**Lipolysis inhibition as a treatment of clinical ketosis in dairy cows: Effects on adipose tissue metabolic and immune responses**

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**ABSTRACT**

Dairy cows with clinical ketosis (CK) exhibit excessive adipose tissue (AT) lipolysis and systemic inflammation. Lipolysis in cows can be induced by the canonical (hormonally induced) and inflammatory lipolytic pathways. Currently, the most common treatment for CK is oral propylene glycol (PG); however, PG does not reduce lipolysis or inflammation. Niacin (NIA) can reduce the activation of canonical lipolysis, whereas cyclooxygenase inhibitors such as flunixin meglumine (FM) can limit inflammation and inhibit the inflammatory lipolytic pathway. The objective of this study was to determine the effects of including NIA and FM in the standard PG treatment for postpartum CK on AT function. Multiparous Jersey cows \(n = 18; 7.1 (SD = 3.8)\) DIM were selected from a commercial dairy. Inclusion criteria were CK symptoms (lethargy, depressed appetite, and drop in milk yield) and high blood levels of \(\beta\)-hydroxybutyrate (BHB \(\geq 1.2\) mmol/L). Cows with CK were randomly assigned to one of 3 treatments: 1) PG: 310 g administered orally once per d for 5 d, 2) PG+NIA: 24 g administered orally once per d for 3 d, 3) PG+NIA+FM: 1.1 mg/kg administered IV once per day for 3 d. Healthy cows (HC; \(n = 6\)) matched by lactation and DIM (\(\pm 2\) d) were sampled. Subcutaneous AT explants were collected at d 0 (d0) and 7 (d7) relative to enrollment. To assess AT insulin sensitivity, explants were treated with insulin (INS = 1 \(\mu\)L/L) during lipolysis stimulation with a \(\beta\)-adrenergic receptor agonist (isoproterenol, ISO = 1 \(\mu\)M). Lipolysis was quantified by glycerol release in the media. Lipid mobilization and inflammatory gene networks were evaluated using real-time qPCR. Protein biomarkers of lipolysis, insulin signaling, and AT inflammation, including HSL, AKT, and ERK1/2, were quantified by capillary immunoassays. Flow cytometry of AT cellular components was used to characterize macrophage inflammatory phenotypes. Statistical significance was determined by a non-parametric \(t\)-test when 2 groups (HC vs CK) were analyzed and an ANOVA test with Tukey adjustment when 3 treatment groups (PG vs PGNIA vs PGNIAFM) were evaluated. At d0, AT from CK cows showed higher mRNA expression of lipolytic enzymes \(ABHD5\), \(LIPE\), and \(LPL\), as well as increased phosphorylation of the lipase HSL (pHSL) compared with HC. At d0, INS reduced lipolysis by 41 ± 8% in AT from HC, while CK cows were unresponsive (−2.9 ± 4%). AT from CK cows exhibited reduced Akt phosphorylation compared with HC. CK had increased AT expression of inflammatory gene markers, including \(CCL2\), \(IL8\), \(IL10\), \(TLR4\), and \(TNF\), along with ERK1/2 phosphorylation. AT from CK cows showed increased macrophage infiltration compared with HC. By d7, AT + PG+NIAFM cows had a more robust response to INS, as evidenced by reduced glycerol release (36.5 ± 8% compared with PG, 26.9 ± 7%, and PGNIA, 7.4 ± 8%) and enhanced phosphorylation of Akt. By d7, PG+NIAFM cows presented lower inflammatory markers, including ERK1/2 phosphorylation and reduced macrophage infiltration, compared with PG and PGNIA. These data suggest that including NIA and FM in CK treatment improves AT insulin sensitivity and reduces AT inflammation and macrophage infiltration.

Keywords: adipose tissue, clinical ketosis, lipolysis inhibition

**INTRODUCTION**

The postpartum period in dairy cows is the lactation stage associated with the highest risk for disease and culling. Despite progress in nutritional interventions, management, and preventive medicine programs, 30 to 50% of dairy cows still succumb to at least one disease during the early postpartum (LeBlanc, 2010). Among the numerous metabolic and inflammatory events associated with periparturient disease risk, lipolysis dysregulation in adipose tissues (AT) is well-recognized as a prominent predisposing factor (Ospina et al., 2010, Zachut and Contreras, 2022). When dysregulated (i.e., intense and protracted), lipolysis rapidly overwhelms the liver’s capacity to process the free fatty acids (FA) it releases, resulting in enhanced ketone body production...

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synthesis and development of ketosis. Clinical Ketosis (CK) is characterized by reduced appetite, drop in milk yield, and lethargy along with elevated plasma β-hydroxybutyrate [BHB, > 1.2 mmol/L] and hypoglycemia [<2.0 mmol glucose/L] (Duffield, 2000, Dervishi et al., 2021). Although recent reports identified hepatic gene networks and circulating metabolic and proteomic profiles associated with CK (Wu et al., 2020), the pathogenesis of the disease is not entirely defined, and the role AT plays is poorly understood. Therefore, determining AT’s metabolic and inflammatory responses before and after treatment of CK is important to advance our understanding of the mechanisms for the development and resolution of this disease.

Similar to other mammals, AT is the main energy-reserving organ in dairy cows. AT undergoes physiological adaptations around the time of calving to fulfill the energy requirements of periparturient cows (Contreras et al., 2017b). Among these adaptations, lipogenesis and re-esterification of FA are sharply reduced while lipolysis is upregulated (McNamara, 1991). These homeorhetic adaptations provide sufficient energy for the final stages of pregnancy and the onset of lactation (Bauman and Currie, 1980). One critical mechanism that leads to intense lipolysis postpartum is the reduction in circulating insulin levels. (Mann et al., 2016). Lipolysis is further intensified by decreased insulin sensitivity and heightened responses to β-adrenergic stimulation in AT (Contreras et al., 2017b). During pathologic conditions such as CK and displaced abomasum (DA), systemic insulin sensitivity is drastically reduced, resulting in excessive lipolysis (Pravettoni et al., 2004, De Koster and Opsomer, 2013). Despite the crucial role of AT in the pathophysiology of metabolic diseases, its specific contribution to the pathogenesis of CK remains poorly understood.

Within AT, lipolysis triggers an inflammatory response characterized by the infiltration of adipose tissue macrophages (ATM). Previous studies from our group showed that ATM infiltration is associated with heightened lipolysis during early lactation and dysregulated lipolysis during hyperketonemia and DA (Contreras et al., 2015; De Koster et al., 2018b). Dairy cows’ AT are characterized by the expression of clusters of differentiation (CD) markers, including CD14, CD68, and CD172a. ATMs are classified as M1 (classical) and M2 (alternative) depending on their phenotype and function. M1 ATM express CD14 and CD11c and secrete pro-inflammatory cytokines, including TNF-α and IL-6 (Lumeng et al., 2007). Remarkably, M1 is associated with intense lipolysis as it occurs during CK (Contreras et al., 2015). In contrast, M2 ATM express CD11b and CD163 and produce IL-10, which promotes the resolution of inflammation (Fujisaka et al., 2009; Ferrante Jr, 2013). Although several reports have described the use of flow cytometry in healthy or diseased cows (Contreras et al., 2015; Contreras et al., 2016; Oliveira et al., 2020), no studies have characterized ATM phenotypes before and after treating cows with CK.

