ABSTRACT

The objective of the present study was to quantify the relationships between prepartum nonesterified fatty acid (NEFA) concentrations and the development of subsequent diseases or culling and to identify the optimal thresholds allowing identification of animals at high risk of developing postpartum diseases or being culled. A total of 1,299 Holstein cows from 50 commercial herds located around Saint-Hyacinthe (QC, Canada) were enrolled in this observational study. Blood samples were collected from enrolled cows between 1 and 14 d before calving for serum NEFA quantification. Data concerning postpartum diseases and culling were collected from computerized record systems. The association between prepartum NEFA concentrations and postpartum diseases and culling was quantified using generalized linear mixed models, accounting for parity, season, week of sampling, and herd. Optimal NEFA thresholds were evaluated with receiver operator characteristic curves analysis for all diseases and then confirmed with generalized linear mixed models, considering NEFA as a categorical variable (high or low). Prepartum serum NEFA concentrations were associated with diseases diagnosed during the first 30 d in milk (DIM) and culling within the first 50 DIM. The optimal NEFA threshold associated with diseases was ≥290 µmol/L for retained placenta, ≥300 µmol/L for metritis and abomasal displacement, and ≥280 µmol/L for clinical mastitis and hyperketonemia. The level associated with the occurrence of at least one of these diseases in the first 30 DIM was ≥280 µmol/L, but it was ≥260 µmol/L for culling in the first 50 DIM. No relationship was found between NEFA concentrations and reproductive tract diseases (purulent vaginal discharge or cytological endometritis) or subclinical intramammary infection. Despite the strong relationship between prepartum NEFA concentrations and many diseases, the NEFA optimal threshold accuracy found in our study was low. In conclusion, our results demonstrate a relationship between NEFA concentrations in the 14-d period before calving and the subsequent development of diseases and culling. Prepartum NEFA concentrations thresholds between ≥260 and 300 µmol/L appear to be a strategic choice. However, considering the low accuracy, their use at the cow level should be performed with caution.

Key words: prepartum NEFA, high-risk cows, diseases, culling

INTRODUCTION

The peripartum period is an important challenge in the life of dairy cows (Ospina et al., 2013). Dairy cows face a state of negative energy balance (NEB) during this period, caused by changes in DMI and increased energy needs, mostly for milk production (Ospina et al., 2013; Sordillo and Raphael, 2013). To compensate for the energy deficit, dairy cows mobilize lipids from adipose tissues in the form of nonesterified fatty acids (NEFA; Sordillo and Raphael, 2013). The latter are transported to the liver, where they can be stored, used for energy production, or transformed to ketone bodies, among which BHBA is the most abundant (Ospina et al., 2013; Sordillo and Raphael, 2013). By themselves, the presence of NEFA and BHBA in blood is considered normal in early-lactation dairy cows (Qu et al., 2014; Shin et al., 2015), but when these concentrations exceed certain thresholds, immune functions are impaired, as well as other physiological functions (Sordillo et al., 2009; de Souza Ferreira et al., 2012). As a consequence, dairy cows experiencing excessive NEB during the postpartum period are at greater risk of developing subsequent diseases than cows having lower concentrations of NEFA and BHBA (Sordillo and Raphael, 2013). The occurrence of diseases in the postpartum period is a concern for farmers for many
Concentrations of NEFA and BHBA in serum have been described as good indicators of peripartum energy balance and have been investigated in multiple studies as possible predictors of subsequent postpartum diseases (Dubuc et al., 2010a; Ospina et al., 2010a,c, 2013; Roberts et al., 2012). In these studies, cows were generally sampled for blood or serum from the 2 wk before calving until after calving. Elevated pre- and postpartum NEFA concentrations as well as postpartum BHBA concentrations have been associated with a greater risk of abomasal displacement (AD), hyperketonemia (HK), retained placenta (RP), metritis (MET), and culling (CUL; Dubuc et al., 2010a; Ospina et al., 2010a,b; Chapinal et al., 2011; Seifi et al., 2011). Threshold values of these metabolite concentrations have been proposed for identifying animals at greater risk of developing the aforementioned diseases. However, these values have not always been the same between studies and within diseases of interest (Dubuc et al., 2010a; Ospina et al., 2010a,b). Associations between elevated NEFA concentrations and the incidence of clinical mastitis (CM) have been reported, but no thresholds have been proposed to our knowledge (Holtenius et al., 2004; Schwegler et al., 2013). It also remains unclear whether the NEFA concentration thresholds reported by all these studies are accurate, because performance data have generally not been reported. Such information is crucial for appropriate data interpretation.

