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Associations between ultrasound hepatic measurements, body measures, and milk production traits in Holstein cows

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ABSTRACT

Ultrasound (US) imaging has been proposed as a noninvasive tool for monitoring liver dysfunction in dairy cows. This study, carried out on 306 clinically healthy Holstein cows in the first 120 d of lactation kept in 2 herds in northern Italy, aimed at investigating the association between US imaging-derived traits, namely predicted liver triacylglycerol content (pTAG, mg/g), liver depth (LD, mm), portal vein depth (PVD, mm) and area (PVA, mm^2), and body size measurements, body condition score (BCS), and milk productivity indicators. Transcutaneous US examination, milk sampling, body size measurements (withers height and heart girth), and BCS were collected once from all cows in 10 sampling batches. The body weights (BW) of a subsample of 73 cows were recorded and used together with an existing data set of BW and measures of Holstein Friesian cows (n = 399) to develop a regression equation to predict BW, which was then used to compute productivity indicators by scaling the milk production traits to predicted BW. Body size measures, BCS, milk traits, and productivity indicators were classified (low, medium, and high) in 0.75 units of standard deviation of the residuals generated from a linear model that included the effects of parity, days in milk, and sampling batch. Liver pTAG, PVA, PVD, and LD were analyzed with a sequence of linear mixed models that included the fixed effects of days in milk and parity and the random effect of sampling batch as common terms, whereas the classes of body and milk traits and the productivity indicators were included one by one. The US-related traits were found to be associated with body size measurements and BCS. Specifically, pTAG was inversely related to BCS, whereas PVD and LD increased with increasing heart girth, BCS, and predicted BW. Generally, no relevant associations were observed between the US parameters and milk production traits, including when expressed in terms of productivity. In conclusion, this study suggests that US measures of liver dimensions of clinically healthy cows are related to their size, whereas pTAG concentrations reflect body condition status, with no particular implications for milk production and productivity. Moreover, healthy cows seemed able to counteract the metabolic stress of the first 120 d of the lactation period without straining liver functionality. Finally, US imaging proved to be a promising technique to assess liver metabolic conditions. However, further studies are needed to confirm its potential as a noninvasive tool for monitoring liver conditions in healthy cows.

Key words: liver imaging, body measures, productivity indicators, dairy cows

INTRODUCTION

Metabolic disorders often affect high-producing cows immediately after parturition and during the early lactation period. The most recurrent metabolic alteration worldwide is hepatic steatosis, defined as an accumulation of fat, primarily triacylglycerol (**TAG**), in the liver (Goff and Horst, 1997). Negative nutrient balance in early-lactating cows often leads to the hydrolysis of TAG in body adipose tissue to counteract the acute energy deficit. Lipolysis induces a distinct increase in circulating nonesterified fatty acids, and if their concentrations in the blood remain elevated for prolonged periods, they may accumulate after re-esterification as TAG in the liver (Yue et al., 2017; Shen et al., 2018), causing liver dysfunction and ketosis (Bobe et al., 2004), and giving rise to a generalized lipid mobilization syndrome known as fatty liver syndrome (Gonzalez and Rosendo, 2013). At the herd level, aside from health issues resulting in high culling rates and elevated

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treatment costs, the occurrence of even mild fatty liver syndrome in early lactation not only may affect milk production in the ongoing lactation period but may also reduce reproductive performance, resulting in considerable economic losses (Carpenter et al., 2016; Carvalho et al., 2019). Although in other studies early-lactating cows with different liver fat content did not evidence differences in daily milk yield (Hammon et al., 2009), the hepatic fat content may have variable implications on cows' performances. Indeed, several risk factors have been recognized as being associated with or predisposing to fatty liver syndrome, such as peripartum disease (e.g., displaced abomasum, toxic mastitis, and retained placenta), age, low or imbalanced dietary intake around parturition, sudden changes in BCS, and inadequate environmental and sanitary conditions (Bobe et al., 2004). Coping with this widespread metabolic alteration implies that, first, the ability to prevent the conditions predisposing to hepatic steatosis from arising and, second, the technical capability for early detection of individuals presenting subclinical signs of fatty liver syndrome are important for reducing the negative effects of such disorders.

The gold standard diagnostic test for hepatic steatosis is histological evaluation of TAG content in the liver (Kalaitzakis et al., 2007). However, percutaneous liver biopsy is an invasive procedure with inevitable risks and complications and, as such, is impractical for on-farm screening (Eisenberg et al., 2003; Starke et al., 2011). Ultrasound (**US**) imaging of the liver has therefore been proposed as an alternative noninvasive diagnostic tool, and in the last decade, quantitative methods for extracting indicative features from transcutaneous US imaging have been developed, including texture analysis (Gao et al., 2014). The use of texture analysis of liver US images to predict TAG (**pTAG**) content in dairy cows has been explored (Bobe et al., 2008; Starke et al., 2010; Weijers et al., 2012). At the same time, other studies have investigated the feasibility of US measurements of liver dimensions and liver-related anatomic structures (e.g., liver depth, portal vein diameter and depth) to detect hepatic lipidosis (Haudum et al., 2011; Starke et al., 2011; Fiore et al., 2018).

The utility of US imaging for identifying clinically manifest liver disorders has been widely discussed (Thijssen et al., 2008; Haudum et al., 2011; Weijers et al., 2012). We recently examined the variations in liver dimensions and liver-related anatomic structures as measured by US and pTAG in clinically healthy dairy cows, and their associations with hematochemical parameters (Giannuzzi et al., 2021), but the implications of their putative associations with body size and condition and milk production and productivity indicators have never been evaluated.

This study aimed to assess the associations between a set of liver-related measures obtained through US imaging and a set of traits related to body size, body condition, and milk production and productivity in a population of clinically healthy Holstein Friesian dairy cows in the first 120 d of lactation.

MATERIALS AND METHODS

Animals and Sampling

The present study involved 306 clinically healthy Holstein Friesian cows (185 primiparous and 121 multiparous) in a range of 1 to 120 d after parturition. The cows were kept in 2 herds located in Piacenza, northern Italy, comprising 279 and 27 cows, respectively, with average daily milk yields of 34.6 ± 8.8 and 34.2 ± 9.1 kg/d. All cows were reared in a freestall housing system, fed total mixed rations and milked twice a day. The ingredients and chemical compositions of the diets are reported in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.19447118.v1; Giannuzzi, 2022). Drinking water was available from automatic water bowls.