The optimal treatment for CK should focus on re-establishing appetite, promoting the return to normoglycemia, and reducing ketogenesis by limiting circulating FA availability (Herdt and Emery, 1992). Currently, oral administration of propylene glycol (PG) is accepted as the treatment for CK in dairy practice due to its gluconeogenic properties (Gordon et al., 2013). However, short-term supplementation of PG does not efficiently inhibit AT lipolysis during CK (Gordon et al., 2017). In dairy cows’ AT, lipolysis is activated by the canonical and inflammatory pathways (Chirivi et al., 2022). The canonical pathway of lipolysis involves the activation (i.e., phosphorylation) of hormone-sensitive lipase (HSL) by protein kinase A (PKA). In the inflammatory pathway, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAP/ERK) phos- phorylates HSL, initiating lipolysis (Grisouard et al., 2010). Niacin (NIA) is a strong antilipolytic agent that inhibits PKA activation, resulting in reduced canonical lipolysis (Tunaru et al., 2003). However, as cows with CK are often in a pro-inflammatory state, inhibiting the canonical pathway alone may not be sufficient for reducing lipolysis. Therefore, curtailing inflammation with cyclooxygenase (COX) blockers such as flunixin meglumine (FM) could reduce stimulation of the inflammatory lipolytic pathway (Kovacevic et al., 2019, Inazumi et al., 2020). However, the effects of treating CK cows with NIA and FM on AT function remains unknown.

This work is part of a randomized clinical trial that evaluated the effect of lipolysis inhibitors in treating CK (Chirivi et al., 2023a). In the present study, we characterized AT’s functional and inflammatory profile during spontaneous cases of CK and determined the effects of PG, NIA, and FM treatment on AT function during the treatment of CK. The data presented herein demonstrate that CK cows exhibit inflammation and reduced insulin sensitivity in AT. We provide evidence that combined administration of PG, NIA, and FM improves AT’s sensitivity to insulin and limits AT inflammatory responses, including ATM trafficking.

**MATERIALS AND METHODS**

**Animals**

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University (IACUC:202100139). This study
was performed at a single commercial dairy farm in Michigan, USA. The farm was selected by convenience, given the owner’s voluntary willingness to participate in the study. At the time, the dairy had ~2645 lactating Jersey cows milked twice daily in a double-25 parallel herringbone parlor that averaged 25 kg of milk per cow daily. Animals in this study were part of a larger randomized clinical trial (RCT) conducted from July 2021 through January 2022. Since October 2021, every animal enrolled in the RCT was included in the present study without exception. The RCT analyzed the effects of 1) propylene glycol (PG), 2) PG + niacin (PGNIA), or 3) PGNIA + flunixin meglumine (PG-NIAFM) on BHB concentrations and lipolysis reduction during naturally occurring cases of CK in dairy cows. The full description of the study population is detailed in (Chirivi et al., 2023a).

Animals were housed in free stalls with recycled manure solids as bedding. Prepartum and postpartum diets were fed as total mixed rations, and ingredients included corn silage, alfalfa silage, chopped alfalfa hay, corn grain, soy hulls, soybean meal, heat-treated soybean meal, minerals, and vitamins. The prepartum diet contained (% diet DM) ~38% NDF, 35% forage NDF, 17% starch, and 15% CP. Postpartum cows were examined daily by a trained veterinarian and 2 experienced farm staff members.

Inclusion criteria for CK cases included multiparous dairy cows 2–21 DIM with depressed appetite, reduced rumen fill, and lethargy. If animals fulfilled these criteria, blood BHB was measured with the Precision Xtra® meter following manufacturer instructions and as described by Iwersen et al. (2009). Only cows that presented hyperketonemia (BHB > 1.2 mmol/L) were enrolled in the study.

**Exclusion criteria**

CK cows were excluded if they had at least one concurrent disease diagnosed by a trained veterinarian at enrollment, such as DA, retained placenta, lameness, metritis, pneumonia, or clinical mastitis. Animals that developed any of these diseases within 14 d after enrollment were subsequently removed from the trial.

**Inclusion criteria**

For clinically healthy control cows (HC; n = 6) included multiparous dairy cows 2–21 DIM without any signs of disease and blood BHB <1.2 mmol/L. HC were selected by matching the CK cows by parity and ± 2 DIM. We used HC cows to establish a baseline for AT function and inflammatory markers in non-ketotic animals on the farm. One HC cow was included for every 3 CK cows enrolled. We randomly selected one of the CK cows to match with a healthy cow.

Cows were assessed for eligibility without prior knowledge of their allocation. Cows were consecutively enrolled without any interruption. Whenever a sick cow met the inclusion criteria, an AT biopsy was collected from her. This resulted in varying numbers of cows being sampled on different days, ranging from one or 2 cows to none. All the animals that had biopsies collected are included in the current study. Treatments were assigned using randomization sequences generated using an online random sequence generator (random.org) before the trial commenced, and the assigned sequences were provided to the veterinarian in charge of the trial at the farm. Once eligibility was determined, cows were moved to the chute for sampling. Subsequently, the treatment assignments were revealed.

**Treatments**

Treatments were assigned at the cow level as follows:

- **PG (n = 6):** 310 g (~300 mL) of propylene glycol (PG) administered orally 1 × /d for 5 d (Propylene Glycol, USP Kosher, Interstate chemical company, Inc., USA).
- **PGNIA (n = 6):** PG for 5 d and 24 g of niacin (NIA) administered orally 1 × /d for 3 d (NiaShure™, rumen-protected niacin, Balchem, USA).
- **PGNIAFM (n = 6):** PG for 5 d, NIA for 3 d, and 1.1 mg/kg of BW of intravenous flunixin meglumine (FM) 1 × /d for 3 d (Banamine®, Merck Animal Health, USA).
- **HC (n = 6):** healthy animals with normoketone-mia received no treatment.

Doses of PG and NIA were selected based on previous reports (Piantoni and Allen, 2015, Tienken et al., 2015). The FM dose was selected based on label recommendations. Treatments were administered at ~11:00 a.m. each day as follows: 1) PG: after animal restraint, PG was administered in a single bolus using a cattle drench gun. 2) NIA: 2 gelatin capsules (size 10, Torpac®, Fairfield, NJ, USA) containing 12 g of NIA each were given orally using a bolus gun. 3) FM: following a site preparation that included skin disinfection with 70% ethanol, FM was injected into the jugular vein. Milk from cows treated with FM was discarded for at least 36 h after the last injection. The dairy’s cow health and welfare guidelines did not allow the inclusion of an untreated CK control; therefore, all animals received treatment.

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Sampling

Before treatment administration (~11:00 a.m.), blood samples were collected on d0, d3, d7, and d14 for quantification of albumin, BHB, BUN, calcium, glucose, insulin, magnesium, NEFA, total protein, and inflammatory markers, including endotoxin and acute phase proteins [results reported in (Chirivi et al., 2023a)]. Following blood collection, subcutaneous AT (SCAT) samples were obtained from the right flank on d 0 (d0). SCAT biopsies were collected on d 7 (d7) relative to enrollment and were used to evaluate the impact of lipolysis inhibition on AT function. The second SCAT biopsy was collected 10 cm above the first biopsy. The surgical procedure was performed as described previously (Chirivi et al., 2022). Briefly, hair was removed using a clipper with a 40 mm blade. Surgical disinfection followed a 2-step process involving iodine and 70% ethanol. The local anesthesia technique was an inverted L block nerve performed with lidocaine (2%). The incision for biopsy collection was 2–3 cm long. Five grams of SCAT were collected and the biopsy site was closed using a continuous interlocking suture with Braunamid (USP1, Aesculap, Center Valley, PA, USA). Sutures were removed 7–10 d after each procedure.