Therefore, the objective of the present study was to quantify the relationships between NEFA concentrations in the 2 wk prepartum and the development of subsequent diseases (AD, HK, and uterine and mammary infections) or CUL. A secondary objective was to identify optimal NEFA concentration thresholds that allow identification of animals at high risk of developing postpartum diseases or being culled. Our hypotheses were that prepartum NEFA concentrations are elevated in animals that subsequently develop postpartum diseases or are culled. Furthermore, we hypothesized that optimal thresholds can be used to identify animals at high risk of subsequent disease or culling. This approach should help veterinarians with decision-making concerning the prevention of postpartum diseases in dairy herds.

We conducted a prospective cohort study over a period of 1 yr (November 2018 to December 2019) on 50 commercial Holstein dairy herds that were regular clients of the Bovine Ambulatory Clinic of the Faculté de médecine vétérinaire of the Université de Montréal (St-Hyacinthe, QC, Canada). The study was approved by the Animal Use Ethics Committee of the Université de Montréal (Rech-2059). Reporting of the study was done according to Strobe-Vet guidelines (Sargeant et al., 2016). Recruitment of herds was based on convenience and on the following inclusion criteria: (1) location within 1 h of St-Hyacinthe (QC, Canada); (2) enrollment in a preventive medicine program involving herd health veterinary visits every 14 d; (3) permission for the research team to collect blood samples from cows; (4) enrollment in a regular DHIA program; and (5) standardized disease monitoring and collection of data using electronic health management software. A total of 1,313 cows were targeted for this study. This estimated sample size was arbitrarily (clinical experience of the authors) based on the detection of a difference of 20 percentage points in the incidence of AD (disease with the lowest incidence) between animals with high and low concentrations of NEFA (5% vs. 25%, respectively), with 95% confidence and 80% power (Dohoo et al., 2009), and an estimated incidence of 4%. It also included a 5% loss to follow-up.

**Samples and Data Collection**

Farm visits were performed every 14 d, by the herd veterinarian and an animal health technician. During the visits, animals within 35 d (±7) of subsequent predicted calving date were enrolled in the study. On the day of enrollment and every 14 d after, a blood sample was collected from each cow enrolled in the study for the subsequent evaluation of serum NEFA concentration. Blood samples were collected from the coccygeal vessels in tubes without anticoagulant (Vacutainer, Becton Dickinson and Company). Within 4 h of sampling, tubes were centrifuged at 1,750 × g for 10 min at 20°C, and the serum was harvested and stored at −20°C. All samples were labeled with a unique identification number that included the date of sampling. Once cows calved, these samples were reorganized to determine the real number of days before calving at which they were sampled. All cows enrolled in the study were followed until 50 DIM (Figure 1). Data concerning postpartum diseases were collected from computerized record systems. Hy-
perketonemia, AD, and postpartum reproductive tract diseases (RTD) were always diagnosed by veterinarians during the postpartum period. Hyperketonemia was diagnosed during the first herd health visit after calving, between 1 and 14 DIM. On that occasion a blood sample was obtained from the coccygeal vessels and immediately analyzed with a Precision Xtra portable device (Abbott Diabetes Care). The BHBA concentration in the first 14 DIM was used to define cases of HK, with a validated threshold of ≥1.4 mmol/L (Iwersen et al., 2009). A diagnosis of AD was made when a high-pitched sound was heard in the paralumbar fossa or in the last intercostal spaces on the left or the right side and was confirmed during surgical resolution. Only AD diagnosed in the first 30 DIM were included in the analysis (LeBlanc et al., 2005). Postpartum RTD were identified at 37 (±7) DIM during the routine herd visit. They were defined on the basis of 2 different tests, to recognize both purulent vaginal discharge (PVD) and cytological endometritis (CE; Dubuc et al., 2010a). Vaginal discharge was evaluated and scored using a Metricheck device (Simcro Tech Ltd.). This device consists of a stainless-steel rod with a silicon hemisphere attached at the end. The vaginal content was scored as follows: 0 = absence of discharge; 1 = clear mucus; 2 = some flecks of purulent material observed in the mucus; 3 = purulent material less than the 50% of all discharge; 4 = purulent material more than 50% of all discharge; and 5 = fetid red-brown watery discharge with an odor (McDougall et al., 2007). A score ≥3 was used to diagnose the presence of PVD (Denis-Robichaud and Dubuc, 2015b). Retained placenta, CM, and MET were diagnosed and treated by the farmers based on standardized definitions. Consequently, they received information about how to identify these diseases before the beginning of the study and on several occasions during the study. At each herd health visit (every 14 d), veterinarians discussed the occurrence of such diseases to confirm farmers’ diagnoses. Retained placenta was defined as lack of detachment of fetal membranes within the first DIM (Eiler and Fecteau, 2007). Mild and moderate CM were defined as modification of milk aspects alone or in combination with swelling of the quarter, respectively (Ruegg and Erskine, 2020). Severe CM was defined as the presence of hyperthermia (≥39.5°C), anorexia, or both, in addition to alteration of milk aspect and quarter swelling (Ruegg and Erskine, 2020). Clinical mastitis was included when the occurrence was during the first 30 DIM (Rollin et al., 2015). Metritis was defined as the presence of red-brown fetid vaginal discharge combined with hyperthermia (≥39.5°C) and anorexia in the first 20 DIM (Sheldon et al., 2006).