The project was approved by the ethical committee of the Catholic University of the Sacred Heart (Piacenza, Italy) and the Italian Ministry of Health (Rome; protocol number 510/2019-PR of 19/07/2019), and all procedures were performed in accordance with the relevant guidelines and regulations. Moreover, the study was carried out following the recommendations of the Animal Research: Reporting of In Vivo Experiments (ARRIVE; https://arriveguidelines.org/arrive-guidelines) guidelines.

To enroll a sample of clinically healthy cows, all the animals of both herds within 120 DIM had undergone an upstream selection based on veterinary inspection and US evaluation carried out by a single operator (veterinarian, highly experienced in liver US imaging). Cows with abscesses or neoplastic masses were excluded. In addition, a complete blood metabolic profile for each cow was evaluated to exclude clinical disease (e.g., ketosis, hypocalcemia, hypomagnesemia, infections), as detailed in Giannuzzi et al. (2021). Around 1% of the animals examined were not enrolled in the study.

Body size measures, BCS, milk samples, and liver US measurements were taken once from all the cows enrolled in the study according to the procedures described herein. Measures and milk sampling were carried out on the same day in the period from September 2019 to February 2020. Because the laboratory could process a maximum of 50 samples of fresh milk per day, sampling sessions occurred on 10 different dates, with an average sample size of 31 ± 16 cows. As dates of sampling sessions were partly confused with herd, a specific herd/date effect (**HD**) was defined to classify the experimental records.

Body Size Measures and BCS

All cows were measured once by the same operator after the morning milking and before feeding for heart girth (**HG**, cm), taken around the cow behind the shoulders, and for height at withers (**HW**, cm), taken from the ground to the highest point of the shoulders, and were scored once for BCS according to Edmonson et al. (1989) on a scale ranging from 1 (very lean) to 5 (very fat) in increments of 0.25.

Milk Sampling, Milk Composition, and Cheese-Making Procedure

Individual milk yields (**MY**) were measured, and proportional milk samples (200 mL) were taken once from each cow during the evening milking. After collection, the milk samples were mixed by gentle inversion and stored at 4°C until analysis. All samples were split into 2 subsamples: one (45 mL) was used for composition analysis, the other (50 mL) for cheese-making. Before transfer to the laboratory of the Breeders Association of the Veneto Region (Padova, Italy) bronopol preservative was added to the subsamples for composition analysis, which was carried out within 24 h of collection. Milk composition traits (fat, protein, casein, and lactose percentages, and urea) were measured with an FT6000 Milkoscan infrared analyzer (Foss Electric A/S).

Cheese yield was assessed using fresh milk according to the 9-MilCA method proposed by Cipolat-Gotet et al. (2016), which has already been described for these samples by Pegolo et al. (2021). Briefly, 9 mL of each milk sample, without preservative, were heated at 35°C for 15 min and then poured into a glass tube. To each tube was added 200 μL of rennet solution (Hansen Standard 215 with 80 ± 5% chymosin and 20 ± 5% pepsin; Pacovis Amrein AG) diluted to 1.2% (wt/vol) with distilled water. After incubation at 35°C for 30 min, the first manual cut was made with a stainless-steel spatula, followed by a curd-cooking phase at 55°C for 30 min, during which a further manual cut was made. At the end of the cooking phase, the curds were left to separate from the whey for 30 min at room temperature. The curds were gently pressed to drain the remaining whey, then weighed on a precision scale. Cheese yield was determined as the weight of the fresh curd as a percentage of the milk processed.

Ultrasonographic Evaluation

Transcutaneous US examination was carried out once on all cows enrolled in the study by a veterinarian highly experienced in liver US imaging, using the equipment and procedures described in detail by Banzato et al. (2016) in a study aimed at estimating the degree of fatty infiltration of the liver in Holstein Friesian cows via US, and validating this methodology with transcutaneous biopsies and complete histological examination. Briefly, images of the hepatic parenchyma were captured using a Mylab OneVET portable US scanner (Esaote SpA) with a linear probe (SV3L11 Animal Science Probe; Esaote SpA). Liver US examinations were performed on the right side of the animal in a standing position. The selected skin area was degreased with 90% alcohol, cleaned with water, and smeared with US gel to improve images acquisition. The acoustic window for sound wave penetration was the intercostal space between the 10th and 11th ribs, and the probe was moved dorsal-ventrally to an amplitude of about 15 cm within this space. The US settings, kept constant for visualization of the hepatic parenchyma of all the animals enrolled in the study, were as follows: frequency 2.8 MHz, gain 90% with time gain compensation in a neutral position, and depth 21 cm. Multiple US images were stored in the Digital Imaging and Communications in Medicine (DICOM) format, without compression, for further analysis. The final US image for each animal was selected by a single operator based on its diagnostic capacity.

Liver depth (**LD**, mm), portal vein area (**PVA**, mm^2), and portal vein depth (**PVD**, mm) were measured using MyLab Desk software (Esaote SpA), as reported by Fiore et al. (2018). The hepatic parenchyma was analyzed using MaZda v. 4.6 texture analysis software (Technical University of Lodz, Institute of Electronics), and liver TAG content (mg/g) was predicted using the regression equation proposed by Banzato et al. (2016), which showed a sensitivity ranging from 86.2 to 92.3% and a specificity ranging from 84.2 and 88.6% depending on the degree of TAG hepatic content.

Definition of Explanatory Variables, Statistical Analysis, and Association Studies

Body Weight Prediction. To develop a robust equation aimed to predict cows' BW, different sources of information were used. First, a subsample of 73 cows reared in 1 of the 2 herds enrolled in the study (23 primiparous and 50 multiparous, of all lactation stages) were measured for HG and HW, scored for BCS, and weighed with an electronic scale in one day after the morning milking and before feeding. These data were then merged with those from an already existing database, which originated in studies carried out by the same research group, in which a set of BW, body size, and BCS measures were assessed in 400 Holstein Friesian cows kept in several herds. After a preliminary cleaning step aimed at removing the outliers (1 cow), we obtained a final data set of 472 cows. Cows were grouped into 3 parity (**PAR**) classes (parities 1, 2, and ≥3, with 110, 130, and 232 cows per class, respectively) and 4 DIM classes of 100 d each, from ≤100 to >300 d, with numbers of cows per class ranging from 92 to 143. These categorical data were then coded as dummy variables (0 or 1). The data set was split into 2 subsets: two-thirds (n = 311) were used to develop a calibration equation for predicting BW, and one-third (n = 161) were used for validation of the prediction equation. Pearson correlations were computed to assess multicollinearity among traits treated as predictors in subsequent analysis. A multiple regression model was applied to calibration data set using the PROC REG function of SAS (version 9.4; SAS Institute Inc.) with a stepwise procedure including the following independent variables: HG, HW, and BCS as continuous variables, and DIM and PAR as dummy variables. The best prediction equation was as follows:

$$BW = -682.53 + 33.59 \times PAR_2 + 40.96 \times PAR_3 + 18.70 \times DIM_2 + 12.81 \times DIM_3 + 8.32 \times DIM_4 + 58.78 \times BCS + 5.31 \times HG,$$

where PAR_2 and PAR_3 referred to cows in the second and third parities, respectively, and DIM_2, DIM_3, and DIM_4 refer to cows of 101 to 200, 201 to 300, and >300 DIM, respectively. Height at withers was not retained in the model. As variance inflation factors were <2 for all the parameters retained in the equation, we can assume that traits treated as predictors did not evidence appreciable multicollinearity (Johnston et al., 2018). Coefficient of determination of the prediction equation was equal to 0.80, with a residual standard error of 44.3 kg.

To test its performance, the equation was used to calculate predicted values for the validation data set and to obtain residuals for evaluation. Regressing the residuals created by the equation showed uniform residual patterns, indicating no bias. The coefficient of correlation between observed and predicted BW was 0.88, with an average bias (BW predicted − BW observed) of 8.1 ± 42.9 kg, which is around 1.3% of the mean of measured BW. Finally, the equation was applied to predict the BW of the 306 Holstein Friesian cows involved in the study.

Production and Productivity Indicators. The energy content of milk (NEmilk) was computed according to the following equation (NRC, 2001):

$$\text{NEmilk (MJ/kg)} = 0.3887 \times \text{fat} + 0.2289 \times \text{protein} + 0.1653 \times \text{lactose},$$

where fat, protein, and lactose indicate the percentages of fat, protein, and lactose in the milk, respectively.

Daily yields of fat, protein, energy in milk, and curd were computed for each cow by multiplying the daily MY by the corresponding trait, previously obtained from milk analyses and processing. Five productivity indicators were then computed as the ratios between the amount of milk, fat, protein, milk energy, and curd yielded daily, and predicted BW.

Statistical Analysis. Before inferring the association between ultrasound hepatic measurements (i.e., LD, PVA, PVD, and pTAG) and body measures (i.e., HW, HG, BCS, predicted BW), milk production traits (i.e., MY, fat and protein contents, daily yields of fat, protein, energy in the milk, and curd), and productivity indicators (i.e., daily yields of milk, fat, protein, energy in the milk, and curd, scaled by predicted BW), a preliminary exploratory data analysis was performed to check outliers and assumptions required for model fitting and hypothesis testing. After this, to conduct association analyses, we adopted a conservative approach in which we did not assume any linear relationship between the response and independent variables. To correctly interpret the variations within all independent variables, the latter were discretized according to residuals obtained by a mixed model implemented in the SAS MIXED procedure (SAS Institute Inc., version 9.4), which corrected the data for DIM, parity order, and HD of sampling. The rationale underlying to this approach is attributable to the fact that such individual sources of variations could influence the explanatory variables, their discretization in classes, and consequently also the association with the response variables (i.e., ultrasounds traits).

The model was as follows:

$$y_{ijkl} = \mu + DIM_i + Parity_j + HD_k + e_{ijkl},$$

where y_{ijkl} is the observed trait; μ is the overall mean; DIM_i is the fixed effect of the ith class of days in milk (i = 4 classes; class 1: ≤30 d, n = 47; class 2: 31–60 d, n = 81; class 3: 61–90, n = 85; class 4: 90–120, n = 93); $parity_j$ is the fixed effect of the jth parity (j = 3 classes;

Table 1. Descriptive statistics of liver ultrasound (US) measurements, body size measurements and BCS, milk production traits, and productivity indicators

Trait	N	Mean	SD	Minimum	Maximum
Response variable					
Liver US trait					
Liver depth, mm	289	149.06	13.28	106.80	179.40
Portal vein depth, mm	302	130.46	13.36	90.40	180.80
Portal vein area, mm^2	295	1,111.48	283.43	435.04	1,956.47
Predicted triacylglycerol, mg/g	302	69.58	10.74	36.14	106.54
Independent variable					
Body measurement					
Heart girth, cm	290	206	8.9	181	230
Height at withers, cm	290	142	4.4	131	155
BCS[1]	290	3.09	0.23	2.25	4.00
Predicted BW (pBW), kg	290	611	62.7	459	769
Milk production trait					
Milk yield (MY), kg	289	37.6	8.3	18.8	62.2
Fat, %	294	3.56	0.71	0.90	6.43
Protein, %	294	3.22	0.40	2.26	8.33
Curd yield, %	292	20.30	3.02	0.70	33.74
Milk energy (NEmilk[2]), MJ/kg	290	2.94	0.32	1.84	5.05
Fat yield, kg/d	288	1.32	0.33	0.29	2.26
Protein yield, kg/d	288	1.20	0.26	0.47	2.03
NEmilk yield, MJ/d	284	109.6	22.8	50.9	179.5
Daily curd yield (CY), kg/d	281	7.59	1.85	0.24	15.65
Productivity indicator					
MY/pBW, g/kg/d	284	61.66	12.08	30.69	100.84
Fat yield/pBW, g/kg/d	284	2.16	0.50	0.47	3.67
Protein yield/pBW, g/kg/d	284	1.97	0.36	0.92	2.99
NEmilk yield/pBW, kJ/kg/d	284	179.4	33.4	84.2	264.2
CY/pBW, g/kg/d	281	12.42	2.74	0.36	24.73

[1]BCS was scored on a scale from 1 to 5 with increments of 0.25.

[2]NEmilk = energy content of milk.

class 1: first parity order, n = 185; class 2: second parity order, n = 69; class 3: ≥third parity order, n = 52); HD_k is the random effect of the kth herd/date (k = 10); and e_{ijkl} is the random residual. Herd/date and residuals were assumed to be normally distributed with a mean of zero and variances of σ_h^2 and σ_e^2, respectively. Residuals from this model were used to classify the explanatory variables as low, high, and medium for residuals of <−0.75, >0.75, and between −0.75 and 0.75 standard deviation units, respectively.