Immediately after harvesting, the AT samples were divided into ~100 mg pieces. Lipolysis and flow cytometry samples were collected in Krebs Ringer Bicarbonate HEPES Buffer (KRBB, pH 7.4). Samples for lipolysis assays were transported at 37°C, while those for flow cytometry were maintained at 4°C. The transit time to the laboratory was 1 h. For histological studies, samples were immediately immersed in a 4% paraformaldehyde solution (Electron Microscopy Sciences, Hartfield, PA). Proteomics and genomics samples were immediately snap-frozen in liquid nitrogen upon collection at the farm and stored at −80°C for preservation.

In Vitro Lipolysis Assay. AT lipolysis was assessed as described by Chirivi et al. (2022). Basal lipolysis was established without the addition of any reagent. Lipolysis was induced using the β-adrenergic receptor agonist isoproterenol (ISO, I6504, Millipore-Sigma, Burlington, MA) at 10−6 M concentration. AT explants were pre-incubated (i.e., before ISO) for 1 h with 1 µg/L of insulin from bovine pancreas (I0516, Sigma-Aldrich, St. Louis, MO) to evaluate the inhibitory effect of insulin on lipolysis. After 3 h of incubation, AT explants and medium samples were collected, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

Following the procedure described by Chirivi et al. (2022), lipolysis quantification was performed using a free glycerol reagent (F6428, Millipore-Sigma) to determine the amount of glycerol released in the explant media. The intra- and inter-assay coefficients of variation were 3.9% and 6.8%, respectively. Glycerol release was normalized by the weight of the AT explant (nmol/mg), and the results are expressed as a percent of lipolysis reduction compared with ISO-induced lipolysis. All samples were run in duplicate.

RNA Extraction from Adipose Samples. RNA was extracted from SCAT samples using a TRIzol-based method, as previously described in Chirivi et al. (2022). RNA was stored at −80°C, and the concentration and integrity of total RNA were evaluated using a NanoDrop OneC spectrophotometer (Thermofisher Scientific, Waltham, MA, USA). All samples had a 260:280 nm ratio between 1.9 and 2.02. Reverse transcription was performed with 400 ng of RNA using 4 µL of the qScript cDNA SuperMix (95048 Quantabio, Beverly, MA, USA) for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. cDNA was stored at −20°C.

Gene expression analysis. Transcriptional studies were performed using the QuantiStudio™ & Flex System (Applied Biosystems Inc., Waltham, MA). SYBR gene expression primers were used for qPCR assays and were either commercially available or designed from bovine sequences and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Samples were assayed in duplicate, each 10 µL PCR reaction contained 1X (5 µL) of SYBR™ Green PCR Master Mix (4309155, Applied Biosystems), 400 nM of primer assays [sequences listed in Supplemental Table S1 (Chirivi et al., 2023b)], and 5 ng of sample cDNA. A non-template control and a non-reverse-transcriptase control monitored contamination and primer-dimer formation that could produce false-positive results and validated the absence of genomic DNA. The following cycling conditions were used: initial enzyme activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, and annealing at 60°C for 60 sec. Housekeeping genes with the lowest pairwise variation values, including eukaryotic translation initiation factor 3 subunit K (EIF3K), ribosomal protein lateral stalk subunit P0 (RPLP0), and ribosomal protein S9 (RPS9) were used. The expression of genes of interest was normalized against the geometric mean of selected housekeeping genes’ CT. Quantification cycle values were extrapolated using the 2−ΔΔCT method. Gene expression data are presented as geometric means ± 95% CI.
Protein analysis. Proteins were extracted from ~100 mg of snap-frozen SCAT samples as described previously (Chirivi et al., 2022). Protein quantification was performed in the Simple Protein Wes system (SM-W004; ProteinSimple, Santa Clara, CA, USA) using capillary electrophoresis with 12–230 kDa Wes Separation Module capillary cartridges (Nelson et al., 2017). An optimal concentration of 0.75 mg/mL of protein was determined and used on all antibodies tested. Rabbit monoclonal antibodies were obtained from Cell Signaling (Danvers, MA, USA). Akt [(1:50), Cat#9272s], ERK1/2 [(1:50), Cat# 9102], phosphorylated (Ser563) HSL [(1:25) Cat#4139], phosphorylated (Ser473) Akt [(1:25), Cat#9271t], phosphorylated (Thr202/Tyr204) ERK1/2 [(1:50), Cat#9101]. Anti-HSL was from ThermoFisher Scientific [1:25; Cat#PA5–17196]. The anti-rabbit-detection module for the Wes kit (DM-001, ProteinSimple) included Luminol-S, Peroxide, antibody diluent 2, streptavidin-HRP, and anti-rabbit secondary antibody. Sample proteins were allowed to separate by microcapillary electrophoresis and the chemiluminescence signal peaks were generated for analysis. These signal peaks were transformed into digital images depicting bands as observed in Western blot analysis. These signal peaks were transformed into digital images depicting bands as observed in Western blot analysis. Using Compass software (ProteinSimple), the peak areas of proteins were estimated. The peak areas are directly proportional to the amount of target protein quantified. The normalized data are expressed as a ratio of phosphorylated protein over total protein: pHSL(Ser563):HSL, pAkt:Akt, and pERK1/2:ERK1/2. To validate protein detection, overexpression lysates were used as positive controls for Akt (LC401580, OriGene Technologies, Rockville, MD, USA), HSL (LC417354, OriGene Technologies), and ERK1/2 (042–488, ProteinSimple).

Flow Cytometry. Cells from SCAT’s stromal vascular fraction (SVF) were obtained by collagenase digestion. Briefly, SCAT (1–2 g) was minced in 4% BSA dissolved in Hank’s balanced salt solution, 10mM HEPES, and Liberase™ TL Research grade (5401020001, Roche, Indianapolis, IN) and incubated at 37°C for 60 min. Samples were then resuspended in 15 mL of 4% BSA solution, passed through 70µm (22363548, Fisherbrand, Pittsburgh, PA) and 40µm (22363547, Fisherbrand) cell strainers, and centrifuged at 300 x g for 5 min at 4°C. Next, the SVF pellet was resuspended and incubated in RBC lysis buffer (420301, Biologend Inc., San Diego, CA) for 5 min at room temp and centrifuged 300 x g for 5 min at 4°C. The SVF pellet obtained was then resuspended and incubated for 30 min at 4°C with 100 µg/mL of purified bovine IgG (Sigma-Aldrich) in 1X Dulbecco’s PBS, 2% FBS, 2 mM EDTA, 10 mM HEPES as blocking reagent for non-specific antibody binding. All samples were incubated with Biologend Zombie NIR fixable viability dye (423106, Biolegend Inc., San Diego, CA) diluted 1:500 in FACS solution [1X Dulbecco’s PBS, 2% FBS, and 0.1% sodium azide (26628–22–8, ThermoFisher Scientific)] to exclude dead cells. Afterward, SVF-derived cells were incubated with conjugated monoclonal primary antibodies for 30 min. Before conducting the experiments, the optimal concentrations of all primary antibodies were determined through titration [antibodies list and dilutions described in Supplemental Table S2 (Chirivi et al., 2023b)]. Following primary incubation, cells were washed, fixed with 2% paraformaldehyde in PBS, washed, and resuspended in Dulbecco’s PBS, 2% FBS, 2 mM EDTA, and 10 mM HEPES.