Individual SCC from the last DHIA test before dry-off and the first after calving were retrieved for each cow from DHIA records. Only cows that had their first test after calving within 50 DIM were included in the analysis. Cows that had SCC ≥200,000 cells/mL at first test after calving were considered to have a subclinical IMI (Fauteux et al., 2014). Cows that had their last test before dry-off with SCC <200,000 cells/mL and ≥200,000 cells/mL at their first test after calving were considered to have a new subclinical IMI (NIMIc; Fauteux et al., 2014). New subclinical IMI were also computed for nulliparous cows by including animals that had ≥200,000 cell/mL at their first test.
after calving (NIMIh; Fauteux et al., 2014). Cows that had their last test before dry-off with SCC ≥200,000 cells/mL and SCC <200,000 cells/mL at their first test after calving were considered to be cured (CIMI; Fauteux et al., 2014). Data concerning CUL during the first 50 DIM were also collected.

Laboratory Analysis

All serum samples collected between 1 and 14 d before calving were submitted to the Centre de diagnostic vétérinaire of the Université de Montréal (St-Hyacinthe, QC, Canada) for quantification of NEFA concentration. When ready to be analyzed, the samples were thawed at room temperature and analyzed using a Beckman DxC 600 automatic analyzer (Beckman Coulter Corp.), with reagent supplied by Randox Laboratories Ltd. Individual cow measures of SCC were quantified according to their regular monthly DHIA program offered by Lactanet (Ste-Anne-de Bellevue, QC, Canada). Once delivered, the samples were analyzed with a CombiFoss 7 DC instruments (Foss) to quantify SCC concentrations.

Statistical Analysis

Statistical analyses were performed using a freeware statistical software package R v.3.4.1 (R Foundation for Statistical Computing). Descriptive statistics were computed. Each disease was included only once for each cow (lactational incidence). Lactational incidence of diseases were reported as frequency (%), and NEFA serum concentrations were reported as median (minimum, maximum).

To quantify the relationships between prepartum serum NEFA concentration and subsequent disease development, generalized linear mixed models with logit link (“glmer” using the “bobyqa” optimizer) were computed using the lme4 package (Wang et al., 2022).

Step 1. The associations between NEFA concentrations (continuous variable) and diseases or culling were investigated. An individual multivariable model was computed for each disease (RP, HK, AD, MET, CM, PVD, CE, IMI, NIMIc, NIMIh, CIMI), and CUL, which were considered as the dependent variable of each model. Another dependent variable (dummy variable) was created for having at least one disease in the first 30 DIM (AL1D) within the following: RP, HK, AD, CM, and MET. Serum NEFA concentration was included as a numerical independent variable, and parity (first lactation vs. subsequent lactation), season (January–March; April–June; July–September; October–December), and week of sampling (wk 1: 1–7 d, wk 2: 8–14 d before calving) were forced in the models as possible confounding factors. Herd was included in the models as a random variable to account for herd clustering. All models were offered the 2-way interaction term NEFA concentration × week of sampling. Significance was declared at a P-value <0.05.

