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Variation in protein metabolism biomarkers during the transition period and associations with health, colostrum quality, reproduction, and milk production traits in Holstein cows

N. Siachos,12* V. Tsiamadis,[1] G. Oikonomou,[2] N. Panousis,[3] G. Banos,14 I. Sampsonidis,[5] S. Kalogiannis,[5] G. Arsenos,[1] and G. E. Valergakis[1]
[1]Laboratory of Animal Husbandry, Faculty of Veterinary Medicine, School of Health Sciences, Aristotle University of Thessaloniki, Box 393, GR-54124, Greece
[2]Department of Livestock and One Health, Institute of Infection, Veterinary & Ecological Sciences, Faculty of Health and Life Sciences, University of Liverpool, Neston CH64 7TE, UK
[3]Clinic of Farm Animals, Faculty of Veterinary Medicine, School of Health Sciences, Aristotle University of Thessaloniki, GR-54124, Greece
[4]Scotland's Rural College, Edinburgh, Midlothian EH25 9RG, Scotland, UK
[5]Department of Nutritional Sciences and Dietetics, International Hellenic University, Sindos Campus, GR-57400, Thessaloniki, Greece

ABSTRACT

The aims of this study were to assess a) the variation of protein metabolism biomarkers and of factors affecting them during the transition period, b) the association of each biomarker with skeletal muscle reserves and their changes, and c) the association of these biomarkers with postpartum health, colostrum quality, reproduction, and milk production. For this purpose, 238 multiparous Holstein cows from 6 herds were used in a prospective cohort study. Plasma concentrations of 3-methylhistidine (3-MH) and 1-methylhistidine (1-MH) and serum concentrations of total protein (TP), albumin (ALB), urea nitrogen (BUN) and creatinine (SCR) were determined for each cow at −21d; −7d; 7d; 21d and 28d relative to calving. Clinical diseases were recorded during the first 28 d post-calving, and presence of subclinical ketosis (scKET) was investigated at 7d and 21d. Colostrum quality was estimated by Brix refractometry. Reproduction data by 150 d-in-milk (DIM) and milk production records were also available. Linear mixed models including the fixed effects of time point, herd, parity, body condition score (−21d), duration of dry period and post-parturient diseases were fitted to assess the variation in each biomarker's concentration. The association between the biomarkers' concentration during the pre-partum period with the odds for each post-parturient disease and for a combined trait (CD_1–28), defined as the presence of at least one clinical condition during the first 28 d after calving, were assessed with separate binary logistic models for time points −21d and −7d. The relationship of each biomarker's concentration with *longissimus dorsi* thickness (LDT) and the changes in LDT (ΔLDT) was assessed with pairwise correlations. Separate general linear models were used to assess the association of each biomarker with colostrum Brix values and milk production traits. Finally, the associated hazard for 1st artificial insemination (AI) and for pregnancy by 150 DIM (PREG_150DIM) was assessed with Cox proportional hazard models, while odds for pregnancy to the 1st AI (PREG_1stAI) were assessed with binary logistic models. 3-methylhistidine was affected mainly by herd, time points and their interaction. Higher 3-MH was associated with increased odds for metritis and CD_1–28, increased hazard for PREG_150 DIM and with increased milk production. 1-methylhistidine was affected mainly by herd, scKET and occurrence of displaced abomasum. Higher 1-MH was associated with better colostrum quality, increased odds for scKET, increased hazard for 1st AI by 150 DIM and with decreased milk production. Both 3-MH and 1-MH were weakly to moderately negatively correlated with LDT and moderately to strongly negatively correlated to ΔLDT at the corresponding time-periods. Additionally, higher TP was associated with increased odds for metritis and CD_1–28 and increased milk production, while higher ALB was associated with increased odds for scKET and increased milk production. Moreover, higher BUN was associated with decreased odds for scKET, increased odds for PREG_1stAI and increased milk production. Higher SCR was associated with decreased odds for retained fetal membranes, metritis, and CD_1–28. Peri-parturient protein metabolism is significantly associated with postpartum health, colostrum quality, reproduction, and milk production; mechanisms involved require further investigation.
Keywords: amino-acids, dairy cattle, methylhistidine, mobilization, peri-parturient

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*Corresponding author: nektarios.siachos@liverpool.ac.uk

INTRODUCTION

The transition from late gestation to early lactation, usually defined as the period 3 weeks before to 3 or 4 weeks after calving, is characterized by increased nutrient requirements and decreased dry matter intake in most cases, which along with hormonally regulated and genetically driven metabolic adaptations lead to mobilization of body nutrient reserves (Grummer, 1995; Drackley, 1999; Hayirli et al., 2002). Excessive mobilization of fat, protein, glycogen, and minerals is often accompanied by an immune dysfunction, making cows susceptible to both metabolic and infectious diseases postpartum (Sordillo and Raphael, 2013; Bradford and Swartz, 2020).

Dairy cows face negative protein balance around calving, with nadir being reported at about 7 d after calving (Bell et al., 2000). Muscle tissue represents about 60% of the total metabolically active body mass. Therefore, despite its relatively low basal metabolic rate, any changes in composition or size of muscles can influence whole-body metabolism (Davis and Fiorotto, 2005). Recently, it has been shown that about 35 kg of fat-free body mass corresponding to 7.6 kg of body protein were mobilized during the first 4 weeks of lactation, and only one third of these losses were restored by wk 16 (Daniel et al., 2022).

The ultrasonographic assessment of *longissimus dorsi* muscle thickness (**LDT**) and the measurement of plasma or serum creatinine (**SCR**) are both considered reliable non-terminal methods of measuring a cow's skeletal muscle reserves (McCabe and Boerman, 2020; Sadri et al., 2022). The decrease in muscle depth during transition has been described by several researchers with an overall conformity across studies (van der Drift et al., 2012; Megahed et al., 2019; Hatfield et al., 2022). Our group has also described the variation of LDT measurements in multiparous cows from multiple farms and of the factors affecting them (Siachos et al., 2022a). Furthermore, we have assessed the simultaneous association of LDT and backfat thickness measurements with the risk for several post-parturient diseases and with reproductive and milk production traits (Siachos et al., 2022b).

3-methylhistidine (**3-MH**), or τ-methylhistidine (tele-methylhistidine), is a post-translational methylated product of histidine following the breakdown of the myofibrillar proteins actin and myosin (Chibisa et al., 2008). As 3-MH is not reutilized for anabolic purposes, its formation is an irreversible phenomenon. Measuring 3-MH concentration is considered a reliable indicator of skeletal muscle tissue mobilization in cattle (Doepel et al., 2002; van der Drift et al., 2012; Pires et al., 2013). The 3-MH:creatinine ratio is considered to adjust for the total fat-free mass of a cow (Pires et al., 2013). When investigating skeletal muscle protein catabolism, the analytical separation of 3-MH from its isomer 1-methylhistidine (**1-MH**), or π-methylhistidine (pros-methylhistidine), another methylated end product of histidine which results from the breakdown of anserine, is essential. The latter is also not reutilized and is recovered from bovine plasma samples at similar concentrations to those of 3-MH (Houweling et al., 2012).

Despite the growing body of research in this field, the variation of 3-MH in transition dairy cows has not been described adequately; all previous studies were performed in a single farm without examining the affecting factors. Regarding 1-MH, we are not aware of any publication providing information for dairy cows. Moreover, whether these biomarkers are associated with skeletal muscle reserves and postpartum health and productivity of a cow remains unknown. A common approach in practice, has been the measurement of total protein (**TP**), albumin (**ALB**) and urea nitrogen (**BUN**) in blood samples as indicators of proper liver function, circulating labile AA reserves and dietary protein intake (Russell and Roussel, 2007; van Saun, 2023). However, an integrated description of protein metabolism biochemistry during transition period is lacking.

The objective of this study was 3-fold: a) to investigate the variation of biomarkers indicative of protein metabolism (3-MH, 1-MH, TP, ALB, BUN and SCR) in transition Holstein cows raised in commercial herds and any potential affecting factors, b) to assess the association of each biomarker with skeletal muscle reserves and their changes, as estimated by measuring the *longissimus dorsi* muscle thickness with ultrasonography, and c) to assess the association of these biomarkers with postpartum health, colostrum quality, reproduction and milk production.

MATERIALS AND METHODS

The study was conducted in compliance with ethical and institutional guidelines set by the Research Committee of the Aristotle University of Thessaloniki, Greece (approval protocol number 62/15–12–2015). All farmers gave informed consent for their animals to be included in the study.

Farms' characteristics, animals and study design

Details about farm characteristic, animals and study design have been presented in previous publications (Siachos et al., 2022a; 2022b). Briefly, collection of data and samplings were conducted from September 2016

to October 2019. Six commercial dairy farms, designated as A, B, C, D, E and F, in Central Macedonia prefecture, Greece, were selected. Farms had 110 to 360 milking cows with an average milk yield ranging from 9,000 kg to 12,000 kg per cow per lactation. Nutritional management and formulated rations were recorded, and nutrient content was calculated according to the INRA (2007) tables using the **UFL** (forage unit for lactation, net energy) and **PDI** (truly digestible protein in the small intestine) system and provided in Table 1. Housing and dietary management of transition cows at each farm are described in detail in Siachos et al. (2022a) and provided here in Supplemental material S1 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023).

From each farm, several cows representing at least 15% of the milking herd was prospectively included in the study (farm A: n = 32; farm B: n = 39; farm C: n = 20; farm D: n = 41; farm E: n = 51 and farm F: n = 55), resulting in a total of 238 purebred multiparous Holstein cows in different parities (2nd: n = 101; 3rd: n = 72, and ≥ 4th: n = 65). Only clinically healthy cows at selection day (21 d before expected calving day) were included. Farms were visited by the first author 3 times per week, after the morning milking, for data collection, clinical examination, and blood sampling of cows. The study-period took place mainly during winter months in farms A, C and E, and mainly during summer months in farms B, D and F.