To investigate the association between PVA, PVD, LD, and pTAG with explanatory variables (EXPL_var), liver US measurements were treated as dependent variables and analyzed using the following linear mixed model, where EXPL_var previously described and listed in Table 1 were included one at time to avoid potential multicollinearity problems:

$$y_{ijklm} = \mu + DIM_i + Parity_j + EXPL\_var_k + HD_l + e_{ijklm},$$

where y_{ijklm} is the observed trait (i.e., liver US measurements); μ is the overall mean; DIM_i is the fixed effect of the ith class of days in milk ($i = 4$); $Parity_j$ is the fixed effect of the jth parity ($j = 3$); $EXPL\_var_k$ is the fixed effect of the kth class of the explanatory variable classified on the basis of residuals, as previously described; HD_l is the random effect of the lth herd/date ($l = 10$); and e_{ijklm} is the random residual. Herd/date and residuals were assumed to be normally distributed with a mean of zero and variances of σ_h^2 and σ_e^2, respectively. A given effect was declared significant at $P < 0.05$. Polynomial contrasts ($P < 0.05$) were estimated to describe the pattern of variation in liver US measurements using the variation of EXPL_var, considering the first- and second-order comparisons to measure linear and quadratic relationships, respectively. Only significant results are displayed in the figures.

RESULTS AND DISCUSSION

Cows in early lactation are prone to experience severe negative energy balance, which can lead to excessive mobilization of adipose tissue. This increases their risk of developing various metabolic disorders (Grummer, 1993; Roche et al., 2009), resulting in reduced productivity and fertility, with considerable economic losses for the farm (Fourichon et al., 1999; Overton et al., 2017). Tools that are able to monitor liver health at the herd level and detect metabolic alterations would therefore be useful to the dairy industry by decreasing negative effects of such disorders. Ultrasound imaging of the liver has yielded promising results in detecting liver alterations, but standard values for large samples of healthy individuals are still lacking (Starke et al., 2011; Gao et al., 2014; Fiore et al., 2018). Our study therefore aimed to investigate the associations between hepatic traits obtained from liver US imaging, and a set of body and production traits and their combination (productivity indicators), to establish baseline data on cows in healthy physiological condition.

Descriptive Statistics and Characterization of the Sample

Descriptive statistics for the traits investigated are reported in Table 1. On average, LD and PVD were 149 and 130 mm, respectively, with a coefficient of variation (CV) lower than or close to 10%, whereas PVA and pTAG contents approached 1,111 mm^2 and 70 mg/g, respectively, with greater variation (CV of 25% and 15%, respectively). The liver measures, expressing LD, PVD, and PVA, can provide an indication of the onset of hepatic lipidosis and liver dysfunction (Fiore et al., 2018). In dairy cows with severe hepatic lipidosis, a significant increase in liver fat content has been associated with an increase in the size of the organ and its thickness over the portal vein (Haudum et al., 2011). Furthermore, PVD and PVA measures showed positive patterns of association with nonesterified fatty acids and BHB both in cows with hepatic lipidosis and in clinically healthy cows (Fiore et al., 2018; Giannuzzi et al., 2021). In addition, assessment of portal hypertension is crucial to evaluating the severity of liver disease, one of the determining signs of which is a widening of the portal vein (Partington and Biller, 1995; Lessa et al., 2010). Although the hepatic measurements fluctuated greatly during the first 50 d postpartum due to the intensive nutrient requirements of high-yielding cows (Tharwat et al., 2012), the average LD, PVD, and PVA measurements in our population are indicative of a healthy group of animals, according to previous studies (Haudum et al., 2011; Starke et al., 2011; Fiore et al., 2018).

We predicted liver TAG content using the texture analysis of US images developed by Banzato et al. (2016), which was highly accurate (area under the curve >0.95) in discriminating the various classes of fatty liver compared with the gold standard hepatic biopsy. The majority of the cows in our population had a liver pTAG content ranging between 50 and 100 mg/g, with an average of 70 mg/g. Up to 65% of dairy cows in a herd are reported to be affected by moderate (50–100 mg/g) or severe (>100 mg/g) fatty liver during the early lactation (Jorritsma et al., 2001; Raoofi et al., 2001; Bobe et al., 2004). It is difficult to identify and manage individuals with moderate fatty liver because often no reduction in their milk production or feed intake occurs, but they nonetheless have an increased risk of progressing to severe fatty liver (Gerloff et al., 1986; Veenhuizen et al., 1991). In any case, individual cows may differ in their response to the same TAG concentration and in their ability to adapt to metabolic stress and negative nutrient status in early lactation (Ingvartsen et al., 2003; Hammon et al., 2009; van Dorland et al., 2009). Appropriate feeding management could prevent progression to overt disease (Ingvartsen et al., 2003). Other warning signs are frequently concurrent with moderate fatty liver infiltration as the animal's health deteriorates, such as a rapid reduction in BCS, low glucose, and high nonesterified fatty acid and BHB blood concentrations (Starke et al., 2010).

Three animals in our sample (within 4 DIM) exhibited pTAG contents close to 100 mg/g, but, as no other alterations in clinical signs and hematochemical parameters were found (Giannuzzi et al., 2021), their data were retained. On the whole, the observed liver US parameters along with the clinical examinations reflected the healthy condition of the sampled cows and suggests that the husbandry and feeding practices adopted by the farms enabled the cows to maintain metabolic homeostasis during the transition period and early lactation.

Body measures (HG and HW) showed CV lower than 5%, whereas BCS (average 3.09 points) and predicted BW (average 611 kg) showed CV ranging from 7% to 10%. The cows' average HG in the current study was comparable with measures reported by Gallo et al. (2001) and Saha et al. (2018).

On average, the cows yielded 37.6 kg/d of milk, with 3.56% fat and 3.22% protein, and exhibited a huge variation in MY and fat content (CV $\geq$ 20%) and a medium variation in protein content (CV = 12%), while average milk energy content approached 2.94 MJ/kg. On the basis of daily MY and milk nutrient contents, the daily yields of fat, protein, and curd were calculated as 1.32, 1.20, and 7.6 kg/d, respectively, with CV always greater than 20%.

The cows in this study belonged to 2 farms in northern Italy, operating according to the regulations governing the production of Grana Padano protected denomination of origin (PDO) hard cheese, a major dairy production system in Italy. The average production of the cows and herds sampled was much greater than the officially recorded level for Holstein cows in

Italy (around 31 kg/d; AIA, 2020), even though the cows in our sample were in the first 120 d of lactation (average DIM: 68 ± 32 d). The higher production level and the early stage of lactation may also help explain the lower milk fat and protein contents compared with the average national values for Holstein cows (3.80% for fat and 3.33% for protein content; AIA, 2020).