For gating selection, a fluorescence minus one (FMO) control was made for all markers using one sample in each experiment. Compensation beads were used as single-stained control samples. Data acquisition and compensation were performed in Cytex® Aurora System (Cytek Biosciences, Fremont, CA) using the SpectroFlo® software (Cytek Biosciences) and analyzed in FCS express V.7 (DeNovo Software, Pasadena, CA). UltraComp eBeads beads were used for compensation. A gate was drawn to allow the exclusion of aggregates/doublets and another to exclude cellular debris. Gating strategies are summarized in Supplemental Figure 1S (Chirivi et al., 2023b).

Hematopoietic cells (CD45+) are reported as % of total SVF cells. ATM were selected from CD45+ cells and defined by CD172a and MHC class II (MHCII) expression. Macrophage phenotype populations were defined as: M1 (CD45+, CD172a+, MHCII+, CD14+), M2 (CD45+, MHCII+, CD11b+), and TREM2 metabolically active ATM (CD45+, TREM2+). The results are expressed as the percentage of the specified population relative to the total hematopoietic cell count.

Histology. The SCAT biopsies were fixed in 4% paraformaldehyde, blocked in paraffin, and then sectioned into 4 µm slices by the Michigan State University Investigative Histopathology Laboratory (East Lansing, MI, USA). Following pretreatment protocol, standard micro-polymer staining was performed at room temperature on the IntelliPATH automated stainer (Biocare Medical, Pacheco, CA). Rinses followed all staining steps in TBS Auto-wash Buffer (Biocare Medical). Non-specific protein was blocked using Background Punisher (Biocare Medical) for 10 min. A mouse monoclonal antibody against CD172a (1:50; DH59B, Washington State University Monoclonal Antibody Center, Pullman, WA) detected macrophages and mononuclear immune cells in AT. Anti-CD172a was diluted in Normal Antibody Diluent (Cytex Laboratories, Inc. Logan, UT), and slides were incubated with the solution overnight at 4°C. Sections were counterstained with...
CATHE Hematoxylin diluted 1:10 (Biocare Medical) as described previously (Abou-Rjeileh et al., 2023). Digital images were collected from entire tissue sections using the Olympus VS200 Research Slide (Olympus Corporation, Tokyo, Japan). The areas of adipocytes in 8 randomly selected fields per section were measured using the Adiposoft plugin (v. 1.15) for ImageJ Fiji (v. 2.0.0) as described by Abou-Rjeileh et al. (2023). Adipocyte areas were divided into 7 bins ranging from smaller (<1499 µm²) to larger (>9000 µm²) adipocytes. CD172a signal intensity was measured in the same images using ImageJ as described in (Crowe and Yue, 2019). All image captures and signal quantification were conducted with the analyst blind to treatments.

Statistical analysis.

The sample size was calculated on JMP Pro16 (SAS Inst., Inc., Cary, NC) following the guidelines for the superiority of one treatment in clinical trials (Zhong, 2009). Based on the percent of macrophage infiltration, previously observed in studies by our laboratory (Contreras et al., 2015; De Koster et al., 2018b), assuming a power of 80%, a confidence level of 95%, and a minimum difference of 18.5% in ATM infiltration between 2 groups, a minimum sample size of 6 cows was calculated. All variables were analyzed in JMP Pro16. A non-parametric Mann-Whitney test determined the statistical differences between CK and HC at d0. The protein, PCR, and IHC data of the CK treatment at d0 was normalized by the HC data on the same day and is reported as “relative to HC” in the figures. Data from d7 is expressed as a fold change relative to the corresponding values from the HC group on that same day. We used this approach to convey both the extent of change and the impact of treatments unless specified otherwise. RNA data were transformed (Log10). Results are presented as mean ± SEM unless stated otherwise. Significance was declared at \( P \leq 0.05 \). At d7, treatment effects were evaluated by Friedman’s 2-way ANOVA test, and Tukey’s post hoc adjustment was used for pairwise comparisons.

RESULTS

Descriptive statistics of the study population are summarized in Supplementary Table S3 (Chirivi et al., 2023b). At enrollment, DIM, parity, number of cows allocated, and previous lactation days dry were equally distributed among treatments, including the HC group [Supplementary Table S3 (Chirivi et al., 2023b)]. At the time of enrollment, no meaningful differences existed in any of the parameters examined among the CK cows allocated to the 3 treatment groups.

CK Increases AT Lipolysis Markers

As expected, blood BHB, NEFA, and haptoglobin were higher in CK cows at d0 compared with the HC group [Supplemental Table S3 (Chirivi et al., 2023b)]. Consistent with this observation, the transcription of AT neutral lipases, including \( \beta \)-hydrolase domain containing 5 (ABDH5), hormone-sensitive lipase (Lipe), and lipoprotein lipase (LPL), increased in CK cows compared with HC (Figure 1A). Genes related to FA synthesis and transport, including perilipin, proliferator-activated receptor α (PPARA) and gamma (PPARG), were upregulated in CK cows. In contrast, CK did not affect the expression of the mitochondrial FA transporter CPT1 (Figure 1A). To characterize AT lipolytic activity, we quantified HSL and its phosphorylation (pHSL). At d0, pHSL: HSL in CK cows increased 4.03 ± 1.38-fold compared with HC (Figure 1B). Adipocyte size distribution is a proxy for lipolysis and triacylglyceride storage within the fat cells. As such, AT from CK presented a higher frequency of smaller adipocytes (<1499 µm²) compared with HC (Figure 1C).

AT Insulin Sensitivity is Reduced During CK

Compared with HC, CK cows showed higher transcription of the genes encoding the fatty acid transporter FA binding protein (FABP4) and the triglyceride synthesis enzyme phosphatidic acid phosphodiesterase lipin1 (LIPIN1) at d0. In contrast, the expression of the solute carrier family 2-member 4 (SLC2A4) was reduced in CK compared with HC (Figure 2A). No changes were observed in the expression of insulin receptor substrate 1 (IRS1). We quantified Akt protein phosphorylation to evaluate adipocyte insulin signaling activity. At d0, pAkt: Akt was reduced by 60 ± 6.4% in CK compared with HC (Figure 2B). AT insulin sensitivity was also measured ex vivo by assessing the capacity of the pancreatic peptide to reduce adipocytes’ lipolytic response upon \( \beta \)-adrenergic-stimulation (ISO). As expected, insulin reduced glycerol release by 41% in SCAT from HC. In contrast, SCAT from CK cows were unresponsive to insulin (−9.5%; Figure 2C).