BCS estimation and longissimus dorsi muscle thickness measurements

For the purpose of this study, we used the estimations of BCS and live bodyweight (**BW**) at enrollment (21 d before expected calving) from our previous publication (Siachos et al., 2022a). From the same publication (Siachos et al., 2022a), we used the predicted values derived from linear mixed models for the repeated ultrasound LDT measurements (at 6 time points relative to the day of calving (0d): −21d; −7d; 0d; 7d; 21d and 28d) and for changes in LDT between time points (**ΔLDT**) during transition.

Blood sampling

Two blood samples were collected from each cow by coccygeal venipuncture at −21d; −7d; 7d; 21d and 28d relative calving (0d). The first sample was collected into 6 mL sterile glass vacuum tubes with Lithium-Heparin as anticoagulant (BD Vacutainer®; Plymouth, UK) and the second one into 10 mL sterile glass vacuum tubes without anticoagulant (BD Vacutainer®; Plymouth, UK). Samples were placed in a portable cooler

Table 1. Chemical composition of dry-cow (DC), far-off (FO), close-up (CU), and fresh-cow / lactating (FC) diets used in six farms, as calculated according to the recorded ingredient composition using the INRA (2007) feeding tables

Chemical Composition[1]	Farm A			Farm B			Farm C			Farm D			Farm E			Farm F		
	FO	CU	FC	DC	FO	FC	FO	CU	FC	FO	CU	FC	DC	FC	FO	CU	FC
UFL /kg DM	0.65	0.70	0.90	0.79	0.79	0.98	0.79	0.87	0.95	0.73	0.79	1.01	0.72	1.01	0.76	0.82	0.99
NE_L Kcal /kg DM	1.11	1.19	1.53	1.34	1.34	1.66	1.34	1.48	1.62	1.25	1.35	1.71	1.23	1.72	1.29	1.39	1.69
CP % of DM	6.0	11.2	15.2	11.1	11.9	16.8	11.9	14.1	14.6	11.1	12.5	17.4	12.3	15.7	12.1	16.0	17.6
CF % of DM	2.2	2.2	3.2	2.6	2.8	4.6	2.8	3.0	3.9	2.4	2.6	4.9	2.6	4.1	2.5	2.5	5.2
PDIN g/kg DM	33.3	72.8	101.8	71.8	73.3	111.9	73.3	92.5	98.3	66.8	78.9	114.1	72.0	104.1	77.6	106.0	117.2
PDIE g/kg DM	54.7	78.0	94.5	78.1	76.0	100.0	76.0	89.0	95.3	70.8	78.4	108.9	68.5	104.0	79.0	95.7	101.9
NDF % of DM	62.9	56.5	36.8	48.5	48.4	34.4	48.4	41.3	35.2	52.3	43.5	33.9	55.2	32.2	51.0	45.2	32.6
ADF % of DM	36.2	32.6	19.9	27.5	27.5	19.0	27.5	21.7	18.5	29.8	24.6	18.3	33.2	16.8	29.7	26.1	18.1

[1]UFL: Forage unit for lactation, net energy; NEL: Net energy for lactation; CP: crude protein; CF: crude fat; PDIN: truly digestible protein in the small intestine when degradable nitrogen limits microbial growth; PDIE: truly digestible protein in the small intestine when available energy limits microbial growth, the actual metabolizable protein supply; NDF: neutral detergent fiber; ADF: acid detergent fiber.

immediately after collection. Plasma and serum were obtained by centrifugation (3000 $g \times 15$ min) within 2 h of collection and stored into 1.5 mL polyethylene tubes at −40°C pending analysis.

Plasma concentrations of 3-methylhistidine and 1-methylhistidine

Heparinized plasma samples were analyzed for determining simultaneously the concentration of 3-MH and 1-MH, using hydrophilic interaction ultra-high performance liquid chromatography coupled to tandem mass spectrometry (**HPLC-MS/MS**) by a method developed and described by Sampsonidis et al. (2023), allowing for labor- and cost-efficient analyses of large number of samples. A total of 1,101 paired plasma 3-MH and 1-MH measurements were available from HPLC-MS/MS.

Serum biochemistry

Serum samples were analyzed by photometry with Siemens ADVIA 1800 Chemistry System. Specific reagents were used for determining the concentrations of TP (biuret method), ALB (colorimetry – bromocresol green dye binding), BUN (Roch-Ramel enzymatic reaction) and SCR (enzymatic reaction – creatininase). Samples collected on time points 7d and 21d were additionally analyzed for determining (enzymatic reaction – β-hydroxybutyrate dehydrogenase) the concentration of β-hydroxybutyrate (**BHB**). Intra- and inter-assay coefficients of variation were 0.40% and 1.13% for TP, 1.00% and 1.80% for ALB, 1.00% and 2.20% for BUN, 0.50% and 0.90% for SCR, and 0.36% and 1.39% for BHB, respectively. A total of 1,083 serum TP, ALB, BUN and SCR, and 439 BHB measurements were available.

Colostrum quality

A composite colostrum sample of the first milking, after completely milking all 4 quarters within 6 h after calving, was collected from each cow into 50 mL plastic vials and placed in a portable cooler. In none of the farms in this study were the calves allowed to suckle their dam. The immunoglobulin concentration of each sample was indirectly assessed within 2 h from collection, by measuring total solids with a hand-held Brix digital refractometer (PAL-1, ATAGO, Fukaya-shi, Saitama, Japan) on a 0–53% scale. Colostrum samples from Farm C were not collected.

Post-parturient diseases

A thorough clinical examination was performed at each time point by the first author (qualified veterinarian). Disease definition used in this study are provided in Supplemental material S2 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023). Briefly, clinical cases of retained fetal membranes (**RFM**), milk fever (**MF**), metritis (**MET**), mastitis (**MAST**), ketosis (**KET**), left displacement of abomasum (**LDA**) and pneumonia (**PN**) during the first 28 d post-calving were diagnosed and recorded by the first author on visit days (3 times per week), while subclinical ketosis (**scKET,** serum BHB ≥1,200 μmol/L) was investigated at 7d and 21d; cases of clinical diseases between-visit days were either diagnosed by the farms' veterinarians or recorded by farm personnel using pre-defined protocols and confirmed by the first author. Additionally, a combined trait defined as the presence of at least one clinical disease during the first 28 d post-calving (**CD_1–28**) was created. All diseased cows received proper treatment, based on the established farm protocols without any intervention on the regimen by the authors. The recorded prevalence of post-parturient diseases among the cows in our study per each farm is described in Siachos et al. (2022a).

Reproduction data

A voluntary waiting period of 50 d was implemented on all herds. Estrus detection was performed by combination of visual observation and use of leg-mounted accelerometers. No hormonal interventions were used for the first AI on any farm. Initial pregnancy diagnosis was performed by farm veterinarians by ultrasonography or rectal palpation at 32–50 post-AI. Computerized records were retrieved from the farms' herd management software. All AI outcomes and pregnancy status by 150 DIM were available; time to 1st AI by 150 DIM (**1stAI_150DIM**), pregnancy to 1st AI (**PREG_1stAI**) and pregnancy by 150 DIM (**PREG_150DIM**).

Milk production traits

Four of the enrolled farms had daily individual milk records, and 2 of them were recording monthly with the Greek Holstein Association milk recording scheme; the 30d, 100d and 305d mature equivalent milk production (**30ME**; **100ME** and **305ME**, respectively) were available, as described in Siachos et al. (2022b).

Statistical analysis

All analyses were performed with IBM SPSS v.25 (Armonk, NY: IBM Corp.). Statistical significance level was set at $P \leq 0.05$ and tendency for significance was considered at $P \leq 0.10$.

Variation in plasma and serum concentrations of protein metabolism biomarkers during the transition period

Linear mixed models (**LMM**) were used to assess potential predictors of 3-MH, 1-MH, TP, ALB, BUN and SCR, and the ratios 3-MH: SCR and 1-MH: SCR. Explanatory variables assessed as fixed effects included time point relative to calving (5 levels), herd (6 levels), parity (3 levels: 2nd; 3rd and $\geq$ 4th), initial BCS class (BCS at −21d, 3 levels: low <3.00; medium 3.00 – 3.50; high >3.50), presence of each post-parturient disease (each tested disease as a binary variable), dry period duration (**DPD**, continuous variable) and initial BW (BW at −21d, continuous variable), as main effects and all 2-way interaction terms. Time points were used to specify within-subjects repeated observations. An initial screening on the independent variables was performed with univariate analysis. For consistency reasons facilitating comparisons between dependent variables, all explanatory variables yielding statistically significant effects in the univariate analysis with any biomarker, were inserted across all final LMMs as fixed effects. Each cow nested within-herd was considered as random effect. A first-order autoregressive covariance structure with heterogenous variances was selected across all models since it yielded the lowest Akaike's information criterion value. The quartile-quartile and the predicted values vs. residuals plots were visually observed to assess the assumptions of normal data distribution and homogeneity of variances, respectively. Where these assumptions were not met, a transformation of the data was performed with the natural logarithm. Results are presented as back-transformed values. Multicollinearity among the explanatory variables was evaluated by performing a series of diagnostic tests (correlation matrix; variance inflation factor; condition index at the lowest eigenvalue row and variance proportion). At significant F values for categorical variables with more than 2 levels, pairwise comparisons between the estimated marginal means (**EMM**) were performed using Bonferroni's confidence interval adjustment.

Association of protein metabolism biomarkers and longissimus dorsi thickness ultrasound measurements

To determine any linear relationship between each protein metabolism biomarker with LDT measurements Pearson correlation coefficients were calculated between the predicted LDT values on each time point and the predicted ΔLDT values during specific time-periods, as derived from the LMMs described in Siachos et al. (2022a), and the predicted values of each protein metabolism biomarker as derived from the LMMs described in the previous sub-section.

Association of protein metabolism biomarkers with post-parturient diseases

Any post-parturient disease yielding a statistically significant effect on the concentration of at least one biomarker in the LMMs, either as main effect and/or interacting by time points, along with CD_1–28, was considered for further analysis as a binary outcome.