The average BCS values were comparable to those reported by Gallo et al. (2001) for Italian Holstein cows in early lactation, and those reported by Cozzi et al. (2005) from a sample of multiparous Holstein cows within 90 d of lactation. Fiore et al. (2015) also found a mean BCS for healthy Holstein cows within 60 d postpartum close to that found in the present study. This confirms that our sample comprised clinically healthy cows and suggests that the management and nutritional regimens of the herds were good enough to support both the production level and the body condition of the cows.

In terms of productivity indicators, cows produced on average 62 g/d of milk, 2.16 g/d of fat, 1.97 g/d of protein, 179 kJ/d of milk energy, and 12.4 g/d of curd per kilogram of predicted BW, with CV close to or greater than 20%. The milk production traits of individual cows express the animal's absolute production levels, whereas milk productivity indicators, scaling production traits to the cows' BW, are more related to the intensity of production. Therefore, in this study we decided to also relate productivity indicators to US liver measurements, to obtain a more complete picture of the dynamics of liver traits as the level of production increases, and possibly also the metabolic load that high-yielding dairy cows experience in the first 120 d of lactation.

Associations Between Liver US Measurements and Body Traits

Results from the mixed model for liver US-related traits, as affected by body size measures, BCS, and milk production and productivity traits, are shown in Table 2. The HD random effect explained from 1 to 17% of the total variation of the US parameters (data not shown in table), and it was included in the statistical model to manage the differences in size of the 2 herds, which could have been a potential limitation of the study. Liver depth and PVD were significantly associated ($P < 0.01$) with HG and BCS, and consequently with predicted BW classes. Indeed, both LD and PVD linearly increased with increasing HG and BW (Figure 1a); the same relationships were found between liver size US measurements and BCS, as LD and PVD linearly increased with increasing BCS (Figure 1b). Also the relationships between LD and PVD with BW were linear and positive (Figure 1c). By contrast, PVA was not associated with any of the body traits considered. As expected, LD and PVD tended to show the same patterns of association, due to their large correlation ($r = 0.81$). Indeed, given the anatomical location of the portal vein, both measures are an expression of liver size, which, in animals in a healthy physiological condition, is to some extent related to their size. As in humans, this relationship needs to be taken into account to avoid a false diagnosis of hepatomegaly (Siddiqui et al., 2014). Moreover, PVD signals the shape and thickness of the dorsal part of the liver, which is related to the cows' nutritional status and hence to BCS.

Unlike liver US dimension measures, pTAG was not associated with HG and predicted BW, whereas it showed a slight linear increase with increasing HW (Figure 2a). Moreover, pTAG liver content was strongly related to BCS ($P < 0.01$), and it increased in a linear trend as the cows' condition scores decreased (Figure 2b).

The results from this study provide evidence that in clinically healthy cows the size of the liver, as measured by US, increases with increasing size and mass of the animals. Heart girth and BCS were the major determinants of these positive associations, alone or combined, to predict the BW of the cows. Body weight is known to be associated with body size and body condition, and these measures can be used to provide valid estimates of BW for use in field studies and in dairy herd management (Enevoldsen and Kristensen, 1997; Yan et al., 2009). Withers height seems to be more related to the skeletal development of cows (Enevoldsen and Kristensen, 1997), and it seems to have a weaker correlation with BW than with HG (Yan et al., 2009), such that in the present study it was not retained in the stepwise procedure for estimating BW from body measures, and it was not found to be associated with US liver size.

Associations between US liver traits and body measurements have scarcely been investigated, particularly in healthy cows. Starke et al. (2011) reported that the diameter of the portal vein and some portal blood flow traits assessed by Doppler ultrasonography were not significantly correlated with BW, HG, and backfat thickness. These findings seem consistent with our observations concerning PVA, which is an expression of the diameter of the portal vein and is directly related to portal blood flow. Unfortunately, no other studies have analyzed HG and BW in relation to hepatic US measurements, so further studies are needed to confirm these findings.

The lack of association between (predicted) BW and pTAG in the present study is consistent with the findings of Starke et al. (2010), who reported a low correlation between (measured) BW and TAG. In con-

Table 2. Results from the mixed model (*F*-values and significance) for liver depth (LD), portal vein depth (PVD) and area (PVA), and predicted triacylglycerol content (pTAG), as affected by body size measures, BCS, predicted BW, milk production traits, and productivity indicators[1]

Item	LD, mm		PVD, mm		PVA, mm^2		pTAG, mg/g	
	F-value	RMSE[2]	*F*-value	RMSE	*F*-value	RMSE	*F*-value	RMSE
Body size measure								
Heart girth, cm	6.76**	11.87	8.20**	11.87	0.46	282.91	1.00	9.82
Height at withers, cm	1.55	12.08	0.41	12.18	—	283.36	3.31*	9.74
BCS	4.11*	11.96	4.78**	11.98	2.11	281.31	6.23**	9.64
Predicted BW (pBW), kg	5.37**	11.92	7.26**	11.90	0.30	283.06	2.01	9.78
Milk production trait								
Milk yield (MY), kg	0.40	12.13	0.21	12.19	3.06*	280.39	0.32	9.84
Fat, %	1.99	12.05	1.02	12.15	0.58	282.79	1.47	9.80
Protein, %	1.94	12.07	1.09	12.15	0.84	282.53	1.21	9.81
Fat yield, kg/d	1.24	12.09	0.49	12.17	1.04	282.34	1.08	9.81
Protein yield, kg/d	1.09	12.11	1.37	12.15	1.92	281.48	1.89	9.80
NEmilk yield, MJ/d	1.55	12.09	1.00	12.16	1.17	282.22	0.60	9.83
Cheese yield, kg/d	0.32	12.13	0.60	12.17	1.06	282.32	0.31	9.84
Productivity indicator								
MY/pBW, g/kg/d	1.13	11.94	2.09	12.03	1.22	285.46	1.51	9.89
Fat yield/pBW, g/kg/d	1.00	12.10	2.41	12.09	0.83	282.54	1.23	9.81
Protein yield/pBW, g/kg/d	1.33	11.93	2.36	12.02	1.41	285.48	0.75	9.92
NEmilk yield/pBW, MJ/kg/d	2.16	11.89	3.75*	11.96	3.16*	283.62	0.83	9.92
Cheese yield/pBW, g/kg/d	0.37	11.98	0.94	12.09	1.98	284.33	1.50	9.89

[1]NEmilk = energy content of milk. Before analysis, traits were classified in 3 classes (low, medium, high) according to 0.75 SD units of residuals derived from a linear mixed model, which included parity and DIM as fixed effects and herd/date as random effect.

[2]RMSE = root mean square error.

