disease diagnosis, farm identity, breed, parity, weeks after calving, sample date, haemolysis, and treatment.$\text{Biomarker level} = \beta_0 + \beta_1(Control) + \beta_2(Diagnosis) + \cdots + \beta_k(Covariates) + \varepsilon$, where β_I represents the estimated effect size of each predictor and ε represents the residual error.

RESULTS AND DISCUSSION

Figure 1 and Table 1 summarise differences between the "Control" and "Diseased" groups. Mean magnesium, NEFA, total protein, and triglycerides concentrations showed no statistically significant differences. Higher biomarker concentrations in comparison to "Control" were identified for BHB, bilirubin, globulin, urea and glucose, and lower range for albumin-globulin ratio and albumin. Calcium, while showing lower concentrations for sick animals, also had outliers in the upper range.

Overall statistical and visual analyses indicated that clinically ill cows' metabolic profiles follow patterns of systemic inflammation, including elevated globulins, reduced albumin, altered lipid metabolism (cholesterol and NEFA), and liver markers like bilirubin. These patterns suggest that the "Diseased" group exhibits a systemic inflammatory response rather than localised stress. Globulins increase during inflammation as they contain antibodies and inflammatory proteins. Reduced

albumin and albumin-globulin (A/G) ratio occur as the liver prioritises Acute Phase Protein synthesis over albumin. Changes in lipid metabolism during systemic inflammation are characterised by reduced cholesterol concentrations. Elevated bilirubin indicates liver stress or red blood cell breakdown during inflammation. Higher BHB levels suggest an energy imbalance, commonly seen in inflammatory conditions with negative energy balance.

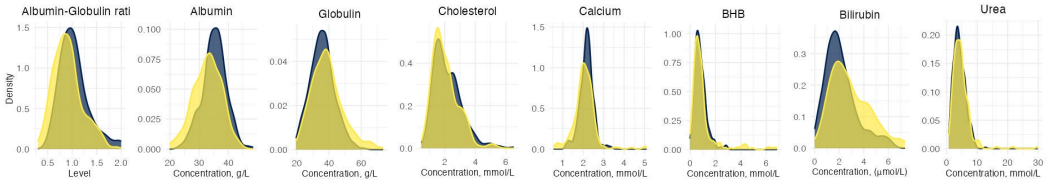

Figure 1. Density distribution histogram of diseased (yellow) and control (blue) groups
Albumin-globulin ratio, albumin, globulin, cholesterol, calcium, BHB, bilirubin, urea (from left to right).

Table 1. Statistical analysis of biomarker differences between control and diseased groups

Biomarker	Diagnosis	Mean	SD	Min	Max	SE	p-value	Normal range
A/G	Control	1.04	0.30	0.50	2.00	0.02	<0.001	0.70 - 1.1
	Sick	0.91	0.29	0.30	1.90	0.02		
Albumin	Control	35.16	4.12	23.00	48.50	0.23	<0.001	25.0 - 38.0 (g/L)
	Sick	32.82	4.77	19.80	43.90	0.33		
BHB	Control	0.82	0.48	0.00	2.88	0.03	<0.05	< 1.2 (mmol/L)
	Sick	1.03	1.14	0.00	6.85	0.08		
Bilirubin	Control	2.14	1.31	0.00	6.33	0.07	<0.001	0.00 - 24.0 (µmol/L)
	Sick	2.88	1.58	0.00	7.35	0.11		
Urea	Control	4.22	1.89	0.70	12.70	0.10	<0.05	2.1 - 10.7 (mmol/L)
	Sick	4.71	3.04	1.10	29.9	0.21		
Calcium	Control	2.16	0.33	1.14	3.37	0.02	<0.05	2.00 - 2.75 (mmol/L)
	Sick	2.07	0.55	0.55	5.10	0.04		
Cholesterol	Control	2.20	0.93	0.48	6.51	0.05	<0.05	1.73 - 7.73 (mmol/L)
	Sick	2.04	0.84	0.65	5.34	0.06		
Globulin	Control	35.80	7.46	19.50	61.50	0.41	<0.001	30.0 - 45.0 (g/L)
	Sick	38.80	9.71	20.80	73.60	0.68		
Glucose	Control	3.25	0.49	1.30	4.50	0.03	<0.001	2.5 - 4.2 (mmol/L)
	Sick	3.72	1.29	1.70	9.90	0.09		
Magnesium	Control	0.99	0.16	0.40	1.62	0.01	0.87	0.74 - 1.44 (mmol/L)
	Sick	0.99	0.29	0.29	2.89	0.02		
NEFA	Control	0.74	0.29	0.16	1.72	0.02	0.46	0.2 - 0.8 (mmol/L)
	Sick	0.76	0.32	0.12	1.59	0.02		
Total protein	Control	70.96	7.23	45.40	93.30	0.40	0.36	60.0 - 85.0 (g/L)
	Sick	71.62	9.07	51.80	102.80	0.64		
Triglycerides	Control	0.20	0.09	0.02	0.68	0.01	0.26	0.00 - 0.5 (mmol/L)
	Sick	0.21	0.08	0.08	0.52	0.01		

Linear models were fitted to assess the effect of disease diagnosis on serum biomarkers, suggesting that these biomarkers serve as indicators of distinct physiological states. Statistically significant associations ($P < 0.05$) are shown in a Table 3. Magnesium was strongly associated with RFM, subclinical hypocalcaemia with low cholesterol concentrations, and hyperketonemia with increased urea concentrations. Milk fever demonstrated a positive association with glucose in

untreated animals while clinical signs persisted. Both subclinical conditions were associated with positive NEFA and bilirubin levels. Overall, the linear model results highlighted that energy metabolism biomarkers (BHB, NEFA, glucose) were strongly influenced by metabolic disorders, while inflammatory biomarkers albumin, albumin-globulin ratio, and globulin, as well as NEFA, were associated with infection-related diseases.

Table 2. Linear model p-values for biomarker-disease associations

Biomarker	Adj r^2	Metritis	Endometritis	Mastitis	Milk fever	Ketosis	RFM	HK	HCa
Albumin	0.39	0.004		0.030					
Urea	0.34							0.012	
Magnesium	0.20	0.022					0.007		
Cholesterol	0.52								0.012
BHB	0.57					<0.001		<0.001	
Globulin	0.40			0.009					
Bilirubin	0.31					0.028		0.001	0.001
NEFA	0.34		0.048					0.012	0.009
Glucose	0.38				<0.001	<0.001		0.020	0.036
Calcium	0.64				<0.001				<0.001
AG	0.42	0.043		0.027					

Cell colours represent the effect's direction: red for positive (increased biomarker concentrations), blue for negative (decreased concentrations), and white for non-statistically-significant effects, with statistically significant effect p-values displayed within each cell. HK denotes subclinical hyperketonemia, HCa subclinical hypocalcaemia, and RFM retained foetal membrane.

CONCLUSION

These results highlight that certain serum biomarkers have the potential to be used as intermediate phenotypes, linking the biological changes in metabolic and inflammatory pathways to clinical disease (ketosis, RFM, and milk fever). Elevated NEFA and BHB, and decreased glucose concentrations, suggest a systemic energy deficit, reinforcing their potential as predictive markers. Decreased A/G ratio and increased globulin concentrations indicate a systemic inflammatory response, suggesting that protein markers can serve as early indicators of infection. Our findings demonstrate that serum biomarkers related to energy metabolism and inflammation can serve as valuable intermediate phenotypes for disease prediction in transition cows

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