The association of protein metabolism biomarkers during the dry period (time points −21d and −7d) with the odds of post-parturient disease traits was assessed with generalized linear models for a binary logistic response (**GLMB**). Separate models were fitted for each time point and for each disease trait. Models included each biomarker, herd, herd × calving season, parity, DPD, initial BCS class and coexisting diseases.

Association of protein metabolism biomarkers with colostrum quality

The association of protein metabolism biomarkers during the dry period (time points −21d and −7d) with Brix colostrum values was assessed with general linear models (**GLM**), separately for each time point. Models included each biomarker, herd, herd × calving season, parity, DPD and initial BCS class.

Association of protein metabolism biomarkers with reproduction traits

The association of protein metabolism biomarkers during the whole study period with the hazard for 1stAI_150DIM and the hazard for PREG_150DIM was assessed with separate multivariable Cox proportional hazards models for each time point. As described in detail in Siachos et al. (2022b), there were 95 right-censored cases for PREG_150DIM. Models included each biomarker, herd, herd × calving season, parity, DPD, initial BCS class and post-parturient disease

traits (RFM; MET; MAST; LDA; KET; scKET and CD_1–28) and were adjusted for DIM at 1st AI.

The association of protein metabolism biomarkers during the whole study period with the odds for PREG_1stAI was assessed by fitting separate GLMBs for each time point. Models included each biomarker, herd, herd × calving season, parity, DPD, initial BCS class, post-parturient disease traits and DIM at 1st AI.

Association of protein metabolism biomarkers with milk production

The association of protein metabolism biomarkers during the whole study period with 30ME, 100ME and 305ME was assessed with GLMs, separately for each time point. Models included each biomarker, herd, herd × calving season, parity, DPD, initial BCS class and post-parturient disease traits (RFM; MET; MAST; LDA; KET; scKET and CD_1–28).

Final GLMBs and Cox proportional hazard models were built following a backward likelihood ratio elimination method. The saved predicted probabilities of each GLMB were entered as test variables in a receiver operating characteristic (ROC) analysis and the area under the curve (AUC) was calculated to assess the reliability of each model. Final GLMs were built by manually eliminating variables with a $P > 0.10$. To meet the assumption of linearity, values were transformed into natural logarithms. Results are shown as back transformed estimates. Herd, herd × calving season and parity were forced to remain in all models described above where significant associations with any biomarker were detected with any of the dependent variables assessed.

RESULTS

The prevalence of the recorded post-parturient diseases in the cows of our study was 13.9% for RFM, 29% for MET, 0.8% for MF, 5.5% for MAST, 3.4% for LDA, 2.9% for KET, 0.8% for PN and 13.9% for scKET. Twelve cows were involuntarily culled during the postpartum period; data before culling for these cows remained in the analysis. Final data set available for statistical analyses included colostrum Brix measurements for 192 cows, 1stAI_150DIM records for 210 cows, PREG_1stAI and PREG_150DIM records for 204 cows, and milk yield records for 218 cows.

Biomarker concentration variation during the study period is shown in Figure 1, as EMMs (±95% confidence interval) and fixed effects results are provided in Supplemental Table S3 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023). Statistically significant associations of post-parturient diseases with the variation in the concentration of all studied biomarkers are detailed in Supplemental Material S4 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023) and summarized in the results below.

Statistically significant correlations observed between the LMM-derived predicted single LDT and ΔLDT and the predicted values of protein metabolism biomarkers at corresponding time points are presented in Supplemental Table S5 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023).

All statistically significant associations with the risk for post-parturient diseases are summarized in Table 2 as odds ratios and shown in detail in Supplemental Table S6 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023). Regarding SCR, we considered that displaying odds ratios by one-unit (1.0 mg/dL) change in concentration was biologically unrealistic; therefore, odds ratios were re-calculated and are reported in the text and the tables at the 0.1 mg/dL scale.

Statistically significant associations with natural log-transformed Brix % colostrum values are shown in Supplemental Table S7 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023) and in the text below as back-transformed estimates.

All statistically significant associations with reproductive outcomes are summarized in Table 3 and shown in detail in Supplemental Table S8 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023) as hazard ratios and odds ratios.

All statistically significant associations with natural log-transformed milk production outcomes are summarized in Table 4, shown in detail in Supplemental Table S9 (https://data.mendeley.com/drafts/f9w9gwscvt; Siachos, 2023) and described below as back-transformed estimates.

3-methylhistidine

Herd, time point, time point × herd and time point × scKET interactions were identified as statistically significant predictors of 3-MH concentration, on a descending F-value order. Estimated marginal means differed significantly across time points, following an increasing trend from −21d up to 11.99 μM at 7d, and decreasing thereafter to 4.90 μM at 28d (Figure 1A).

Estimated marginal means for 3-MH per farm are depicted in Figure 2A. A significant variation was observed among farms within each time point. Farms B and E were in the upper and lower extreme, respectively. Estimated marginal means for 3-MH in Farms A, C, D, E and F followed a common evolution pattern, they increased at 7d and decreased thereafter until 28d to levels lower than those at −21d. On the other hand, EMMs for Farm B demonstrated a sharp increase dur-

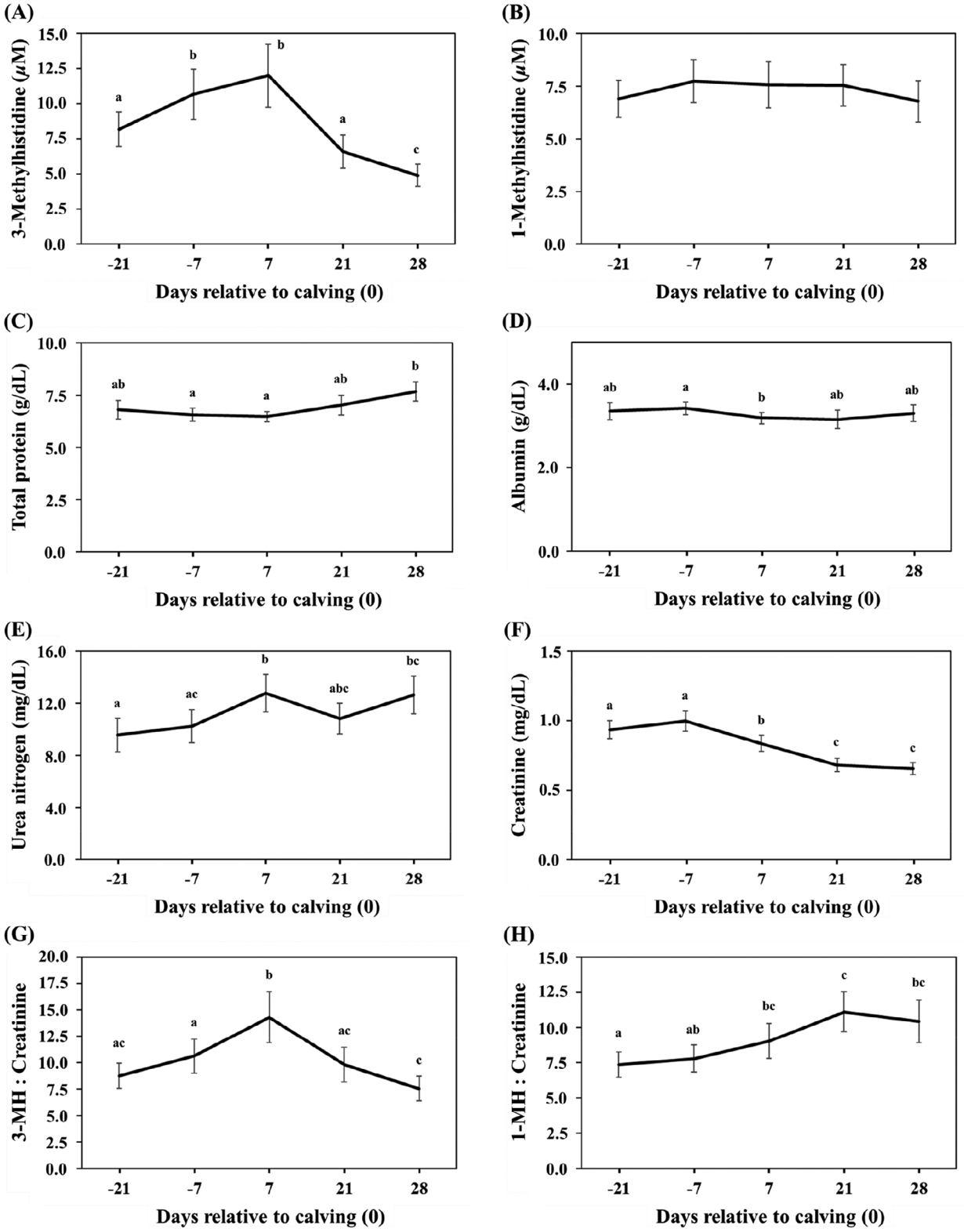

Figure 1. Back transformed (from natural logarithm transformation) estimated marginal means (±95% confidence interval) derived from linear mixed models, showing the variation during the transition period of plasma concentrations of: (A) 3-methylhistidine (3-MH) and (B) 1-methylhistidine (1-MH), the variation of serum concentrations of: (C) total protein, (D) albumin, (E) urea nitrogen, (F) creatinine, and the variation of (G) 3-MH: creatinine ratio, and (H) 1-MH: creatinine ratio, measured in 238 multiparous Holstein cows of 6 commercial dairy farms at 5 time points from 21 d pre-partum to 28 d postpartum. [a-c] Different superscripts denote statistically significant differences at $P < 0.05$.