*$P < 0.05$; **$P < 0.01$.

tradiction to our results, those authors found that TAG and HW were substantially independent.

Starke et al. (2010) also reported a weak positive association between hepatic TAG and US-measured back fat thickness, which reflects the amount of subcutaneous fat stored in the body and should therefore be related to BCS. We should point out, however, that the Holstein cows in the study by Starke et al. (2010) were not healthy, as they had had surgery to correct left-sided abomasal displacement, and probably did not have high MY (milk production records were not reported in that paper). This may explain the different trend in the associations between BCS and pTAG found in our study compared with the results of Starke et al. (2010). It is well known that after calving and during early lactation cows often experience a period of negative energy balance, driven by the concomitant reduced feed intake and increased MY; this results in variable degrees of body fat mobilization, which may be monitored by changes in BCS (Gallo et al., 1996; Roche et al., 2009), although variation occurs among cows in the rate of increase of dry matter intake after calving and in the amount of visceral adipose tissue (Lucy et al., 1992; Drackley et al., 2014). Increased body fat mobilization has many consequences, including increased fat storage in the liver, mainly in the form of hepatic TAG, when the extent of body lipid mobilization overcomes the capacity of fatty acid oxidation (Drackley, 1999; Starke et al., 2010). Our study was carried out with healthy cows in the first 120 d of lactation; thus we may expect an inverse association between BCS and TAG in the liver, as cows with lower BCS are expected to have mobilized more body reserves since calving, due also to higher MY. In fact, when analyzed as a dependent variable with the same model as that used for the statistical analysis of pTAG, daily MY linearly increased as BCS class decreased ($P < 0.01$), with a production difference between cows of the highest and the lowest BCS classes greater than 10% (data not shown in table).

Associations Between Liver US Measurements and Milk Production and Productivity Traits

Considering the associations between liver US measurements and milk production traits (Table 2), the size and variability of the sample allowed us to assign cows to classes ranging over large differences in MY traits (i.e., the cows in the highest yield class had daily yields of milk, nutrients, energy, and curd that were 55% to 80% greater than those of the cows in the lowest class, which means, for instance, a production of nearly 18 kg more milk per day). Nonetheless, the variations in LD and PVD did not appear to be associated with daily milk, nutrient, and curd yields, nor with the fat and protein contents of milk. Also, pTAG was not related to milk production traits and exhibited erratic values with the variations in daily milk, nutrient, and curd yields and the fat and protein contents of milk. Conversely, PVA was significantly ($P < 0.05$) associated with MY. Namely (Figure 3a), PVA linearly increased with increasing MY class, and the cows with the greatest daily MY had PVA nearly 10% greater than those of cows with the lowest daily MY. This association suggests

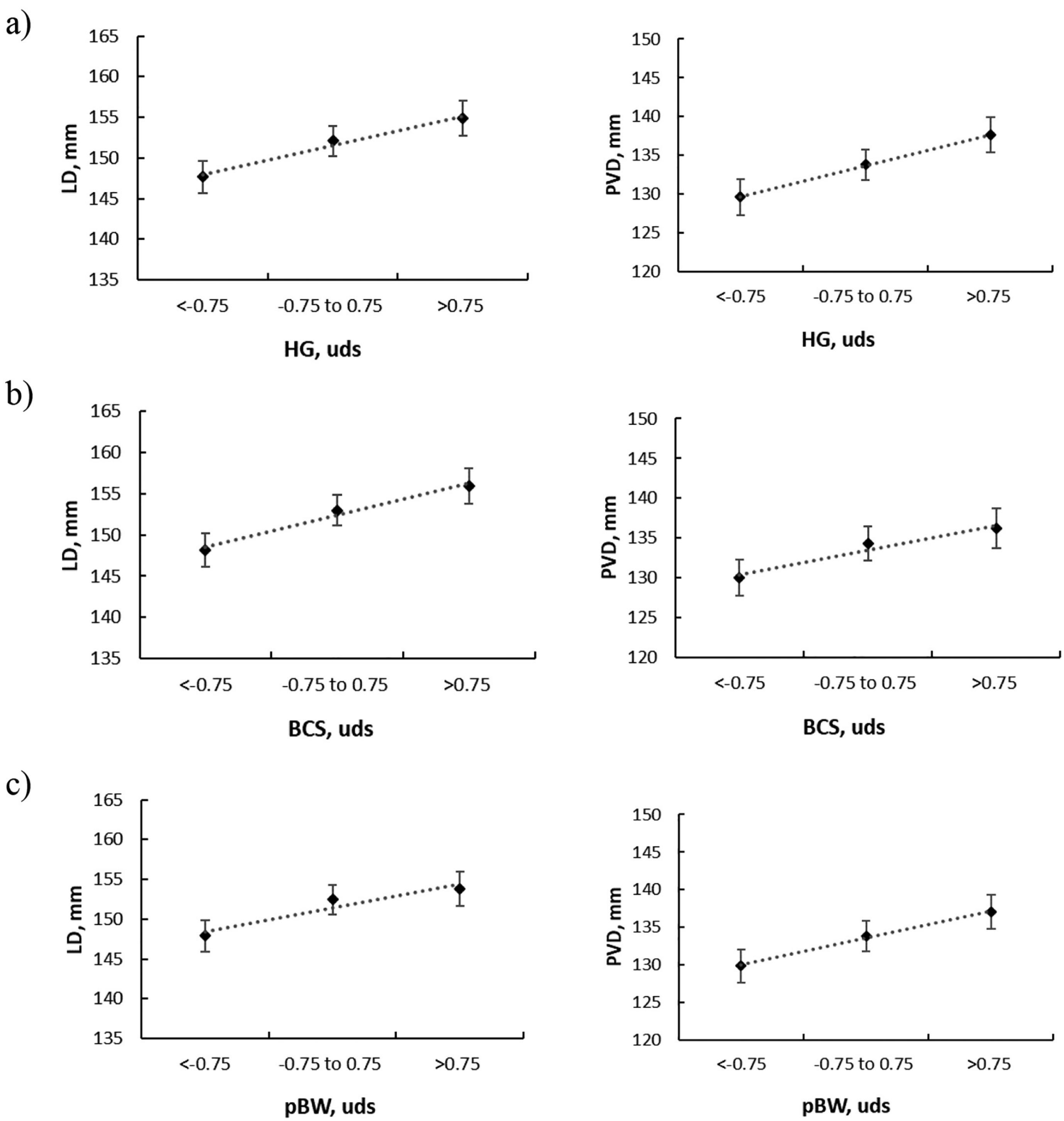

Figure 1. Least squares means for liver depth (LD, mm) and portal vein depth (PVD, mm) across (a) heart girth (HG), (b) BCS, and (c) predicted BW (pBW) classes obtained by the classification of the residuals [according to 0.75 units of standard deviation (uds)] from a mixed model that included the fixed effects of parity and DIM, and the random effect of herd/date. Only significant results are displayed ($P < 0.05$). Black dots indicate LSM, and error bars indicate SE. The dotted line describes the linear pattern of variation of the dependent variable with increase of the independent one.