CK Induces AT Inflammation

AT d0, CK cows exhibited upregulation of inflammatory gene markers such as interleukin 8 (IL8) and 10 (IL10), toll-like receptor 4 (TLR4), and tumor necrosis factor (TNF) compared with HC cows (Figure 3A). On the same day, genes associated with macrophage chemotaxis, including chemokine (C-C motif) ligand 2 (CCL2) and osteopontin (SPP1), and the macrophage marker signal regulatory protein α (SIRPA), which
encodes for CD172A, were upregulated in AT from CK cows (Figure 3A). The transcription of \( \text{LOC527744} \), encoding the BHB receptor HCAR2, was upregulated in CK vs. HC (Figure 3A). Unexpectedly, \( \text{IL6} \) was downregulated in CK compared with HC cows (Figure 3A). We quantified pERK1/2:ERK1/2 protein content as a marker of inflammatory signaling in AT. At d0, AT’s pERK1/2:ERK1/2 in CK cows was 1.6 ± 0.42 fold higher than in HC (Figure 3B).

**CK Increases ATM Infiltration**

Flow cytometry of live SVF cells from SCAT showed no differences in the frequency of hematopoietic cells (CD45+) between CK and HC at d0 (29.91% vs. 21.14%; \( P = 0.16 \); Figure 4A). ATM were selected from live cells expressing CD45+ [gating strategies and immune cell phenotyping details are described in Supplemental Figure S1 (Chirivi et al., 2023b)]. At d0, CK had a higher ATM frequency than HC (22.96% vs. 13.24%; Figure 4B). On the same day, M1 ATMs were more abundant in CK vs. HC (7.38% vs. 2.66%; Figure 4C). Similarly, the percentage of M2 ATM at d0 was elevated in CK vs. HC (6.3% vs. 2.4%; Figure 4D). CK cows had fewer TREM2 anti-inflammatory metabolically active ATM compared with HC (1.27% vs. 3.98%; Figure 4E). To complement flow cytometry analyses, ATM infiltration was evaluated by immunohistochemistry using an antibody against the ATM marker CD172a with horse-radish peroxidase (HRP) labeling in SCAT sections. Compared with HC, CK cows had a 2.3-fold higher HRP signal (Figure 5).
Next, we evaluated the impact of lipolysis inhibition on AT function on SCAT biopsies collected at d7. On this day, AT from PGNIAFM showed 1.3- and 1.9-fold higher pAkt:Akt compared with PG and PGNIA, respectively (Figure 6A). In line with enhanced Akt signaling, lipolysis assays at d7 indicated insulin reduced lipolysis by 36.5% in SCAT from PGNIAFM (Figure 6B). In contrast, SCAT from PG and PGNIA remained unresponsive to the antilipolytic effect of insulin, showing no lipolysis reduction (−16.97% and −7.39% respectively; Figure 6B). By d7, PGNIAFM cows presented a higher distribution of large adipocytes (7499–8999 µm²) and tended to have fewer small adipocytes (<1499 µm²) vs. PG and PGNIA (Figure 6C). No treatment effect was observed in the expression of genes related to insulin sensitivity and FA metabolism (Table 1). AT from PGNIAFM cows showed the lowest expression of the LOC5527744 gene by d7 (Table 1). LPL gene expression was reduced in AT from PGNIAFM vs. PG and PGNIA (Table 1). There were no treatment effects on pHSL: HSL (Figure 6D).

**PGNIAFM Improves AT Insulin Sensitivity in CK Cows**

Figure 2. Cows with CK have reduced adipose tissue insulin sensitivity. Subcutaneous adipose tissue (SCAT) samples were collected from healthy (HC) or clinical ketosis (CK; BHβ > 1.2 mmol/L) periparturient dairy cows at enrollment (d0). (A) Fold change of FABP4, LPIN1, SLC2A4, and IRS1 gene expression relative to HC (2−ΔΔCT). Gene expression was normalized by reference genes EIF3K, RPL19, and RPS9. (B) Protein abundance of Akt and phosphorylated Akt (pAkt) at Ser473 relative to HC. Bands were detected at 60 kDa. (C) Percentage of lipolysis reduction relative to isoproterenol (ISO: 1 µM) induced lipolysis in SCAT explants exposed to insulin (ISO+INS: 1 µg/L) for 3h. Bars with * (P < 0.05), ** (P < 0.01), *** (P < 0.001) are different. Gene expression data are geometric means ± 95% CI. Protein data are means of pAkt:Akt. Each dot represents an individual animal. Lipolysis reduction data are means ± SEM. HC n = 6, CK n = 18.

**PGNIAFM Reduces AT Inflammation and ATM Trafficking**

Although treatments had no effect on the expression of genes encoding cytokines (Table 1), the pERK1/2:ERK1/2 in AT from PGNIAFM was reduced by 54.1% and 60.4 ± 18% when compared with PG and PGNIA, respectively (Figure 7). Immunohistochemical evaluation of ATM (CD172a⁺) abundance in SCAT shows no HRP signal difference among treatment groups (Table 2). By d7, the flow cytometry analysis demonstrated no differences in the number of hematopoietic cells among the 3 treatment groups (Table 2).
The frequency of ATM (CD45⁺, CD172a⁺, MHCII⁺) was reduced in SCAT from PGNIAFM cows, compared with PG and PGNIA groups (\( P = 0.03 \); Table 2). The frequency of M1 ATM (CD45⁺, CD172a⁺, MHCII⁺, CD14⁺) tended to be lower in PGNIAFM compared with PG and PGNIA cows (\( P = 0.09 \); Table 2). Similarly, PGNIAFM had a lower frequency of M2 ATM (CD45⁺, MHCII⁺, CD11b⁺) compared with PG and PGNIA (\( P = 0.01 \)). Finally, there were no differences in the abundance of anti-inflammatory metabolically active ATM (CD45⁺, TREM2⁺) among groups.

**DISCUSSION**

Despite major advances in the management and prevention of CK, the effects of the disease on AT function are not entirely understood and represent a significant knowledge gap. Furthermore, PG, the most widely recommended treatment for CK, is not completely effective in controlling the disease, having a resolution rate of 42% after one week of treatment (Gordon et al., 2017). The reasons behind this poor efficacy are likely associated with the minimal effect of PG on AT lipolysis and systemic inflammation, which are significant factors in...
CK pathogenesis. The present study demonstrates that cows with CK have impaired AT function, as evidenced by lipolysis dysregulation, inflammation, and insulin resistance. Our results also demonstrate that using the lipolysis inhibitors NIA and FM combined with PG improved AT function by recovering its insulin sensitivity and reducing inflammation. These AT responses observed after treatment with PGNIAFM align with the reduction in systemic inflammation, circulating ketone bodies, and dyslipidemia biomarkers reported in the companion paper (Chirivi et al., 2023a).

**AT dysfunction during CK**

Previous studies demonstrate that AT lipolysis is dysregulated and becomes intense and protracted during CK (Contreras et al., 2015). Lipolysis dysregulation is defined as the impaired response of adipocytes to insulin-induced antilipolytic and pro-lipogenic effects (Contreras et al., 2017b). Accordingly, CK cows in the present study had higher adipocyte triglyceride hydrolysis, which was reflected in a higher frequency of smaller adipocytes (<1499 µm²) and an increase in lipolysis biomarkers NEFA and BHB [described in detail in the companion paper (Chirivi et al., 2023a)]. Our findings are in line with the reduced adipocyte diameter observed in cows with DA and ketosis (Contreras et al., 2015). The present study investigated the underlying mechanisms contributing to CK cows' lipolysis dysregulation. Our findings demonstrate that this dysregulation is driven by enhanced activity of neutral lipases (e.g., HSL, ATGL), limited lipogenesis, and AT insulin resistance and inflammation.