Table 2. Associations of plasma and serum concentrations of protein metabolism biomarkers during the dry period with the odds of being diagnosed with post-parturient diseases during d 1 to 28 postpartum in 238 multiparous Holstein cows of 6 commercial dairy farms, presented as odds ratios (OR) with 95% confidence interval (CI), adjusted for the effects of herd, herd × calving season, parity, initial BCS and concurrent diseases, derived from generalized linear models for a binary logistic response. Only statistically significant ($P < 0.05$) associations are presented. Odds ratios refer to one-unit change, except for creatinine where they refer to change of 0.1 mg/dL

		RFM[1]	MET[2]	CD_1–28[3]	scKET[4]
Time point	Biomarker	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
−21d	3-MH[5] (μM)				0.70 (0.53–0.91)
	1-MH[6] (μM)				2.05 (1.34–3.13)
	ALB[7] (g/dL)				3.62 (1.19–11.04)
	BUN[8] (mg/dL)				0.79 (0.65–0.96)
−7d	3-MH (μM)		1.13 (1.04–1.23)	1.13 (1.03–1.21)	
	TP[9] (g/dL)		1.97 (1.18–3.27)	1.77 (1.13–2.79)	
	SCR[10] (mg/dL)	0.62 (0.42–0.92)	0.77 (0.59–1.00)	0.76 (0.60–0.95)	

[1]RFM: retained fetal membranes.
[2]MET: metritis
[3]CD_1-28: at least 1 clinical disease during the first 28 days post-partum
[4]scKET: subclinical ketosis
[5]3-MH: plasma 3-methylhistidine
[6]1-MH: plasma 1-methylhistidine
[7]ALB: serum albumin
[8]TP: serum total protein
[9]BUN: blood urea nitrogen
[10]SCR: serum creatinine

Table 3. Associations of plasma and serum concentrations of protein metabolism biomarkers during the transition period with hazard for 1st artificial insemination until 150 d-in-milk (1stAI_150DIM) as hazard ratios (HR), with odds for pregnancy to 1st artificial insemination (PREG_1stAI) as odds ratios (OR), and with hazard for pregnancy by 150 d-in-milk (PREG_150DIM) as HR, adjusted for the effects of herd, herd × calving season, parity, duration of dry period, days-in-milk at 1st artificial insemination, and post-parturient diseases, derived from Cox proportional hazard models and generalized linear models for a binary logistic response in 210 multiparous Holstein cows of 6 commercial dairy farms. Only statistically significant associations ($P < 0.05$) are shown

		1stAI_150DIM	PREG_1stAI	PREG_150DIM
Time point	Biomarker	HR (95% CI[1])	OR (95% CI)	HR (95% CI)
−21d	1-MH[2] (μM)	1.08 (1.01–1.15)		
−7d	3-MH[3] (μM)			1.03 (1.01–1.06)
7d	BUN[4] (mg/dL)		1.22 (1.01–1.25)	
21d	3-MH (μM)			1.03 (1.01–1.04)

[1]CI: confidence interval.
[2]1-MH: plasma 1-methylhistidine.
[3]3-MH: plasma 3-methylhistidine.
[4]BUN: blood urea nitrogen.

ing the pre-partum period, reaching a peak of 22.67 μM at −7d, and decreasing thereafter significantly to 5.86 μM at 28d, at levels comparable to those in the other farms. In Farm E, EMMs ranged from 8.16 μM at 7d to 3.23 μM at 28d. Finally, EMMs for cows diagnosed with scKET were higher compared with healthy ones at 7d ($P = 0.010$) and 21d ($P = 0.019$) (Figure 3A).

Weak to moderate negative correlations were detected between LDT and 3-MH at −21d, −7d, 7d and 28d, with the highest coefficient (in absolute values) being produced between LDT and 3-MH at 7d. Strong negative correlations were detected between ΔLDT [-21d to −7d] and 3-MH at −21d and at −7d and weak to moderate negative correlations between ΔLDT [7d to 21d] and [21d to 28d] and 3-MH at the corresponding time points.

A 1 μM-higher 3-MH concentration at −21d was associated with decreased odds for a cow being diagnosed with scKET by 30.5% (OR = 0.695, 95% CI: 0.65 – 0.96; $P = 0.009$) and with increased 305ME by 168.7 L (95% CI: 15.9 – 316.2, $P = 0.031$). Moreover, a 1 μM-higher 3-MH at −7d was associated with higher odds for a cow being diagnosed with MET by 12.8% (OR = 1.128, 95% CI: 1.04 – 1.23; $P = 0.004$), higher odds for a cow being diagnosed with CD_1–28 by 11.3% (OR = 1.113, 95% CI: 1.03 – 1.21; $P = 0.009$), higher hazard for PREG_150 DIM by 3.4% (HR = 1.034, 95% CI: 1.01 – 1.06; $P = 0.021$) and increased 30ME and 100ME by 3.2 L (95% CI: 1.2 – 5.2, $P = 0.004$) and 15.3 L (95% CI: 6.5 – 26.2, $P = 0.003$), respectively. Finally, a 1 μM-higher 3-MH concentration at 21d was associated with higher hazard for PREG_150 DIM by 2.5% (HR = 1.025, 95% CI: 1.01 – 1.04; $P = 0.010$).

1-methylhistidine

Herd, scKET and LDA were the most important predictors of 1-MH concentration, on a descending F-value order. Estimated marginal means for 1-MH were generally similar across time points (Figure 1B).

Estimated marginal means for 1-MH per farm are depicted in Figure 2B. Farms A, C, D and F had similar 1-MH concentrations during the whole study period, except for −21d. On the contrary, Farms B and E, as with 3-MH, were in the upper and lower extreme, respectively. Cows in Farm E had constantly the lowest values, with EMMs ranging from 4.19 μM to 4.94 μM, while cows in Farm B had the highest, reaching a

Table 4. Natural-logarithm transformed estimates for 30d (30ME), 100d (100ME) and 305d (305ME) mature equivalent milk production, for 218 multiparous Holstein cows of 6 commercial dairy farms, associated with plasma and serum concentrations of protein metabolism biomarkers during the transition period, adjusted for the effects of herd, herd × calving season, parity and post-parturient diseases, derived from general linear models. Only statistically significant ($P < 0.05$) associations are presented

		30ME	100ME	305ME
Time point	Biomarker	(95% CI[1])	(95% CI)	(95% CI)
−21d	3-MH[2] (μM)			0.021 (0.002 – 0.039)
	1-MH[3] (μM)		−0.024 (−0.040 – −0.007)	−0.037 (−0.067 – −0.007)
	ALB[4] (g/dL)			0.116 (0.028 – 0.203)
−7d	1-MH (μM)	−0.016 (−0.031 – −0.002)		
	TP[5] (g/dL)	−0.037 (−0.073 – −0.001)		
7d	3-MH (μM)	0.008 (0.003 – 0.013)	0.007 (0.003 – 0.012)	
	TP (g/dL)	0.073 (0.022 – 0.125)		
	ALB (g/dL)		0.114 (0.039 – 0.190)	0.145 (0.004 – 0.287)
	BUN[6] (mg/dL)			0.015 (0.001 – 0.028)
21d	BUN (mg/dL)	0.011 (0.003 – 0.018)		

[1]CI: confidence interval.
[2]3-MH: plasma 3-methylhistidine.
[3]1-MH: plasma 1-methylhistidine.
[4]ALB: serum albumin
[5]TP: serum total protein
[6]BUN: blood urea nitrogen