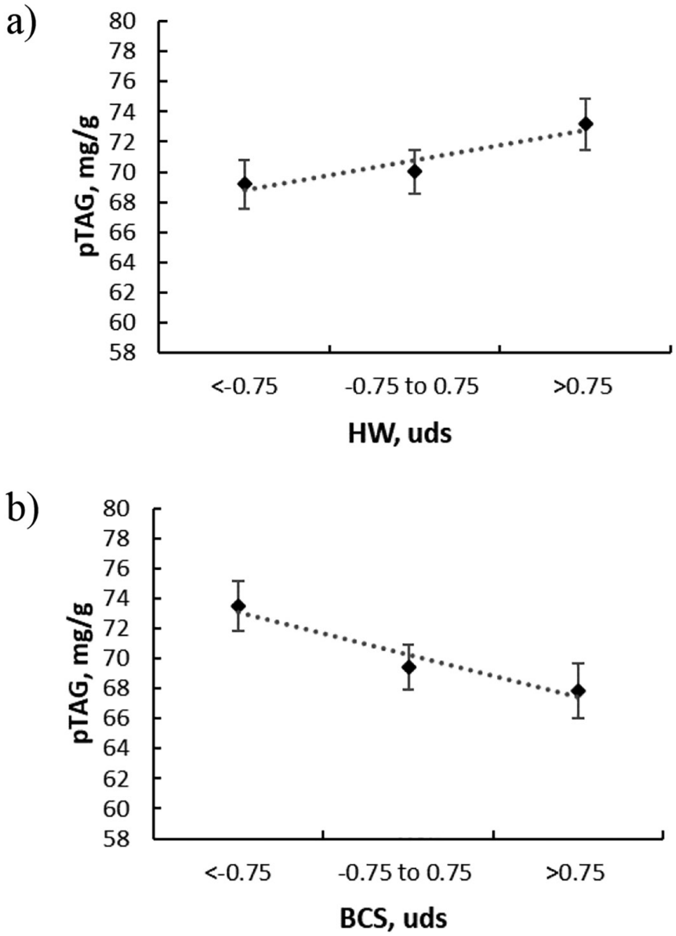

Figure 2. Least squares means for predicted triacylglycerol content (pTAG, mg/g) across (a) height at withers (HW) and (b) BCS classes obtained by the classification of the residuals [according to 0.75 units of standard deviation (uds)] from a mixed model that included the fixed effects of parity and DIM, and the random effect of herd/date. Only significant results are displayed ($P < 0.05$). Black dots indicate LSM, and error bars indicate SE. The dotted line describes the linear pattern of variation of the dependent variable with increase of the independent one.

that cows with higher MY also have a greater hepatic blood flow (or, rather, the more productive cows yield more milk because they have greater hepatic blood flow). The liver is known to be physiologically involved in milk production: Reynolds et al. (2003) reported that the daily metabolic activity per gram of liver nearly doubles between 11 d prepartum and 11 d postpartum. Indeed, the liver is the main site for the uptake of serum-free fatty acids, increased lipid β-oxidation, and increased gluconeogenesis at the beginning of lactation, when the requirements for milk synthesis are greater (Drackley et al., 2001; Roche et al., 2009). After parturition, as digestible energy intake markedly increases (Huntington, 1990), hepatic blood flow increases by up to 84% of its flow before parturition (Reynolds et al., 2003). Because PVA is a measure of hepatic blood flow, a positive association between PVA and MY is to be expected in healthy lactating cows.

Several authors have addressed the implications of fatty liver disease for milk production (Fourichon et al., 1999; Mulligan and Doherty, 2008; Carvalho et al., 2019), whereas the association between the size and TAG content of the liver and the milk production traits of cows in healthy physiological condition has scarcely been investigated. Similar to our findings, Starke et al. (2011) found no significant differences in daily MY among cows with a range of fatty liver conditions from mild (<50 mg/g TAG) to very severe (>150 mg/g TAG). However, all the cows in that study had left displaced abomasum and an average MY of 9 kg/d, so comparison with our results may be inappropriate. Also, Hammon et al. (2009), in a study involving high-yielding dairy cows and therefore with conditions comparable with those of our experiment, reported similar energy-corrected daily MY in 2 groups of 10 early-lactating cows greatly differing for average mean fat content in liver (174 vs. 77 mg/g). Compared with those with leaner livers, cows with fatty livers in that experiment tended also to produce milk with a higher fat content in the first weeks after calving, probably because of greater body fat mobilization due to lower dry matter intake, induced by their greater body reserves at calving.

Liver dimensions and area also appeared to be mostly unrelated to productivity indicators (Table 2), which are a combination of the cows' milk production traits and BW. The only exception was the significant ($P < 0.05$) association between portal vein dimensions and daily energy yielded per unit of body weight, as PVA linearly increased and PVD linearly decreased with the increase in energy produced per kilogram of BW (Figure 3b). As it has been suggested that PVD is greater in animals at greater risk of hepatic lipidosis (Fiore et al., 2018), it is possible that the negative relationship between intensity of milk energy production and PVD could be partially due to liver straining in cows with greater PVD. In fact, symptoms associated with fatty liver disease include reduction in the production of energy precursors, such as glucose, due to decreased gluconeogenesis activity (Bobe et al., 2004). Glucose is the main metabolic energy source and the primary precursor of lactose synthesis (LeBlanc, 2010), and lactose content contributes to the energy content of milk. As a consequence, the milk energy production of cows with greater PVD could be lower because of reduced liver functionality, although this hypothesis needs further support. The average pTAG in the cows sampled for our study was 70 mg/g; only 3 cows exceeded (slightly) 100 mg/g, and in none was it above 150 mg/g, the sug-