**Enhanced lipase activity.** Lipolysis is regulated by the transcription of neutral lipases LIPE, LPL, and PNPLA2. Post-translationally, kinase activity (i.e., phosphorylation) on HSL and the activation of ATGL’s coactivator CGI-58 (encoded by ABHD5) modulate lipolysis intensity (Grabner et al., 2021). In healthy periparturient cows, lipases’ gene transcription is reduced, but lipase activity remains high, as indicated by pHSL: HSL levels (Koltes and Spurlock, 2011; De Koster et al., 2018a). In the present study, lipase gene transcription was upregulated in CK cows, indicating that lipolysis dysregulation in these animals may originate at the transcriptional level. Likewise, dysregulated lipolysis at the transcriptional level has been observed in animal models of lipodystrophy, where higher transcription of

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**Figure 4.** Cows with CK have increased adipose tissue macrophage trafficking. Flow cytometry analysis of stromal vascular fraction (SVF) cells from subcutaneous adipose tissue (SCAT) collected from healthy (HC) or clinical ketosis (CK; BHB > 1.2 mmol/L) periparturient dairy cows at enrollment (d0). Hematopoietic cells (CD45+) in total live SVF cells (A). Macrophages [CD45+ CD172a+ , and MHC class II (MHCII+) ] in total hematopoietic cells (B). Classically activated (M1) macrophages (CD45+, CD172a+, MHCII+, CD14+) in total hematopoietic cells (C). Alternative activated (M2) macrophages (CD45+, MHCII+, CD11b+) in total hematopoietic cells (D). Foamy cells (CD45+, TREM2+) in total hematopoietic cells (E). Bars with * (P < 0.05) or ** (P < 0.01) differ. Each dot represents an individual animal. Data are means ± SEM. HC n = 6, CK n = 18.
Figure 5. Cows with CK have increased macrophage infiltration in adipose tissue. Subcutaneous adipose tissue (SCAT) samples were collected from healthy (HC) or clinical ketosis (CK; BHB > 1.2 mmol/L) periparturient dairy cows at enrollment (d0). Representative images of tissue sections after immunohistochemical staining using CD172a antibody to detect adipose tissue macrophages (ATM) in HC (A-B) or CK cows (C-D). Bovine spleen (SP) was used as positive control (E-F). Arrows point to crown-like structures and asterisks indicate a sole ATM throughout the tissue. (G) Abundance of CD172a antibody signal in adipose tissue sections relative to HC. Bars with * are different (P < 0.05). Each dot represents an individual animal. Bars are means ± SEM. HC n = 6, CK n = 18.
and PNPLA2, along with an increase in ATGL and HSL abundance, are associated with intense lipolysis (Dettlaff-Pokora et al., 2016). Limited lipogenesis. In healthy periparturient cows, adipocytes adapt to increased release of FA by enhancing CPT1A and CPT2 transcription (Elis et al., 2013). The proteins encoded by these genes facilitate FA transport into the mitochondria for oxidation. Our results provide evidence that CK affects adipocyte capacity to transport FA into mitochondria. Despite the higher availability of FA, CK cows did not have changes in CPT1A transcription. Lipolysis products activate pro-lipogenic and adipogenic pathways, such as PPARs, in adipocytes. These pathways serve as negative feedback loops to limit the excessive depletion of triglyceride reserves (Mottillo et al., 2012). In the present study, we found that CK cows had increased transcription of PPAR genes, but the lipogenic responses triggered by these nuclear transcription factors were not activated. AT insulin resistance. Defined as the reduced biological response to the pancreatic peptide, including stimulation of lipogenesis and reduction of lipolysis (Ronald Kahn, 1978). To reduce lipolysis, Akt phosphorylation activates phosphodiesterase 3b, the enzyme involved in the degradation of cAMP, which consequently lowers HSL activity and lipolysis (Degerman

Figure 6. PGNIAFM treatment improves adipose tissue insulin sensitivity during clinical ketosis. Subcutaneous adipose tissue (SCAT) samples were collected from dairy cows that were healthy (HC) or had clinical ketosis (BHB > 1.2 mmol/L) after 7 d of enrollment (HC) or treatment with: PG = Propylene Glycol, PGNIA = PG + Niacin, or PGNIAFM = PGNIA + flunixin meglumine. (A) Protein abundance of Akt and phosphorylated Akt (pAkt) at Ser473. Bands were detected at 60 kDa. (B) Percentage of lipolysis reduction relative to isoproterenol (ISO; 1 µM) induced lipolysis in SCAT explants exposed to insulin (ISO+INS; 1 µg/L) for 3h. (C) Frequency distribution of adipocyte area. (D) Protein abundance of HSL and phosphorylated HSL (pHSL) at Ser563 relative to HC. Bands were detected at 81 kDa. Bars with different letters (a-b) differ significantly \( (P < 0.05) \). Bars with * differ \( (P < 0.05) \). Each dot represents an individual animal. Data are means ± SEM. Protein data are means of the phosphorylated: total protein relative to HC. n = 6 cows per treatment.
et al., 1998). To the best of our knowledge, this study is the first to evaluate AT lipolytic responses in cows with CK. We report that CK cows had reduced Akt phosphorylation, which was evident in a diminished antilipolytic effect when AT was incubated with insulin. Our results are consistent with previous AT proteomic analyses in cows with insulin resistance, which showed decreased Akt signaling and increased HSL and ERK/MAPK activity (Zachut, 2015). The mechanisms leading to the development of AT insulin resistance appear to be multifactorial and likely involve a combination of lipid overflow, inflammation, adipokine dysregulation, genetic traits, and mitochondrial dysfunction (Li et al., 2022). In the present study, we identified systemic and AT inflammation as factors that will directly impair insulin sensitivity in AT.

**AT inflammation.** In this study, CK cows had increased transcription of pro-inflammatory cytokines (CCL2, IL8, IL10, IR3P, SPP1, TLR4, TNF) in AT. This response may be triggered by the presence of lipolytic products in AT and endotoxia. AT inflammation is characterized by the infiltration of myeloid immune cells, proliferation of resident immune cells, including AT, and increased secretion of inflammatory mediators (Kawai et al., 2021). Lipolysis is another important trigger of AT inflammation, as it enhances the biosynthesis of lipid mediators of inflammation derived from linoleic and arachidonic acids that enhance macrophage recruitment (Contreras et al., 2017a). Therefore, the excessive AT inflammation observed in this study in CK cows is likely due to intense lipolysis during CK.