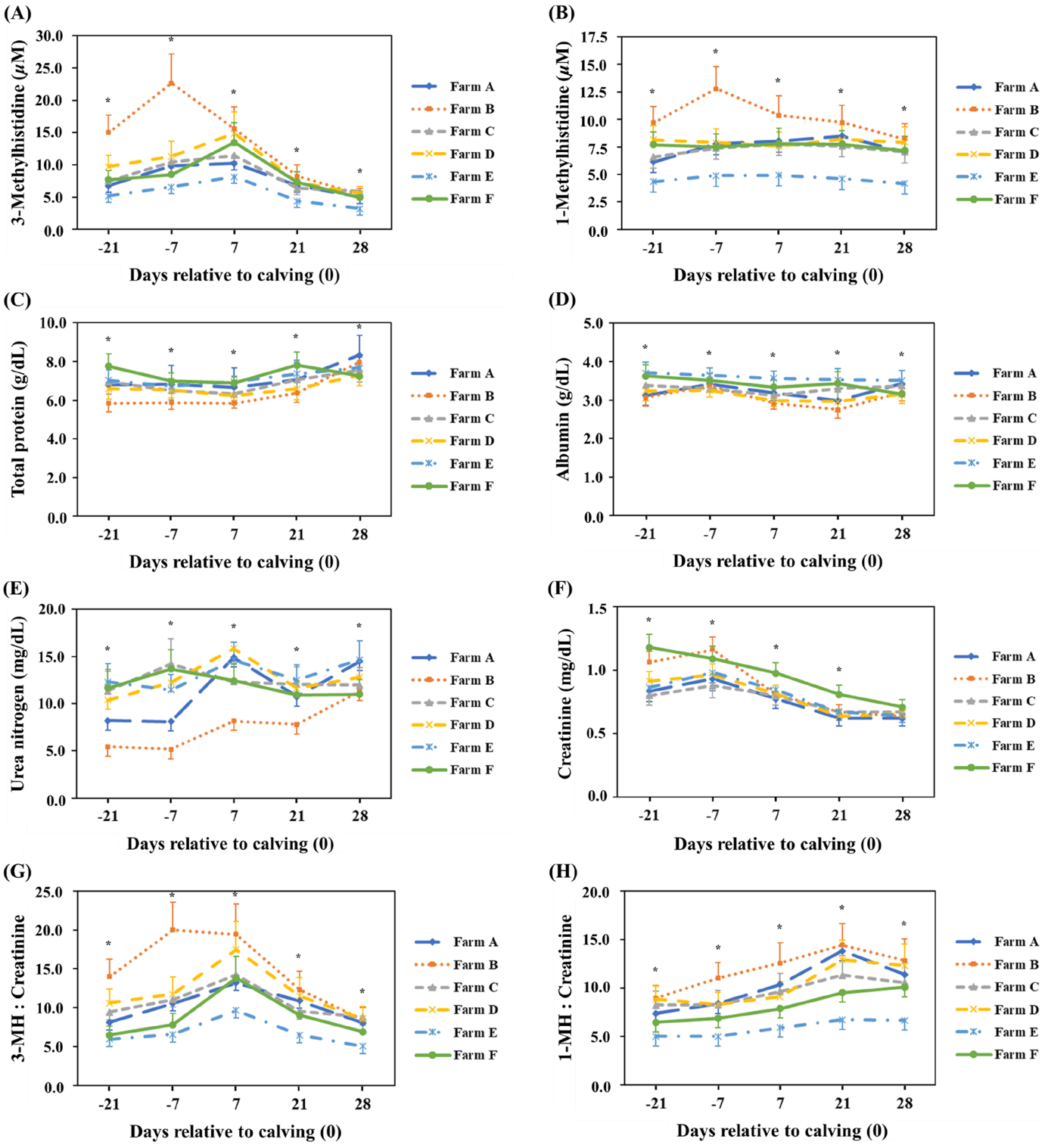

Figure 2. Back transformed (from natural logarithm transformation) estimated marginal means (±95% confidence interval) derived from linear mixed models, showing the variation per farm of plasma concentrations of (A) 3-methylhistidine (3-MH), and (B) 1-methylhistidine (1-MH), and the variation of serum concentrations of: (C) total protein, (D) albumin, (E) urea nitrogen, (F) creatinine, as well as the (G) 3-MH: creatinine and (H) 1-MH: creatinine ratios, measured in 238 multiparous Holstein cows of 6 commercial dairy farms at 5 time points from 21 d pre-partum to 28 d postpartum. * Denotes statistically significant differences between farms within each time point at $P < 0.05$. Pairwise comparisons were made using Bonferroni's confidence interval adjustment.

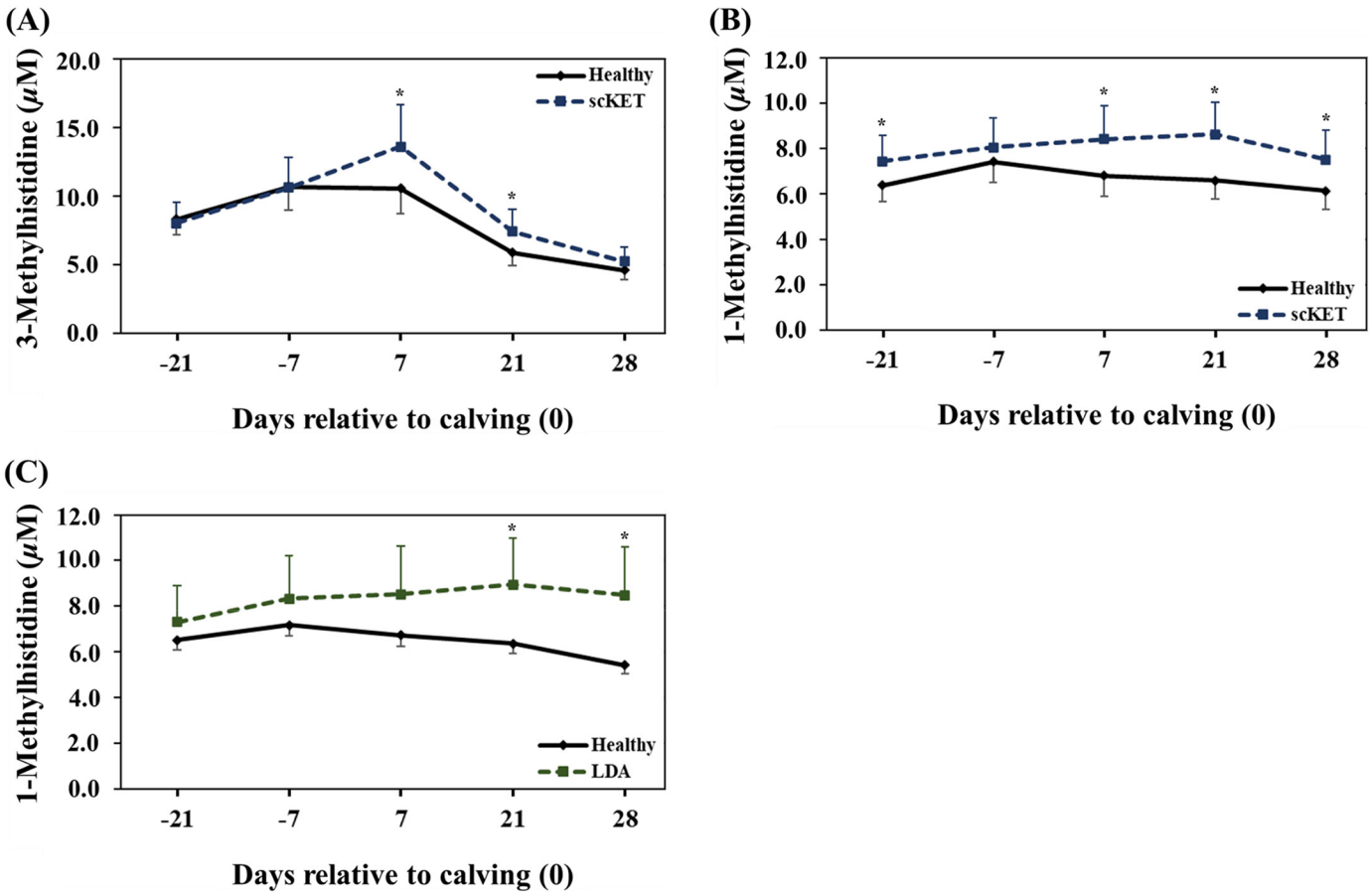

Figure 3. Comparison of plasma (A) 3-methylhistidine and (B) 1-methylhistidine concentrations between cows diagnosed with subclinical ketosis (scKET, dash lines) and healthy (solid lines) ones, and of plasma (C) 1-methylhistidine concentrations between cows diagnosed with left displaced abomasum (LDA, dash lines) and healthy (solid lines) ones, measured in 238 multiparous Holstein cows of 6 commercial dairy farms at 5 time points from 21 d pre-partum to 28 d postpartum. Results are shown as back transformed (from natural logarithm transformation) estimated marginal means (±95% confidence interval) derived from linear mixed models. The recorded prevalence of scKET and LDA in the cows of our study was 13.9% and 3.4%, respectively. *Denotes statistically significant differences within a time point at $P < 0.05$. Pairwise comparisons were made using Bonferroni's confidence interval adjustment.

peak of 12.79 μM at −7d. Estimated marginal means for 1-MH in Farms C, D, E and F followed a common evolution pattern, they remained unaffected by time during the study period, while a statistically significant increase from −21d to −7d was observed only in Farms A and B.

Cows diagnosed with scKET had higher 1-MH at −21d, 7d, 21d and 28d compared with healthy ones, with the biggest difference in EMMs being observed at 21d ($P < 0.001$) (Figure 3B). Moreover, cows diagnosed with LDA had higher 1-MH compared with healthy ones at 21d ($P = 0.008$) and 28d ($P = 0.002$) (Figure 3C).

Weak negative correlations were detected between LDT and 1-MH at −7d, 7d and 28d. A moderate and a strong negative correlation was observed between ΔLDT [-21d to −7d] and 1-MH at −21d and at −7d, respectively, while weak to moderate negative correlations were detected during the other time-periods examined.

A 1 μM-higher 1-MH concentration at −21d was associated with higher odds for a cow being diagnosed with scKET by 2.05 times (OR = 2.051, 95% CI: 1.34 – 3.13; $P < 0.001$), increased Brix colostrum value by 0.25% (95% CI: 0.02 – 0.51, $P = 0.027$) and a higher proportional hazard for 1stAI_150DIM by 8.1% (HR = 1.081, 95% CI: 1.01 – 1.15; $P = 0.020$). Moreover, a 1 μM-higher 1-MH concentration at −7d was associated with decreased 30ME, 100ME and 305ME by 18.0 L (95% CI: 2.2 – 35.2, $P = 0.025$), 86.4 L (95% CI: 25.0 – 145.2, $P = 0.006$) and 299.7 L (95% CI: 55.9 – 550.9, $P = 0.015$), respectively.

Total protein

Herd, time point and time point × herd interaction were the most important predictors of TP concentration, on a descending F-value order. Estimated marginal means for TP increased during the postpartum period from 6.48 g/dL at 7d, up to 7.67 g/dL at 28d ($P < 0.001$) (Figure 1C).

Estimated marginal means for TP per farm are depicted in Figure 2C. A significant variation was observed among farms within each time point. Cows on all farms, except for Farm F, followed the same trend, with gradually increasing values during the postpartum period. Cows in Farm B had constantly the lowest values.

Cows at 2nd parity had lower TP at 7d compared with both 3rd ($P = 0.004$) and ≥ 4th parity ($P < 0.001$). Cows diagnosed with RFM had lower TP compared with healthy ones at 7d ($P < 0.001$).

Total protein was moderately to strongly positively correlated with LDT at −21d, −7d, 7d and 21d, with the highest coefficient being produced at 21d. Strong and moderate positive correlations were detected between ΔLDT [-21d to −7d] and TP at −21d and at −7d, respectively, while weak to moderate negative correlations were detected during the other time-periods examined.

A 1 g/dL-higher TP at −7d was associated with higher odds for a cow being diagnosed with MET and with CD_1–28 by 1.97 times (OR = 1.969, 95% CI: 1.18 – 3.27; $P = 0.010$) and by 1.77 times (OR = 1.774, 95% CI: 1.13 – 2.79; $P = 0.013$), respectively, and with decreased 30ME by 42.2 L (95% CI: 1.1 – 84.7, $P = 0.047$). On the other hand, a 1 g/dL-higher TP at 7d was associated with increased 30ME by 30.0 L (95% CI: 8.8 – 52.8, $P = 0.006$).

Albumin

Herd, RFM and time point × RFM interaction were the most important predictors of ALB concentration, on a descending F-value order. Estimated marginal means for ALB displayed a periparturient decrease from −7d to 7d ($P = 0.048$) (Figure 1D).