Step 2. To identify the optimal thresholds for prepartum serum NEFA concentration, a receiver operating characteristic (ROC) analysis was performed using the “cutpointr” function of the “cutpointr” package (Thiele and Hirschfeld, 2021). Selection of the optimal threshold was based on the highest Youden’s J statistic index (to maximize the sum of sensitivity and specificity). The performance of the threshold was determined based on its area under the ROC curve (AUC), where it ranged from 0.50 to 1.00; AUC of 0.50 was considered non-informative, 0.5 to 0.7 was considered poor, 0.7 to 0.8 was considered acceptable, 0.8 to 0.9 was considered excellent, and greater than 0.9 was considered outstanding (Mandrekar, 2010; Hosmer et al., 2013). Sensitivity, specificity, and AUC confidence interval were estimated with the bootstrap procedure (1,000 interactions) using the “boot_ci” function of the “cutpointr” package (Thiele and Hirschfeld, 2021).

Step 3. To identify the optimal threshold for prepartum NEFA concentration accounting for the possible effect of parity, season, week of sampling, and herd clustering, mixed logistic regression models were computed with serum NEFA concentration as a categorical variable (high or low). The dependent variables of these models included all the diseases that were found significantly associated in the model, with NEFA concentration as a continuous variable (step 1). The interaction term NEFA concentration × week of sampling was included in the model if it was significant in the models built at step 1. Serum NEFA concentrations were categorized into several different thresholds. They were selected by subtracting and adding 10, 20, 30, 40, 50, 60, 70, 80, and 90 µmol/L to the threshold values obtained in step 2. A model was then computed for each disease, CUL, and AL1D when considering each individual NEFA threshold. The threshold with the lowest Akaike information criterion (AIC) was retained as optimal (Dohoo et al., 2003). Significance was declared at a P-value <0.05.

RESULTS

Descriptive Statistics

The 50 participating farms ranged from 30 to 300 lactating cows, with a median of 65. These farms were mostly tiestall barns (54%; n = 27), but some were freestall barns (38%; n = 19); 3 herds (6%) had their dry cows housed in bedded pack and their lactating...
cows housed in tiestalls, and 1 herd (2%) had its dry cows housed in tiestalls and its lactating cows housed in freestalls. None of the herds had access to pasture. The majority of the farms (64%; n = 32) fed their cows TMR, whereas the others (36%; n = 18) used component-fed rations. Cows were milked twice a day on most of the farms (82%; n = 41), whereas the others were milked 3 times a day (18%; n = 9).

A total of 1,351 cows were sampled during the study, based on expected calving dates, but only 1,299 could be used for statistical analyses after adjustment of the real calving date. In the latter, the median interval between blood sampling and real calving date was 7 (min–max: 1–14). The median number of cows sampled by herd was 14 (min–max: 1–128). All enrolled cows were Holstein; 19% (244/1,299) were nulliparous, and 81% (1,055/1,299) were parous cows.

Health Data

A total of 39 animals were culled between calving and 50 DIM. Furthermore, data concerning HK and CE were missing for 126 and 449 animals, respectively. Missing HK data in a few herds were caused by a problem in having regular BHBA testing implemented. Missing CE data were caused by the impossibility of collecting these data in some herds (too complicated for research staff to restrain cows properly in the freestalls). Post-calving DHIA testing was performed within 50 DIM in 904 cows that were used for the evaluation of the incidence of post-calving IMI. Out of the 904, 169 were nulliparous and were used for computing incidence of NIMIh. Among remaining cows (n = 735), data about dry-off DHIA were missing for 79 cows; 445 cows had SCC <200,000 cells/mL at dry-off and were used for the calculation of prevalence of NIMIc; and 211 cows had SCC ≥200,000 cells/mL at dry-off and were included in the calculation of CIMI. The exact numbers of animals included for each disease frequency calculation and culling are reported in Table 1.