A second reported mechanism involved in AT inflammation is endotoxemia (Clemente-Postigo et al., 2019). In the companion paper, we report that over 70% of CK cows exhibited endotoxemia (Chirivi et al., 2023a), which aligns with the findings of the present study showing CK cows had increased transcription of TLR4 and heightened phosphorylation of ERK1/2 in AT. The ERK1/2 cascade plays a role in various cellular processes, including stress response, inflammation, and lipolysis. In humans and rodents with diabetes, ERK1/2 signaling has been associated with AT insulin resistance (Carlson et al., 2003). Despite limited research on AT function during CK, our findings align with previous proteomic analyses, indicating increased inflammatory signaling through the NFκB and ERK1/2 pathways, resulting in elevated cytokine expression in AT during ketosis (Xu et al., 2019). Taken together, these results suggest that circulating LPS may potentially trigger

### Table 1. Relative gene expression in subcutaneous adipose tissue from cows treated for clinical ketosis

<table>
<thead>
<tr>
<th>Gene Network</th>
<th>Gene</th>
<th>Treatment allocation¹</th>
<th>PG</th>
<th>PGNIA</th>
<th>PGNIAFM</th>
<th>P-Value²</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>GM² LCI UCI</td>
<td></td>
<td>GM² LCI UCI</td>
<td>GM² LCI UCI</td>
<td></td>
</tr>
<tr>
<td>Lipid mobilization</td>
<td>ABHD5</td>
<td>0.59 0.14 2.49</td>
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<td>0.32 0.06 1.70</td>
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<td></td>
<td>CPT1A</td>
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<td>0.74 0.32 1.73</td>
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<td></td>
<td>LIPE</td>
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<td></td>
<td>LOC527744</td>
<td>2.63 0.79 8.68</td>
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<td>0.24 0.06 0.92</td>
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<tr>
<td></td>
<td>LPL</td>
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<td>0.06 0.02 2.00</td>
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<td></td>
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<td>0.28b 0.09 0.81</td>
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<tr>
<td>Insulin sensitivity</td>
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<td>0.08 0.01 0.76</td>
<td>0.09 0.01 0.89</td>
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<tr>
<td></td>
<td>GLUT4</td>
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<td></td>
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<td>LPIN1</td>
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<td>0.11 0.02 0.68</td>
<td>0.33</td>
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</tr>
<tr>
<td>Inflammation</td>
<td>CCL2</td>
<td>2.39 0.73 7.83</td>
<td>2.97 0.91 9.72</td>
<td>0.88 0.27 2.86</td>
<td>0.29</td>
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<td></td>
<td>IL-6</td>
<td>0.17 0.06 0.51</td>
<td>0.34 0.10 1.13</td>
<td>0.21 0.07 0.61</td>
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<td></td>
<td>IL-8</td>
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<td>0.43 0.07 2.86</td>
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</tr>
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<td></td>
<td>IL-10</td>
<td>0.65 0.07 5.94</td>
<td>1.11 0.12 10.09</td>
<td>1.09 0.12 9.90</td>
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</tr>
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<td></td>
<td>IRF3</td>
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<td>0.90 0.38 2.09</td>
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</tr>
<tr>
<td></td>
<td>SPP1</td>
<td>1.59 0.42 5.96</td>
<td>1.47 0.39 5.53</td>
<td>0.37 0.08 1.62</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>0.09 0.01 0.81</td>
<td>0.30 0.03 2.67</td>
<td>0.08 0.01 0.80</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNFA</td>
<td>0.84 0.12 6.58</td>
<td>1.06 0.16 7.20</td>
<td>0.15 0.02 1.31</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

¹Clinical ketosis (CK, BHB > 1.2 mmol/L) treatments: PG = Propylene Glycol, PGNIA = PG + Niacin, PGNIAFM = PGNIA + flunixin meglumine.
²Geometric mean of Logⁱ₀ transformed data. LCI-UCI = Lower and upper 95% CI. Gene expression values were calculated using 2ΔΔCt method. ΔΔCt = ΔCtCalibrator sample - ΔCtTarget sample. ΔCt values were normalized to the geometric mean of EIF3K, RPLPO, and RPS9 housekeeping genes. HC samples were the calibrator for the calculation of the ΔΔCt. Data is fold change compared with HC.
³P-values refer to the main effect of treatment. P-values are from log₁₀ transformed data analysis.
⁴Mean values in the same row with different superscripts differ (P < 0.05) adjusted by Tukey. n = 6 cows per group.
metabolic dysfunction and inflammation in AT during CK. In the present study, CK cows exhibited increased ATM infiltration, including cells of both M1 and M2 phenotypes. This finding coincides with previous reports in cows with DA and CK (Contreras et al., 2015). Enhanced M1 polarization in CK cows may be due to TLR4 stimulation and TNF pathway activation. M1 ATM produce pro-inflammatory cytokines and chemokines that enhance the migration of monocytes (CCL2, IL10, SPP1), neutrophils, and basophils (IL8, SIRPA) into AT, resulting in enhanced phagocytic activity (Atri et al., 2018). To our knowledge, this is the first study identifying TREM2 ATM in postpartum cows. We observed a decrease in the frequency of TREM2 ATM during CK. TREM2 cells are specialized macrophages that contain lipids and have beneficial effects such as promoting the resolution of inflammation, lipid uptake, and removal of dying cells (Liebold et al., 2023). The reduction of TREM2 is associated with impaired macrophage ability to uptake oxidized lipids and cholesterol, which can lead to insulin resistance (Coats et al., 2017). While the decreased expression of TREM2 ATM during CK suggests a diminished capacity to clear excessive lipids, the specific role of TREM2 in metabolic inflammation in dairy cows remains to be determined. In summary, while AT inflammation serves a physiological purpose in tissue remodeling, dysregulated inflammation, as seen in CK, may initiate a deleterious cycle that links processes such as lipolysis, ATM infiltration, inflammation, and insulin resistance. It is important to note that CK cows also show signs of inflammation resolution in the present study. For example, M2 ATM trafficking increased in CK cows, suggesting an active inflammation resolution process within the AT. The M2 phenotype is responsible for resolving inflammation by increasing phagocytosis of apoptotic cells and producing angiogenic and chemotactic products that promote tissue repair (Fujisaka, 2021).

### Figure 7. PGNIAFM treatment reduced adipose tissue inflammation
Subcutaneous adipose tissue (SCAT) samples were collected from dairy cows that were healthy (HC) or had clinical ketosis (BHB > 1.2 mmol/L) after 7 d of enrollment (HC) or treatment with: PG = Propylene Glycol, PGNIA = PG + Niacin, PGNIAFM = PGNIA + flunixin meglumine. Protein abundance of extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphorylated ERK1/2 (pERK1/2) at Thr202/tyr204 relative to HC. Bands were detected at 44 kDa. Bars with different letters (a-b) differ significantly (P < 0.05) adjusted by Tukey. n = 6 cows per group.

### Table 2. Macrophage quantification in subcutaneous adipose tissue from cows treated for clinical ketosis

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Marker</th>
<th>Treatment Allocation</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>Macrophages</td>
<td>CD172a</td>
<td>2.96</td>
<td>4.06</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Hematopoietic</td>
<td>CD45+</td>
<td>59.04</td>
<td>47.97</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Macrophages</td>
<td>CD45+ CD172a+ MHCII+</td>
<td>13.61</td>
<td>17.55</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>M1</td>
<td>CD45+ CD172a+ MHCII+ CD14+</td>
<td>9.76</td>
<td>11.06</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>M2</td>
<td>CD45+ MHCII+ CD11b+</td>
<td>7.86</td>
<td>5.75</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>TREM2</td>
<td>CD45+ TREM2+</td>
<td>4.40</td>
<td>7.14</td>
</tr>
</tbody>
</table>

1Clinical ketosis (CK, BHB > 1.2 mmol/L) treatments:
PG = Propylene Glycol, PGNIA = PG + Niacin, PGNIAFM = PGNIA + flunixin meglumine.
2P-values refer to the main effect of treatment.
3IHC = Immunohistochemistry. Data are fold change ± SEM of signal intensity of CD172a antibody in tissue sections relative to HC.
4Data are the mean distribution (%) ± SEM of cells identified. Hematopoietic cells distribution is from the total of live stromal vascular cells. Macrophages, M1, M2 and TREM cells frequencies are from the total of hematopoietic cells.
5Mean values in the same row with different superscripts differ (P < 0.05) adjusted by Tukey. n = 6 cows per group.
cows may be associated with increased IL10 production, a cytokine known for activating anti-inflammatory M2 macrophages (Lopes et al., 2016). M2 polarization is also promoted by BHB in a STAT6-signaling pathway-dependent manner (Huang et al., 2022). Therefore, the increased concentration of BHB during CK likely induces the M2 phenotype (as observed in the present study). Growing evidence suggests ketogenesis could be an endogenous protective mechanism to reduce inflammation and regulate macrophage activation. (Zhang et al., 2022).