Estimated marginal means for ALB per farm are depicted in Figure 2D, showing a significant variation among farms within each time point. On all farms, except for Farm B, there were no differences detected among time points. Cows in Farm B had decreasing ($P < 0.001$) ALB concentrations from −7d to 21d.

Cows at ≥4th parity had lower ALB than cows at 3rd parity at 7d ($P = 0.040$), and lower ALB than both 3rd parity and 2nd parity ones at 28d ($P = 0.008$). Cows diagnosed with RFM had lower ALB compared with healthy ones at 7d ($P < 0.001$), 21d ($P = 0.005$), and 28d ($P = 0.023$). Moreover, cows diagnosed with LDA had lower ALB compared with healthy ones at 28d ($P = 0.001$).

Albumin was moderately to strongly positively correlated with LDT across all time points, with the highest coefficient being produced at 21d. A strong and moderate positive correlation was detected between ΔLDT [-21d to −7d] and ALB at −21d and at −7d, respectively.

A 1 g/dL-higher ALB at −21d was associated with higher odds for a cow being diagnosed with scKET by 3.62 times (OR = 3.622, 95% CI: 1.89 – 11.04; $P = 0.024$) and with increased 305ME by 977.9 L (95% CI: 255.8 – 1,789.5, $P = 0.010$), while a 1 g/dL-higher ALB at 7d was associated with higher hazard for PREG_150 DIM by 2.4 times (HR = 2.406, 95% CI: 1.18 – 4.91; $P = 0.016$) and with increased 100ME and 305ME by 262.4 L (95% CI: 86.4 – 454.8, $P = 0.003$) and 931.1 L (95% CI: 23.9 – 1,938.6, $P = 0.045$), respectively.

Urea nitrogen

Herd, time point × herd interaction and time point were the most important predictors of BUN concentration, on a descending F-value order. Estimated marginal means for BUN were increased significantly during the postpartum period compared with the pre-partum period (Figure 1E), from 9.54 mg/dL at −21d, up to 12.76 mg/dL at 7d ($P = 0.005$).

Estimated marginal means for BUN per herd are depicted in Figure 2E, showing a significant variation among farms within each time point. Cows in Farm B had the lowest values at −21d, −7d, 7d and 21d. Cows in Farms A, D and E displayed a peak in BUN values at 7d, while cows in Farms C and F followed a decreasing trend after reaching a peak at −7d. Cows diagnosed with scKET had decreased BUN compared with healthy ones at −21d ($P = 0.010$).

Weak to moderate positive correlations were observed between BUN and LDT at −21d, −7d, 7d and 21d, with the highest coefficient being produced at −7d. Strong positive correlations were detected between ΔLDT [-21d to −7d] and BUN at −21d and at −7d.

A 1 mg/dL-higher BUN at −21d was associated with decreased odds for a cow being diagnosed with scKET by 21.0% (OR = 0.790, 95% CI: 0.65 – 0.96; $P = 0.015$). On the other hand, a 1 mg/dL-higher BUN concentration at 7d was associated with higher odds for PREG_1stAI by 12.2% (OR = 1.122, 95% CI: 1.01 – 1.25; $P = 0.038$) and with increased 305ME by 90.2 L (95% CI: 6.0 – 169.4, $P = 0.039$), while a 1 mg/dL-higher BUN concentration at 21d was associated

with increased 30ME by 8.1 L (95% CI: 2.2 – 13.4, $P = 0.006$).

Creatinine

Time point, herd and DPD were identified as the most important predictors of SCR concentration, on a descending F-value order. Estimated marginal means for SCR decreased ($P < 0.001$) across time points (Figure 1F), from 1.00 mg/dL at −7d down to 0.66 mg/dL at 28d.

Estimated marginal means for SCR per herd are depicted in Figure 2F, showing a significant variation among farms at −21d, −7d, 7d and 21d. However, the decreasing trend was similar across all farms. Cows in Farm B had the highest periparturient (from −7d to 7d) decrease in SCR. Cows diagnosed with RFM had decreased SCR compared with healthy ones at 7d ($P < 0.001$). Moreover, cows diagnosed with LDA had decreased SCR compared with healthy ones at 28d ($P < 0.001$).

3-Methylhisitidine: SCR ratio increased progressively from −21d to 21d ($P < 0.001$). The same pattern was observed on all farms, with significant differences within time points. Additionally, 1-MH: SCR ratio increased from −21d to 7d ($P < 0.001$) and decreased thereafter to levels similar to those at enrollment day. The same pattern was observed on all farms except for Farm B, where 1-MH: SCR remained at a high plateau from −21d to −7d.

Moderate to strong positive correlations were observed between SCR and LDT across all time points, with the highest coefficient being produced at 7d and 21d. Strong negative correlations were detected between ΔLDT [7d to 21d] and SCR at 7d and at 21d.

A 0.1 mg/dL-higher SCR at −7d was associated with decreased odds for a cow being diagnosed with RFM, MET and CD_1–28 by 37.9% (OR = 0.621, 95% CI: 0.42 – 0.92; $P = 0.019$), 23.3% (OR = 0.767, 95% CI: 0.59 – 1.00; $P = 0.046$) and 24.5% (OR = 0.755, 95% CI: 0.60 – 0.95; $P = 0.017$), respectively.

DISCUSSION

In the present study, we determined the concentration of various biomarkers indicative of protein metabolism in multiparous dairy cows of 6 commercial herds during transition period. We included repeated measurements, covering the period 3 weeks before to 4 weeks after calving, of plasma 3-MH and 1-MH as indicators of myofibrillar protein and anserine degradation, respectively, but also of serum TP, ALB, BUN and SCR. We identified several factors affecting the variation of each biomarker. We found correlations with LDT and LDT changes at the same time points and within corresponding time-periods. Moreover, the association of one-unit increase in the concentration of each biomarker during the pre-partum period with the odds for a cow being diagnosed with RFM, MET, CD_1–28 and scKET and with colostrum quality were defined. Finally, we found associations with several reproductive and milk production traits.

3-methylhistidine

Plasma 3-MH concentrations detected in our study are in accordance with the range of values reported in previous studies (Kokkonen et al., 2005; van der Drift et al., 2012; Pires et al., 2013). Overall, 3-MH concentrations manifested a clear fluctuation during the transition period in contrast to 1-MH. Estimated marginal means for 3-MH were increased one week before and one week after calving, in agreement to the findings of previous studies (Doepel et al., 2002; van der Drift et al., 2012; Pires et al., 2013). The fact that 3-MH decreased significantly from 7d to 21d at values equal to those at −21d, corroborates previous findings showing that muscle mobilization on average is limited within this timeframe (−7d to 21d) in contrast to fat mobilization, which is extended for more than 3 weeks after calving (van der Drift et al., 2012; Megahed et al., 2019; Siachos et al., 2022a).

The highest values were found at 7d in all farms except for Farm B, where 3-MH culminated significantly pre-calving (−7d) and decreased thereafter until 28d to levels similar to those in all farms. Cows in Farm E had the lowest 3-MH values well across the whole study-period. Although we did not include any dietary factors in our analysis, dry cows in Farms A and B were fed diets with the least CP and **PDIE** (truly digestible protein in the small intestine when available energy limits microbial growth, the MP supply) (INRA, 2007) content of all. Dietary protein content during the last 3 weeks before calving was similar, but EMMs for 3-MH differed significantly between these farms. The high 3-MH in Farm B cows is consistent with the significantly lower BUN, probably leading to the low TP and ALB as well, observed particularly in this farm. On the other hand, cows in Farm E had the lowest 3-MH across all time points accompanied by the highest ALB values, indicating that they were able to maintain high reserves of labile AA in their bloodstream with minimal myofibrillar muscle protein turnover throughout the transition period. However, it is noteworthy that measurements were spread during the summer in Farm B and during the winter in Farm A and Farm E. Therefore, a potential influence

of seasonality, either heat or cold stress, on this large difference cannot be overlooked.

Nevertheless, low 3-MH levels on Farm E indicate an achievable target range for transition cows. Cows on this farm mobilized skeletal muscle tissue at a similar rate to cows on all other farms, as indicated by a *ca.* 30% reduction in their LDT from −7d to 21d (Siachos et al., 2022a) and the descending pattern in their SCR values in the present study; this indicates that the decrease in muscle tissue depth and total muscle mass is not proportionate to the degree of myofibrillar protein breakdown. Megahed et al. (2019) proposed that mobilization of intramuscular fatty acids, as estimated by changes in muscle's echogenicity, could contribute to muscle depth loss. The degree of this contribution in dairy cows, if true, has to be investigated.

Cows diagnosed with scKET had higher 3-MH compared with healthy cows at 7d and 21d, time points which define the period where ketone body formation is expected to be more intense. This association appears contradicting the findings of van Der Drift et al. (2012). The latter reported higher 3-MH in cows with lower BHB concentrations (as a relationship between the areas under the curve, after excluding 3 cows with severe hyperketonemia), suggesting restricted ketone body formation in cows with increased muscle protein mobilization. We considered that further examining whether higher 3-MH relates to scKET risk by fitting binary logistic models would be the appropriate approach to answering this question.

Weak negative correlations between 3-MH and single LDT measurements at most time points, indicated a marginally lower degree of muscle protein degradation in cows with higher muscle mass. Changes in LDT from −21d to −7d, a time-period of muscle accretion in most cows (Siachos et al., 2022a), were moderately to strongly negatively correlated with pre-partum 3-MH values, which appears reasonable; the less muscle mass cows mobilized, the lower the 3-MH concentration. During the following time-periods which were characterized mainly by muscle tissue loss, the same negative association, weaker though, was observed, indicating higher 3-MH values with greater LDT losses.