Multivariable Analysis

Step 1. The interaction term between NEFA concentrations × week of sampling was not associated (P > 0.05) with the dependent variable in any models, except for CIMI. Thus, it was kept in models only for the latter. Only some diseases diagnosed during the first 30 DIM and culling within the first 50 DIM (RP, HK, AD, MET, CM, AL1D, and CUL) were associated (P < 0.05) with prepartum serum NEFA concentrations (Table 1). Parity was associated with MET, AD, CM, AL1D, and season was associated with MET, CM, AL1D, PVD, CE, IMI, NIMIh, NIMIc, and CIMI. Week of sampling was not associated with any diseases.
Step 2. For variables that were associated with prepartum serum NEFA concentrations in step 1, ROC curves were built. The ROC thresholds obtained for each disease and their performance (AUC, sensitivity, and specificity) are reported in Table 2.

Step 3. The thresholds obtained in step 2 were used to build multivariable models to find the optimal threshold considering parity, season, week of sampling, and herd. Thresholds with the lowest AIC were retained for RP, MET, AD, CM, AL1D, and CUL (Table 3). Regarding HK, the model with the lowest AIC offered ≥230 µmol/L as a threshold. However, the model with ≥280 µmol/L as a threshold, which was closer to those found for the other diseases, also had a very low AIC, similar to the model with ≥230 µmol/L as a threshold (AIC values: 1,152.7 vs. 1,153.3). Consequently, we decided to retain ≥280 µmol/L as the optimal prepartum NEFA threshold for postpartum HK.

Optimal thresholds obtained from multivariable models are reported in Table 3.

**DISCUSSION**

**Association Between Prepartum NEFA and Postpartum Diseases and Culling**

Our results demonstrate an association between prepartum serum NEFA concentrations and the development of subsequent diseases. These results are in line with other studies investigating similar relationships, such as with RP, HK, MET, AD, CM (Cameron et al., 1998; LeBlanc et al., 2005; Ospina et al., 2010b; Chapinal et al., 2011; Schwegler et al., 2013). These relationships are not surprising, as excessive NEFA concentrations in blood have been recognized as impairing neutrophils’ function, predisposing to postpartum infectious diseases (LeBlanc, 2020). Excessive NEB is also recognized as one of the main risk factors for AD (Nichols and Fecteau, 2018).

No relationship was found between prepartum NEFA concentrations and the subsequent occurrence of RTD (PVD and CE) at 37 ± 7 DIM. Interestingly, some studies have reported an association between high prepartum NEFA concentration and the subsequent risk of developing RTD (Kaufmann et al., 2010; Giuliodori et al., 2013), but one of them underlined that this association was present only for multiparous cows (Kaufmann et al., 2010). In contrast, multiple studies have reported no relationship between prepartum NEFA concentration and the subsequent risk of developing RTD (Dubuc et al., 2010a; Yasui et al., 2014; Bogado Pascottini and LeBlanc, 2020). We hypothesized that prepartum NEFA concentration could be indirectly associated with PVD or CE, considering the association with MET. However, the lack of association observed in the present study could be due to the fact that the 2 events are too distant in time.
from each other. This idea is supported by previous studies in which prepartum NEFA were not associated with PVD and CE, but variation in postpartum NEB markers (NEFA and BHB) were (Dubuc et al., 2010b; Bogado & Pascoletti, 2020). We speculate that the treatment of metritis with antibiotics, which is the most common approach among the clients of the Bovine Ambulatory Clinic, could have created a bias in evaluation of the association between prepartum NEFA and PVD or CE.

No relationship was found in our study between prepartum NEFA concentration and SCC dynamics between dry-off and early lactation (IMI, NIMIc, NIMIh, CIMI), in agreement with previous reports (Moyes et al., 2009; Schwiegler et al., 2013). The absence of association could also be linked with the delay between NEFA concentration evaluation and the identification of IMI, NIMIc, and CIMI, considering that DHIA were included up to 50 DIM. However, it is worth noting that the number of animals used for this analysis was reduced because of the time at which the DHIA test was performed after calving (≤50 DIM) and because of the absence of the DHIA test before dry-off for some animals. Furthermore, the number of nulliparous cows included in the analysis was relatively small and very likely insufficient. It is possible that the number of animals used to test this association may have led to a lack of statistical power and masked a possible biological effect. With the exception of NIMIh, the numerical difference between the prepartum NEFA concentrations of cows that had IMI, NIMIc, or CIMI compared with cows that did not was not very different from the difference found for diseases such as RP or HK. Based on our results, it is difficult to draw conclusions, and these relationships deserve to be further investigated in the future.