We also observed upregulation of LOCS527744, the gene encoding for the BHB receptor HCAR2 in CK cows. Previous reports indicate that HCAR2 mRNA was upregulated in SCAT from postpartum dairy cows, coinciding with the period of higher lipolysis (Lemor et al., 2009). BHB has several effects on AT, including anti-inflammatory effects and the inhibition of lipolysis by HCAR2 signaling (Kenéz et al., 2014; Mielenz, 2016). In rodents, HCAR2 activation enhances AMPK/Nrf2-mediated autophagy, reducing SCAT inflammation (Gao et al., 2021). HCAR2's antilipolytic effect is mediated by PKA/HSL signaling (Kenéz et al., 2014). Although the role of the BHB receptor is not clear in AT during hyperketonemia, our data suggest that the upregulation of this receptor may be a response to inflammation or an attempt to mitigate excessive lipolysis during CK. More research is needed to understand the physiological role of HCAR2 and its ligands during CK in cows.

**PGNIAFM Improves AT Function in CK Cows**

In the larger randomized clinical trial (Chirivi et al., 2023a), cows treated with PGNIAFM recovered from hyperketonemia and had reduced lipolysis and systemic inflammation [Supplemental Figure S2 (Chirivi et al., 2023b)]. Aligning with the clinical observations, the subset of cows treated with PGNIAFM reported in the present study had improved AT insulin sensitivity and reduced AT inflammation. In contrast, treatment with PG alone did not enhance AT insulin sensitivity. This finding may account for the higher lipolysis rates observed in cows treated with PG compared with PGNIAFM-treated ones. In cattle, PG may indirectly reduce lipolysis by increasing blood glucose levels and limiting the utilization of BHB and NEFA as energy sources (Grummer et al., 1994). Nonetheless, in the present study, it appears that PG does not directly influence lipolysis during CK. Our findings align with the limited effect of PG on AT lipolysis during the treatment of CK observed by (Gordon et al., 2017). In a study by Bjerre-Harpøth et al. (2015) that focused on periparturient over-conditioned cows, supplementation of 500 g/day of PG during the first 4 weeks postpartum did not result in any changes in AT proteome when compared with the placebo group. Although research investigating the effects of PG on AT function during ketosis is limited, it was demonstrated that even prolonged periods of PG supplementation do not improve AT function in cows at a high risk of dysregulated lipolysis (Bjerre-Harpøth et al., 2015).

In the present study, combining PG and 24 g of NIA for 3 d (PGNIA) did not improve AT insulin sensitivity. This finding suggests inhibiting the canonical pathway with NIA alone was insufficient in controlling the dysregulation of lipolysis and insulin resistance in AT from cows with CK. Although the mechanisms by which NIA inhibits canonical lipolysis are well described (Tunaru et al., 2003; Hristovska et al., 2017), its effects on insulin sensitivity in healthy cows vary across studies. For example, a study supplementing 120 g/d of unprotected NIA (equivalent to ~12g/day of absorbed NIA) to dairy cows starting 14 d before expected calving date until 14 d after parturition, reported improved systemic insulin sensitivity, as measured by the RQUICKI insulin sensitivity index (Hristovska et al., 2017). However, another study supplementing 24 g/d of unprotected NIA during 21 d before and 21 d after parturition did not alter the expression of proteins related to insulin signaling in AT (Kinoshita et al., 2016).

In the present study, we observed that PGNIAFM promoted the recovery of AT insulin sensitivity by enhancing the phosphorylation of Akt. Consequently, PGNIAFM-treated cows mobilized less fat from AT, resulting in a higher frequency of large adipocytes and a tendency for a reduced number of small adipocytes (<1400 microns). These results align with the reduction in lipolysis and hyperketonemia described in the companion paper (Chirivi et al., 2023a). Combining NIA and FM in PGNIAFM simultaneously blocks the canonical and inflammatory lipolytic pathways. FM inhibits the systemic activity of COX, which in turn suppresses inflammation and specifically the synthesis of prostaglandin E2 (PGE2), a known lipolytic agent, that enhances the activity of ERK1/2 (Bashir et al., 2020; Inazumi et al., 2020). Therefore, it is plausible to speculate that the observed reduction in lipolysis in PGNIAFM-treated cows can be attributed to the downregulation of ERK1/2 expression, likely resulting from the decreased synthesis of prostaglandins. It is important to highlight that FM can alleviate pain, enhance DMI, and elicit systemic effects that ultimately lead to improved AT function. More details about these mechanisms can be found in the companion paper’s clinical responses section. (Chirivi et al., 2023a)
AT function in cows with CK. Previous reports suggest that administering FM for the first 3 DIM in postpartum cows is detrimental to feed intake and milk production (Shwartz et al., 2009). In contrast, a single dose of FM benefits healthy cows by reducing inflammatory markers (Schmitt et al., 2022), suggesting that prolonged administration of inflammatory therapy dampens metabolic adaptations in early lactation cows. The only study evaluating AT function during NSAID therapy demonstrated increased transcription of pro-inflammatory cytokines in AT after administration of carprofen at 1.3 mg/kg for 3 d in early (1, 3, and 5 d postpartum) postpartum dairy cows (Vailati Riboni et al., 2015). While ample evidence exists regarding the beneficial effects of various natural and synthetic anti-inflammatory agents in modulating AT inflammation and ATM infiltration in humans and rodents (da Cruz Nascimento et al., 2022), further research is needed to explore the use of anti-inflammatory therapies during metabolic diseases and its implications for AT function during metabolic diseases.

This study has limitations, including the lack of a PGFM treatment that limits our capacity to differentiate the individual contribution of FM to CK therapy. We also did not evaluate systemic insulin sensitivity and only focused on the responses of AT to insulin. However, based on the known associations between systemic and AT insulin sensitivity, it is plausible that systemic insulin sensitivity may have been enhanced in cows treated with PGNIAFM. This improvement could potentially account for the observed reductions in lipolysis and improved CK recovery. Overall, the results from the ex-vivo lipolysis model used in the present work provide essential information for future studies evaluating systemic insulin responses using glucose clamp techniques.

CONCLUSION

The present study demonstrates that cows with CK exhibit increased AT inflammation, characterized by augmented ATM infiltration of both M1 and M2 phenotypes and activation of the ERK1/2 inflammatory pathway. CK is also associated with reduced insulin sensitivity in AT. Treatment of CK with a combination of PG, NIA, and FM leads to a reduction in AT inflammatory markers and an improvement in AT insulin sensitivity.

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Conflict of Interest Statement The authors declare that there is no conflict of interest.

REFERENCES


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