When assessing risk for scKET as a binary outcome, we found that higher 3-MH at −21d was associated with decreased odds for scKET. This is the first-time risk for scKET has been directly related to muscle protein catabolism. In our previous study (Siachos et al., 2022b) where LDT was simultaneously assessed with backfat thickness, fat-related variables were the only factors found to be statistically significantly associated with odds for scKET; a tendency was only observed with the total LDT mobilization. The association found in the present study corroborates the concept of limited ketone body production when AA from muscle mobilization are available as glucogenic precursors (Drackley et al., 2001; Kuhla et al., 2011), although the actual contribution of AA apart from alanine to hepatic gluconeogenesis has been questioned (Reynolds et al., 2003; Larsen and Kristensen, 2013). Our results suggest that, if needed, early (−21d) mobilization of muscle reserves limits formation of ketone bodies, liver capacity to deal with hyperketonaemia is preserved postpartum and scKET is avoided to a certain extend.

Increased 3-MH at −7d was associated with greater risk for MET and CD_1–28. This time point coincided with the initiation of skeletal muscle mobilization in most cows in our study. Moreover, by performing a univariable ANOVA (data not shown), cows classified at the low LDT tercile at −7d (Siachos et al., 2022a) had higher ($P = 0.002$) mean 3-MH (13.8 μM) compared with cows at the intermediate (10.0 μM) and the upper tercile (9.38 μM), respectively. Therefore, it is not unreasonable to support that either low muscle reserves and/or substantial muscle tissue mobilization is detrimental to postpartum health.

Cows with higher 3-MH at −7d and at 21d had a higher proportional hazard for PREG_150DIM. Skeletal muscle depth decreased at these time points. Mitigation of negative energy balance by providing AA for hepatic gluconeogenesis, as dictated by decreased odds for scKET with higher 3-MH at −21d, is a plausible explanation, although further investigation of potential underlying metabolic pathways directly linked to fertility is needed. In our previous study (Siachos et al., 2022b), we found that for each mm higher LDT at each time point hazard for 1stAI_150DIM increased by 3.0 to 5.9%, while no associations were detected with ΔLDT. At first glance, these findings appear opposing. However, as already mentioned, 3-MH at −7d and 21d was only weakly to moderately negatively correlated to LDT, meaning that level of skeletal muscle reserves and rate of myofibrillar protein degradation are not strongly correlated, but should be interpreted jointly. Hence, combining our previous and present findings, it seems that released AA from muscle protein catabolism through transition favor long-term reproductive performance, while maintaining high muscle reserves through transition shortens the calving to 1st AI interval.

3-Methylhistidine was also associated with milk production; higher rates of myofibrillar protein degradation were associated with increased milk production. Released AA from muscle catabolism during early lactation are considered to support milk protein synthesis and milk yield (Blum et al., 1985; Kuhla et al., 2011; McCabe and Boerman, 2020). Muscle tissue AA profile, especially regarding branched-chain AA, differs from that of milk protein and the amount of released

AA is greater than needed just to support milk protein production during a state of negative protein balance (Kuhla et al., 2011; McCabe and Boerman, 2020).

McCabe et al. (2021) found that cows with higher LDT at the enrollment day in their study (−35d), produced more milk during the first 2 mo of lactation compared with cows with lower LDT. On the other hand, when simultaneously assessing muscle and backfat thickness variables, we found that variables related to backfat were principally associated with milk production, with only a minimal positive association of LDT at calving with milk yield by 30 DIM (Siachos et al., 2022b).

1-methylhistidine

Plasma 1-MH results from the catabolism of anserine, a dipeptide consisting of β-alanine and methylhistidine (Houweling et al., 2012). β-alanine is a non-essential and non-proteinogenic AA. Oral β-alanine supplemented in human athletes had favorable ergogenic effects during short-term exercise of high intensity (Varanoske et al., 2018). In our study, average 1-MH values were similar to those of 3-MH, in agreement with Houweling et al. (2012). To the best of our knowledge, to date, there are no studies considering the variation of plasma 1-MH concentrations in transition cows to compare our findings with.

Occurrence of LDA and scKET among the cows in our study were associated with 1-MH variation. The former was associated with higher postpartum 1-MH (21d and 28d), probably reflecting a higher rate of anserine degradation due to the drop in feed intake following the development of LDA and throughout the recovery period as well. Subclinical ketosis was associated with higher 1-MH during both the pre-partum and the entire postpartum period, showing that anserine degradation accompanies negative energy balance during the transition period.

Concentrations of 1-MH were weakly and negatively correlated with LDT measurements at most time points, indicating a marginally lower rate of anserine catabolism in cows with higher muscle mass. Associations between ΔLDT and 1-MH were similar to those observed with 3-MH.

The association between the pre-partum metabolic status of the cow and colostrum quality has not been investigated extensively. Positive associations have been reported between the pre-partum difference between TP and ALB, representing the globulin (**GLOB**) fraction, and colostrum quality, assessed by Brix refractometry (Immler et al., 2021), and between pre-partum ALB and colostrum IgG content (Costa et al., 2021; Rossi et al., 2022). We found that higher 1-MH pre-partum was associated with greater colostrum Brix values. This specific time point is characterized by a state of neutral or even positive protein balance in most cows in our study, as shown by LDT and SCR variation. Transportation of maternal serum immunoglobulins from blood to the mammary gland peaks 3 to one weeks before calving (Weaver et al., 2000). Hence, this finding possibly indicates either higher rates of normal dipeptide-bound AA turnover in cows with higher lean mass or in positive protein balance during that period, that allowed an increased production of immunoglobulins, or an anabolic prioritization of AA toward immunoglobulin synthesis. Neither can be rejected or validated by our results. The vaccination status of the dam was not recorded and could pose a limitation, although farm effects were accounted for. Further investigation with the inclusion of colostrum yield besides quality is needed.

Interestingly, 1-MH was found to be differently associated than 3-MH with risk for scKET; odds increased with higher 1-MH at −21d. This finding is difficult to interpret. β-alanine can be transaminated in the liver into malonate semi-aldehyde, which is further metabolized into acetyl-coenzyme A (Shetewy et al., 2016; Perim et al., 2019), the component initiating the citric acid cycle, the main energy source of cells. Thus, liberation of β-alanine could theoretically spare glucose molecules and/or nonesterified fatty acids during periods of negative energy balance, by providing an alternative source of acetyl-coenzyme A (Allen and Piantoni, 2013). This mechanism if true, failed to restrict ketone body production. On the other hand, this association could reflect the potentially easier mobilization of peptide-bound AA during periods of negative energy balance, compared with those bound in large polypeptide chains. Since anserine, and carnosine, possess several important properties, such as intramyocellular pH buffering capacity, antioxidant activity and metal ion chelation (Boldyrev et al., 2013), these functions could be impaired in cows with hyperketonemia.

Cows with higher 1-MH at −21d had a higher hazard for receiving their 1st AI by 150 DIM. As already mentioned when interpreting the associations of 3-MH with reproductive performance, this opposes the positive association between LDT and hazard for 1st AI we found in our previous study (Siachos et al., 2022b). Interestingly, cows at the low and upper LDT tercile at −21d (Siachos et al., 2022b) had similar mean 1-MH concentrations (6.81 and 6.98 μM, respectively), numerically higher though than those in the intermediate tercile (6.28 μM). As this positive link of high 1-MH at −21d to fertility contradicts the negative association with risk for scKET, we cannot provide further ground explanations.

Finally, 1-MH and 3-MH were contrariwise associated with milk production. Increased milk production was associated with lower anserine degradation rates, showing that liberation of β-alanine serves different purposes compared with other AA. This disparity, along with their contrariwise association with the risk for scKET, indicates that these isomers appear to be products of distinct metabolic pathways. 1-methylhistidine, in contrast to 3-MH, may reflect the mobilization of a more readily available source of non-carbohydrate carbon substrates during periods of negative nutrient balance. Free AA and small peptide-bound AA (i.e., in anserine) are abundant in bovine muscle (Wu et al., 2016), and probably more easily mobilized than those bound in large polypeptide chains (i.e., actin and myosin).

Total protein

In our study, serum TP was affected by time points. Estimated marginal means decreased only numerically from −21d to 7d but increased thereafter until 28d. A similar increase from −3d to 28d has been described by Megahed et al. (2019). A decrease in serum TP of 5 sampled multiparous cows from −7d to 7d has been reported by Piccione et al. (2011), due to a decrease in GLOB.

Differences were observed between the EMMs of each herd at each time point, although the same evolutionary trend was observed across most farms. Moreover, cows at 2nd parity had lower values at 7d than older ones. Since cows at ≥4th parity had lower ALB than younger cows in our study, this difference could be attributed to increasing GLOB with increasing parity.

Moderate to strong positive correlations with LDT and ΔLDT, showed increased TP in cows with either higher LDT accretion or lower LDT loss in the pre-partum period. While weak to moderate negative correlations during the following time-periods show higher TP in cows mobilizing more LDT, probably as a result of released AA contributing to the circulating pool of total protein.

Increased TP at −7d was also associated with greater risk for MET and CD_1–28. As TP consists mainly of ALB and GLOB, it remains unclear which, either or both, of these fractions is responsible for this observation. According to Burke et al. (2010), cows classified as having a low percentage of polymorphonuclear cells in endometrial cytological samples at 42 d after calving had significantly greater plasma ALB concentrations from 14 d pre-partum to 42 d postpartum with the authors′ explanation being inconclusive. Values of TP, ALB and GLOB can increase collectively due to dehydration (Eckersall, 2008).

Albumin

Serum ALB remained rather constant during the transition period in all farms except for Farm B, where it reached a nadir at 21d. A decreasing trend from −3d to 14d followed by an increase thereafter has been described by Megahed et al. (2019). There were differences among herds at each time point, indicating that peri-parturient ALB levels of cows are subject to manipulation and should optimally be maximized. Low ALB has been associated with risk for transition diseases (van Saun and Sniffen, 2014). In this respect, cows at ≥4th parity are at higher disease risk as having lower ALB than younger cows. However, Megahed et al. (2019) found significantly lower ALB in primiparous compared with multiparous cows, with the authors attributing this to the higher metritis incidence in 1st parity cows.

