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Postpartum Serum Non-Esterified Fatty Acids, Milk Yield, Feed Intake and Plasma Natural Antibodies in Cows with Subclinical Mastitis

Konsentrasi Asam Lemak Non-Esterifikasi Pascapartus, Produksi Susu, Asupan Pakan, dan Antibodi Alami Plasma pada Sapi Mastitis Subklinis

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Abstrak

Jumlah sel somatik (JSS) umumnya digunakan sebagai indikator mastitis subklinis, namun hubungannya dengan performa pasca partus, konsentrasi asam lemak non-esterifikasi (NEFA), dan status kekebalan masih belum jelas. Studi ini mengevaluasi 18 sapi Friesian Holstein yang dikelompokkan berdasarkan SCC susu: rendah (<160.000 sel/ml), sedang (160.000–400.000 sel/ml), atau tinggi (>400.000 sel/ml). Produksi susu harian, asupan pakan, kadar NEFA plasma, dan antibodi alami (IgG dan IgM) terhadap hemosianin limpet lubang kunci (KLH) diukur. ANOVA digunakan untuk mendeteksi perbedaan antar kelompok. Meskipun pola makannya konsisten dan pengambilan sampelnya terstandarisasi, sapi dengan JSS tinggi (4,79) cenderung menunjukkan titer IgG yang lebih rendah daripada sapi dengan JSS rendah (6,16) dan sedang (6,42), yang menunjukkan fungsi imun yang berkurang pada tingkat JSS yang lebih tinggi. Namun, produksi susu, asupan pakan, titer IgM, dan kadar NEFA pasca partus tidak menunjukkan perbedaan yang signifikan di antara ketiga kelompok. Hasil pengamatan ini menunjukkan bahwa JSS yang meningkat dapat mengganggu respons antibodi IgG sistemik tetapi memberikan efek terbatas pada produksi dan parameter metabolik. Sebagai kesimpulan, meskipun JSS tampaknya mempengaruhi status imunitas, hubungannya dengan konsentrasi NEFA, produksi susu, dan asupan pakan kurang jelas dalam kondisi percobaan ini.

Kata kunci: antibodi alami; asam lemak non-esterifikasi; jumlah sel somatik; sapi perah

Abstract

Somatic cell count (SCC) is commonly used as an indicator of subclinical mastitis, yet its association with postpartum performance, non-esterified fatty acid (NEFA) concentration, and immune status remains unclear. This study evaluated 18 Holstein Friesian cows grouped by milk SCC: low (<160,000 cells/mL), moderate (160,000–400,000 cells/mL), or high (>400,000 cells/mL). Daily milk production, feed intake, plasma NEFA levels, and natural antibodies (IgG and IgM) against keyhole limpet hemocyanin (KLH) were measured. ANOVA was used to detect differences among groups. Despite consistent feeding regimens and standardized sampling, cows with high SCC (4.79) tended to exhibit lower IgG titers than those with low (6.16) and moderate (6.42) SCC, suggesting

reduced immune function under higher SCC levels. However, milk yield, feed intake, IgM titers, and postpartum NEFA levels showed no significant differences among the three groups. These findings indicate that elevated SCC may impair systemic IgG antibody responses, but it exerts limited effects on production and metabolic parameters. In conclusion, while SCC appears to influence immune status, its relationship with NEFA concentrations, milk production, and feed intake is less pronounced under these experimental conditions.

Keywords: dairy cows; Natural Antibodies (Nabs); Non-Esterified Fatty Acids (NEFA); Somatic Cell Counts (SCC).

Introduction

In recent years, there has been growing awareness of dairy cow health, particularly udder health, which directly impacts herd quality and milk safety (Burgess, 2010; Schukken *et al.,* 2003). Somatic Cell Count (SCC) serves as a crucial indicator of udder health, with elevated levels often signaling intramammary infections, such as mastitis. Such infections not only degrade milk quality but also adversely affect animal welfare and economic outcomes, underscoring the importance of effective management strategies (Burgess, 2010; Tremetsberger & Winckler, 2015).

SCC reflects the immune system's response to infection, as increased counts indicate an inflammatory reaction, where levels exceeding 160,000 cells/mL reflect heightened immune responses to infection. In comparison SCC levels below 20,000 cells/mL may imply a reduced immune barrier, potentially increasing mastitis susceptibility in low-infection environments (Rainard *et al.,* 2018). As a natural defense mechanism, the immune system's response to infections initiates the production and activation of various somatic cells, such as lymphocytes, macrophages, and epithelial cells (Larsen *et al.,* 2020; Naglik *et al.,* 2017). Therefore, maintaining moderate SCC levels may balance immune readiness and milk quality, as both extremely low and very high SCCs may compromise mammary health (Rainard *et al.,* 2018).