Culling in the first 50 DIM was associated with prepartum NEFA concentrations, in line with previous results (Roberts et al., 2012). Prepartum NEFA concentrations have also been associated with the risk of developing postpartum diseases and culling, with milk yield, and with reproductive performance (Ospina et al., 2010c).

Prepartum NEFA Thresholds and Association with Postpartum Diseases and Culling

In the present study, the optimal prepartum NEFA concentrations thresholds associated with diseases during the first 30 DIM were relatively similar among diseases; they were also similar to those reported in other studies that used a similar prepartum sampling period (2 wk before calving; Ospina et al., 2010b; Chapinal et al., 2012). Ospina et al. (2010b) reported a prepartum optimal NEFA threshold of ≥260, ≥270, and ≥370 µmol/L for HK, AD, and RP, respectively, as well as ≥290 µEq/L for having at least one of these diseases. Chapinal et al. (2012) found that ≥300 µmol/L was the optimal prepartum NEFA threshold for RP and MET. Other studies have reported slightly higher thresholds. LeBlanc et al. (2005) reported ≥500 µmol/L as an optimal prepartum NEFA threshold for prediction of AD; Dubuc et al. (2010b) reported ≥600 µmol/L for MET. Our optimal threshold for the risk of culling was lower compared with another study, which reported NEFA ≥400 µmol/L (Roberts et al., 2012).

Based on our results, a prepartum NEFA concentration threshold of ≥280 µmol/L appears to be relevant to describe the association between prepartum NEFA and the development of various postpartum diseases and culling, when cows are sampled at a 2-wk interval period. When evaluating the thresholds reported in the current study, it is important to notice that even if many diseases were associated with prepartum NEFA concentrations or specific NEFA thresholds, their AUC values were poor (Mandrekar, 2010). Our results demonstrate that sensitivity and specificity are low when used at the cow level, indicating a poor ability to identify animals at high risk. Caution should be used when interpreting these results.

The possibility of performing early identification of cows at high risk of diseases or culling is interesting for veterinary practitioners and farmers. However, the interest in identifying specific individual animals needs to be matched with acceptable accuracy of the used test. On the other hand, early identification of a group or cohort of animals at high risk could potentially help for taking corrective actions sooner on the farm. One example of this approach has been reported by Ospina et al. (2010a, b) who recommended testing prepartum NEFA concentrations in a group of animals to quantify a proportion of cows above a specific threshold and compare it with a herd alarm level. Further studies will be needed to quantify the usefulness of possible corrective action to be applied to animals with elevated NEFA concentrations in the 14-d period before calving. It would also be interesting to determine whether NEFA concentration sampled earlier during the prepartum period (30 d before, for instance) could allow identification of high-risk animals sooner than in the current study.

A possible limitation of the present study is that some of the diagnoses (RP, MET, CM) were not always confirmed by veterinarians. This could have potentially led to an over- or underestimation of the diseases’ incidence. Nonetheless, farmers were trained before the beginning of the study in disease recognition, and a discussion about recent clinical cases was performed.
CONCLUSIONS

The results of the present study confirmed the relationships between NEFA concentration in the 14 d before calving and the subsequent development of AD, HK, RP, MET, and CM in the first 30 DIM, as well as CUL in the first 50 DIM. Optimal thresholds associated with postpartum diseases or culling were between ≥260 and 300 µmol/L. However, the low accuracy of these thresholds should bring caution to their use at the cow level. Further studies are needed to evaluate their potential for use at the herd level.

ACKNOWLEDGMENTS

The authors acknowledge financial support from the Fonds de recherche du Québec – Nature et technologies (Québec, QC, Canada), the Réseau Québécois en reproduction (Université de Montréal, St-Hyacinthe, QC, Canada), the Regroupement Op-flait (Université de Montréal), and the Fonds de recherche clinique Zoetis of the Bovine Ambulatory Clinic of the Université de Montréal. The authors thank Jean-Philippe Pelletier (Université de Montréal, St-Hyacinthe, QC, Canada), Sarah Lescure and Léa Lanogue (École Nationale Vétérinaire de Toulouse, Toulouse, France), as well as the veterinarians from the Bovine Ambulatory Clinic of the Université de Montréal for their technical help during data collection. The authors also express their gratitude to the participating dairy producers for their contribution to this study. The authors have not stated any conflicts of interest.


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