Albumin is a negative acute phase protein, meaning that its liver synthesis is downregulated during infection or inflammation, while GLOB consists of some of the main positive acute phase proteins (Ceciliani et al., 2018). Occurrence of RFM was associated with lower ALB during the whole postpartum period, in accordance with the findings of Seifi et al. (2007) who also found lower ALB in cows with RFM at 7d and 21d postpartum. On the contrary, Moretti et al. (2015) did not find any differences in ALB between affected and non-affected cows by RFM within the first 4 d after calving. This time-related discrepancy could be due to an inflammation establishment in the uterine lumen after that period of the first 4 d after calving, or due to a delayed effect of lower rate in dry matter intake increase after calving, and of dietary protein consequently, in cows with RFM (Dervishi et al., 2016) on the concentration of serum ALB. Albumin has a half-life of 16.5 d in bovine plasma (Cornelius et al., 1962), therefore any alterations in liver synthesis rate require several days to manifest. Finally, the drop in feed intake in cows with LDA is the most plausible explanation of lower ALB at 28d in affected cows.

Albumin was positively correlated with LDT, with the strongest correlation being produced at 21d, signifying the importance of high skeletal muscle reserves postpartum to maintain high blood labile AA reserves. As observed with TP, ALB values at −21d and −7d were strongly positively correlated with ΔLDT [-21d to −7d], showing that the less the depletion or the more the accretion of muscle tissue, the higher the labile AA reserves pre-partum. The similar weak to moderate negative correlations as with TP, were observed with ΔLDT [7d to 21d], showing a potential contribution of AA released from muscle tissue to the blood pool of AA.

Higher ALB at −21d was associated with increased risk for scKET. By looking into our data, mean ALB concentrations among the 3 BCS classes at −21d (low, medium and high) were 3.45, 3.51 and 3.62 g/dL, respectively. By performing a one-way univariable ANOVA (data not shown) this difference was only numerical but was statistically significant for the same comparison at −7d and waned again post-calving. Therefore, the observed higher risk for scKET with higher ALB at −21d, could be explained as a confounding effect of higher BCS at that time and not due to a higher labile AA pool. Further elaboration within each BCS class should be employed but is beyond the scope of this study.

Urea nitrogen

Patton et al. (2014) suggested that higher MUN (same applies for BUN) observed in the first 10 d after calving results from increased mobilization of body reserves of AA, without excluding the possibility of higher RDP intake. The latter is questionable though, as DMI is usually low immediately after calving; it could reflect disproportional levels of ration RDP and net energy. In our study, BUN increased postpartum in agreement with Chibisa et al. (2008) and Megahed et al. (2019). However, 4 out of 6 farms fed pre-partum diets with less than the NASEM (2021) suggested 14.3% CP content and lower BUN values pre-partum may reflect this fact. Regarding the actual metabolizable protein supply though (PDIE), only one farm fed diets below the 6.7% MP requirement (NASEM, 2021) and therefore, AA mobilization pre-partum remains questionable.

The greater the BUN values at −21d and −7d, the less the depletion or the more the accretion of muscle tissue between −21d and −7d. If increased BUN concentrations denote ample protein supply, dietary protein management pre-calving could affect the muscle reserves of a transition cow. Increasing the postpartum dietary MP content and balancing for limiting AA has been shown to decrease 3-MH and muscle mobilization (Carder and Weiss, 2017; Tebbe and Weiss, 2021), while the effects of increasing pre-partum dietary MP content were inconclusive so far (Amirabadi Farahani et al., 2019). On the other hand, the peak at 7d observed in 4 out of 6 farms could be explained by an increased catabolism of mobilized AA the first week after calving, as suggested by Patton et al. (2014) and reported by Daniel et al. (2022). It could also result from low rumen energy availability due to decreased DMI; unfortunately, such data were not available to confirm this hypothesis.

Occurrence of scKET was also a significant predictor of BUN variation, with cows diagnosed with scKET having lower BUN at −21d. This association observed through the LMMs between scKET and BUN, was revealed by the GLMB with risk for scKET as an outcome. Cows with higher BUN at −21d had higher odds for scKET. Interpretation is difficult; for example, higher BUN pre-partum may result from low energy intake and imbalance in energy: protein ratio, low protein utilization efficiency resulting in excess ruminal ammonia, that could lead to lower AA reserves and, consequently lower gluconeogenesis rate postpartum. Alternatively, higher BUN pre-partum may result from increased or adequate DMI, meaning ample energy supply as well, increase in BCS that could lead to low DMI postpartum.

Cows with higher BUN at 7d had higher odds for PREG_1stAI, while higher BUN at 7d and 21d was also associated with higher milk production. These probably reflect the positive effect of increased dry matter intake and subsequently dietary protein intake immediately after calving.

Creatinine

Similarly to our findings, Megahed et al. (2019) reported a 0.24 and 0.30 mg/dL SCR decrease in primiparous and multiparous cows, respectively, within a comparable timeframe. A significant decrease of 0.35 mg/dL between −7d and 7d was observed in Farm B cows; 3-MH and 1-MH values were highest at −7d and still above those of all other farms at 7d, confirming the intense muscle protein mobilization. Farm B cows had the highest SCR at −7d compared with the other farms, but this was not reflected to higher LDT measurements. In fact, the opposite was the case, as these cows had constantly the lowest LDT during the whole study period (Siachos et al., 2022a). Therefore, dehydration or sub-optimal renal function should have contributed to high pre-partum SCR values in Farm B cows. On the other hand, Farm F cows, which had the highest initial SCR and LDT (Siachos et al., 2022a), had an overall decrease of 0.47 mg/dL (from −21d to 28d) and seem to have a different protein mobilization profile, centered on changes of 3-MH concentrations at 7d and not of 1-MH.

Occurrence of RFM and LDA were also associated with lower values of SCR at 7d and 28d, respectively. Disease upregulates muscle autophagy through increased metabolic demands of activated immune system and a reduction in feed intake (Karinch et al., 2001). We consider that muscle atrophy observed during transition diseases should be accounted for when treating, as it may induce weakness, could delay treatment and impair the overall productivity of affected cows.

We also described the variation of 3-MH: SCR and 1-MH: SCR ratios. The evolution of 3-MH: SCR was

influenced by both biomarkers' concentrations; that of 1-MH: SCR was influenced by SCR concentrations, though. The peak in 3-MH: SCR ratio at 7d confirmed that higher rate of muscle protein mobilization occurs the first week after calving. Pires et al. (2013) found a tendency for a BCS effect in 28 multiparous cows, with low-BCS cows having higher 3MH: SCR than medium- and high-BCS ones. In the present study, such an association was not confirmed. At present, the value of ratios' interpretation instead of that of each biomarker remains questionable.

High LDT losses were accompanied by greater decreases in SCR concentrations, confirming that decreases in LDT represent actual loss of skeletal muscle mass. Plasma volume expansion post-calving is considered to contribute minimally to the decrease in SCR values (Megahed et al., 2019), therefore, the utility of SCR monitoring during transition are undeniable as an indicator of muscle mass loss. Higher SCR concentrations at −7d were associated with lower odds for cows being diagnosed with RFM, MET and CD_1–28. This finding, together with constantly moderate to strong positive correlations between SCR concentrations and LDT measurements corroborate our previous findings (Siachos et al., 2022b) regarding the decreased odds for MET per 1 mm-increase of LDT at −7d (OR = 0.93, 95% CI: 0.87 – 1.00). Considering that RFM was also fitted as an explanatory variable in the GLMB to assess risk for MET, we can assume that increased lean body mass before calving assists cows against the occurrence of both RFM and MET. Higher muscle mass pre-partum may facilitate the provision of readily available AA, acting as energy and nitrogen sources, so that the immune system successfully confronts inflammatory challenges in the uterine lumen after calving (Ji and Dann, 2013). The same explanations could apply to the decreased risk for CD_1–28, which includes both infectious and metabolic conditions. The present results add to those of our previous study (Siachos et al., 2022b) and provide strong evidence for the role of ample muscle reserves during the transition period in maintaining postpartum health.

Limitations

The present study has several limitations that should be considered. The study design to enroll each farm consecutively did not allow us to distinguish the effects of farm from potential effects of seasonality on protein biomarkers variation. Recently though, Roths et al. (2023) did not find an effect of heat stress induction in dairy cows in activation of the calpain and ubiquitin-proteasome proteolytic systems, despite a significant increase in plasma urea nitrogen concentration showing significant AA catabolism. Moreover, we could not test for any effects of milk yield potential on protein biomarkers variation due to lack of complete standardized milk production records for the previous lactation or genetic merit indices. An effect of the genomically estimated predicted transmitting ability (**PTA**) for milk production on LDT variation has been shown by Hatfield et al. (2022), with cows classified at the lower PTA tercile having increased LDT compared with those at the intermediate and the upper tercile.

CONCLUSIONS

We found that protein metabolism peri-partum is associated with cow health, fertility, and milk production. A large among-cows variation in the degree of myofibrillar protein degradation was observed with a significant farm effect, implying a potential role of management factors. We highlighted the fluctuation of 3-MH across transition and the contrariwise associations of 3-MH and 1-MH with scKET risk and milk production. For optimal transition, 3-MH and 1-MH levels should be low with minimal variation across time. Moreover, high ALB, BUN, and SCR levels signifying adequate dietary protein intake and ample muscle mass, should be maintained. However, when negative nutrient balance ensues, muscle mobilization and hence higher 3-MH values reduced scKET risk and ameliorated reproductive performance and milk production, confirming the contribution of mobilized AA on maintaining periparturient metabolic homeostasis. Current results suggest that transition cow protein metabolism is more complicated than previously thought and not expressed similarly among cows.

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ORCIDS

N. Siachos https://orcid.org/0000-0001-7670-4950
G. Oikonomou https://orcid.org/0000-0002-4451-4199
N. Panousis https://orcid.org/0000-0002-7584-8191
G. Banos https://orcid.org/0000-0002-7674-858X
G. Arsenos https://orcid.org/0000-0003-3224-5128
G. E. Valergakis https://orcid.org/0000-0002-5219-1166