Another component of the immune system, natural antibodies (NAbs), are essential antibodies that can bind to pathogen-associated molecular patterns (PAMPs) to identify foreign "danger" or "non-danger" threats caused by pathogenic bacteria (Holodick *et al.,* 2017). NAbs are present in normal, healthy individuals without any exposure to external antigens, which trigger a body response when an external antigen present in the body (Avrameas, 1991; Holodick *et al.,* 2017). A previous study reported that NAbs present in bovine milk and plasma (van Knegsel *et al.,* 2007). NAbs titers represent the concentration of circulating antibodies (primarily IgM and IgG isotypes) in the blood with mentioned binding characteristics (Reyneveld *et al.,* 2020), which can provide insight into certain aspects of an individual's immune status, particularly within the innate immune system (Sarrigeorgiou *et al.,* 2024).

In addition to its role in infection, SCC might be linked to other metabolic processes, including non-esterified fatty acid (NEFA) concentrations, milk production, and feed intake. Non-esterified fatty acid (NEFA) is a free fatty acid circulating in the plasma biomarker related to the amount of lipolysis in an animal's body, especially during periods of high metabolic demand, such as parturition and early lactation (Mäntysaari *et al.,* 2019; Nicola *et al.,* 2022), which needs massive amount of energy. It was reported that an increase of plasma NEFA concentration is associated a negative energy balance status in postpartum dairy cows (Adewuyi *et al.,* 2005; Aernouts *et al.,* 2020; Wang *et al.,* 2023).

The increase in required energy in high-yielding dairy cows, especially during the transition period (from parturition to lactation), is met by a high amount of NEFA mobilization (Contreras et al., 2017; Zachut & Contreras, 2022). Given the roles of SCC, NEFA, and natural antibodies (NAbs) in dairy cow health, this study hypothesizes that SCC levels are associated with milk production, feed intake, plasma NAb levels, and plasma NEFA

concentrations, potentially connecting udder health indicators with broader aspects of cow health and productivitycitations.

Materi dan Metode

Animals and Experimental Design

The Ethical Committee of Universitas Padjadjaran, Bandung, Indonesia, has approved the protocol for this experiment with registration number: 0718070998. A total of 18 Holstein Friesian Cows with body weight (400±30 kg) were randomly selected from West Java Regional Dairy Cows Breeding and Artificial Insemination Development Institute (BPPIB-TSP), Bunikasih, Cianjur, West Java, Indonesia. Each cow was individually housed and fed ad libitum forage and concentrates individually (fed twice daily). The ratio of forage:concentrate is 60:40, while the forage consists of napier grass and *Indigofera zollingeriana* with the ratio of 45:15. Offered feed (forages and concentrates) and feed refusal per individual cow were weighed daily. Feed intake was recorded daily by weighing the offered feed (forage and concentrate) and subtracting feed refusals for each cow. Drinking water was provided *ad libitum*. The transition diet is formulated with a similar protein and energy content among groups (CP 16.57% and total digestible nutrients or TDN 59.52% (Table 1). The individual milk yield (morning and evening milking) and were recorded daily.

Table 1. Nutrient content of the experimental diet.

Nutrient Fraction	Content
Dry Matter (DM)	53.50
Ash (%)	13.03
Crude Protein (%)	16.57
Crude Fat (%)	5.15
Crude Fiber (%)	21.45
TDN (%)*	59.52
Ca (%)	0.61
Zn (mg/kg DM)	82.92
Se (mg/kg DM)	0.43

*TDN = Total Digestible Nutrient

Analysis of feed ingredients is conducted at the Ruminant Nutrition and Feed Chemistry Laboratory (Universitas Padjadjaran, West Java, Indonesia) using proximate analysis. Milk SCC levels were determined based on a milk sample taken at the beginning of lactation period (transitional period), using a LactoScan SCC Analyzer (Milkotronic, Bulgaria) in the veterinary Laboratory of Animal Hospital, Lembang, Bandung, West Java, Indonesia.