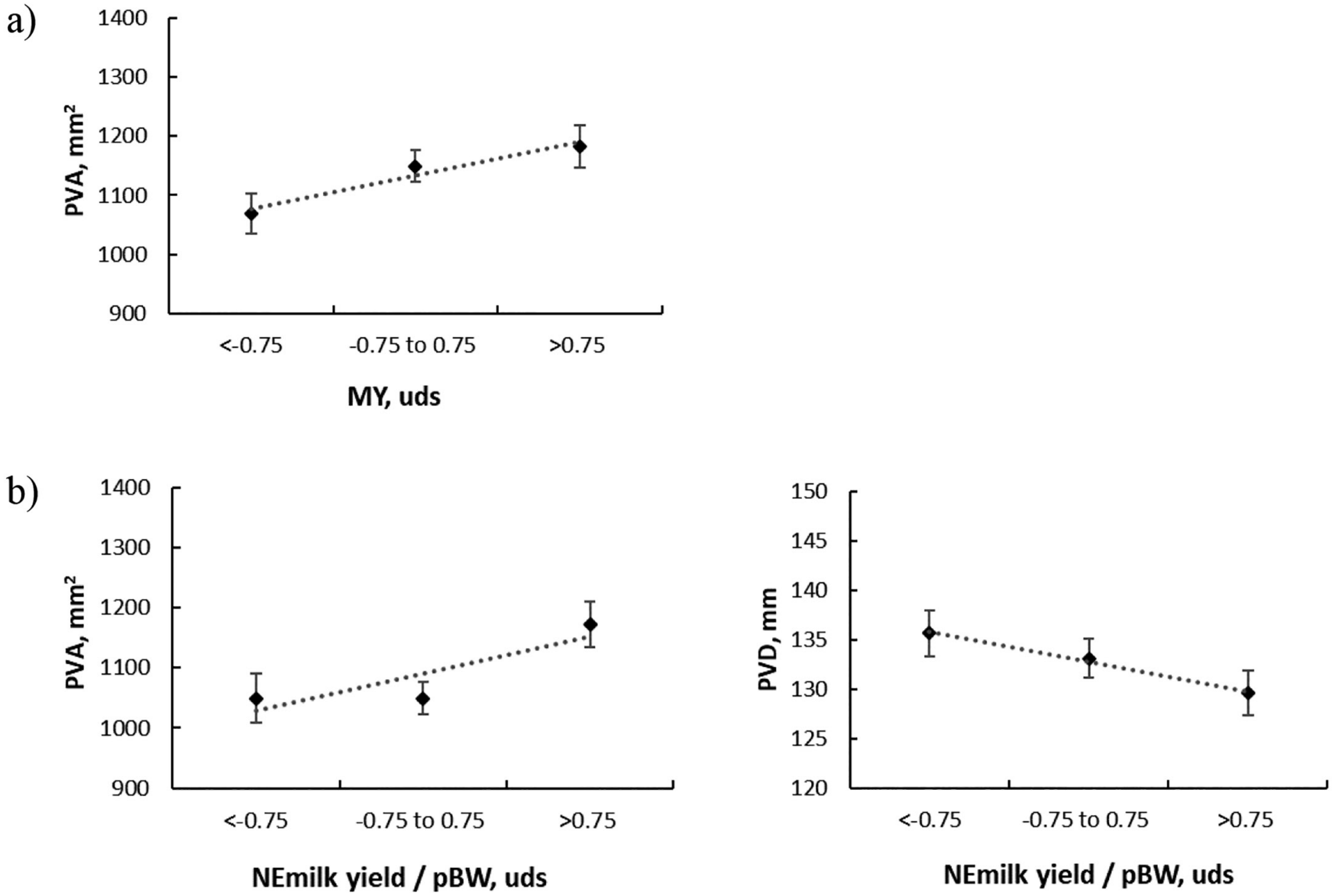

Figure 3. Least squares means for portal vein area (PVA, mm^2) and portal vein depth (PVD, mm) across (a) milk yield (MY) and (b) energy content of milk (NEmilk) yield per kilogram of predicted BW (pBW) classes obtained by the classification of the residuals [according to 0.75 units of standard deviation (uds)] from a mixed model that included the fixed effects of parity and DIM, and the random effect of herd/date. Only significant results are displayed ($P < 0.05$). Black dots indicate LSM, and error bars indicate SE. The dotted line describes the linear pattern of variation of the dependent variable with increase of the independent one.

gested thresholds for classifying hepatic lipid content as severe and very severe, respectively (Starke et al., 2011). Moreover, Haudum et al. (2011) reported that where liver TAG content was lower than 100 mg/g, liver dimensions remained unchanged, but they increased when TAG content exceeded 100 mg/g. An increase in hepatic volume and size has been related to an accumulation of fat in hepatocytes (Reid and Collins, 1980) and a consequent increase in the TAG content, but we found no associations between intensity of milk and milk component production and pTAG. Therefore, it seems unlikely that the cows in our study were under significant liver stress, but, rather, they could be under physiological fluctuations typical of the early lactation phase, which can be faced with a proper metabolic response within few weeks of lactation (Bertoni et al., 2008).

In contrast, the variation in pTAG appeared to be always independent of variations in productivity indicators, so that the liver lipid content was similar in cows exhibiting hugely diverse levels of milk, nutrient, energy, and cheese yields. It appears, therefore, that herd feeding management, in combination with the physiologic and metabolic adaptation capacities of the animals, can help cows overcome stress in the early lactation period without significantly straining liver activity. Metabolic adaptations supporting nutrient partitioning in dairy cattle were reviewed by Baumgard et al. (2017) and recently summarized by McFadden (2020) in a paper dealing with the lipid biology of the periparturient cows. These adaptations involve a decrease in de novo fat synthesis and protein synthesis, and utilization of glucose by the muscle, whereas lipolysis, amino acid mobilization, and hepatic glycogenolysis are in turn accelerated to support milk nutrient synthesis. McFadden (2020) points out that cows with high productive efficiency rely heavily on these adaptations, and, if milk production and health are not compromised, they should not be used to determine metabolic stress, which is instead a consequence of inadequate metabolic adaptation.

CONCLUSIONS

The present study showed that liver and portal vein dimensions were mostly related to the body size and weight of clinically healthy cows, whereas the associations between these dimensions and milk production traits were trivial. Moreover, liver TAG content predicted by US imaging coupled with texture analysis was found to be primarily associated with BCS, reflecting changes in the mobilization of body reserves in the early lactation period. These results provided a first description of baseline US hepatic measures in these animals, confirming the potential of US imaging to monitor liver status. By contrast, in the interval of lactation considered (first 120 DIM), variations in pTAG appeared to be always independent of changes in the cows' production capacity, whether expressed as absolute daily yield or as the production levels of milk, milk nutrients, milk energy, and fresh curd, despite the huge variations in the MY traits of the cows sampled for the study.

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