Blood Sampling

Blood samples were taken at week one after calving. All blood samples were taken in the morning, approximately two hours after feeding, to minimize diurnal variation in NEFA and antibody levels. Blood samples were collected via coccygeal vein, a standard practice that is less invasive and typically causes minimal discomfort compared to other veins, from each cow into blood sample tubes (vacutainer) with a purple cap containing Ethylenediaminetetraacetic acid (EDTA) anti-coagulant agent (BD Vacutainer, Portsmouth, UK) for measurement of plasma NEFA and NAbs. To minimize stress during collection, cows were handled by familiar personnel in a calm environment, adhering to standard animal welfare practices. Additionally, cows were briefly restrained and released immediately after sampling to minimize handling time and associated stress. All procedures adhered to animal welfare guidelines to safeguard the cows' well-being, ensuring that blood sampling and handling protocols complied with ethical standards. Vacutainer tubes containing blood samples were then centrifuged in 1500 rpm for 15 minutes at 4°C. After the centrifugation, the plasma aliquots were transferred into 1.5 mL PCR Tubes (Eppendorf, Hamburg, Germany) and stored frozen at -20 °C till analysis. Before analysis, samples were thawed at room temperature and gently mixed to ensure homogeneity.

NEFA and Natural Antibody Titers Determination

Plasma NEFA concentrations were quantified using the NEFA C quantification kit (Fujifilm Wako Diagnostics, CA, USA), which in principle, is an enzymatic colorimetric method. A spectrophotometer then measured the assay at 550 nm. NEFA was selected as a biomarker because it reflects energy mobilization in dairy cows, particularly during the high-energy-

demanding postpartum period. Elevated NEFA levels are often indicative of negative energy balance, a condition linked to udder health and immune resilience, which may also correlate with increased SCC due to energy diverted towards immune responses (Horst *et al.,* 2021; Mäntysaari *et al.,* 2019).

NAbs titers were estimated by using an indirect enzyme-linked immunosorbent assay (ELISA) against keyhole limpet hemocyanin (KLH), a foreign antigen derived from *Megathura crenulata*, a keyhole limpet that inhabits the coast of California, USA. The use of this antigen was intended to reduce false positives in detecting the NAbs titers because the keyhole limpet is a gastropod, which phylogenetically distant from mammalian proteins. NAbs were chosen as they are part of the innate immune system, providing early defense without prior antigen exposure, which plays a role in udder health. Since SCC elevation reflects immune activity in response to infection, NAbs levels could further indicate immune status and resistance to mastitis in dairy cows (Denholm *et al.,* 2018). The indirect ELISA techniques employed in this research were based on van Knegsel *et al.,* (2012). To maintain biomarker integrity, each sample was processed immediately after thawing. Care was taken to minimize repeated freeze-thaw cycles by preparing single-use aliquots during the initial storage process.

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) in the GLM procedure in SAS Statistics (Ver. 9.4, Cary, NC, USA) to determine the effects of different milk SCC levels on plasma NAbs titers and plasma NEFA concentration. ANOVA was chosen for its robustness in detecting differences between predefined SCC groups (low, moderate, and high) in relation to the dependent variables. This method aligns with our primary objective of assessing group-level effects rather than exploring interactions, which would require multivariate analysis. Although multivariate analysis could address the interconnected nature of immune and metabolic responses, the sample size (n=18) was insufficient for obtaining reliable results with such techniques.

Based on the multifaceted perspectives outlined by Rainard et al. (2018), cows in this study were classified into three SCC-based groups, namely low (SCC<160,000 cells/ml, n=7), moderate (SCC 160,000-400,000 cells/ml, n=7), and high (SCC>400,000 cells/ml, n=4) to reflect varying degrees of mastitis susceptibility and immune response potential. This categorization therefore accounts for the balance between minimizing inflammation and maintaining an immune defense threshold, as recommended by the review.

To better understand the biological relevance of our findings, we calculated the effect size, using Cohen's d, for comparisons among the SCC groups. Cohen's d represents the difference between the averages of two groups, divided by their pooled standard deviation, providing a standardized way measure to gauge the strength of the observed differences. This method allows us to assess the magnitude of group differences independently of sample size. We interpreted these effect sizes using standard guidelines: a d of 0.2 indicates a small effect, 0.5 a medium effect, and 0.8 a large effect, wich helping us to better contextualize the practical significance of the results (Peng & Chen, 2014). The data were expressed as least-squares means (LSM) of the respective parameter with the pooled standard error of the means (SEM). The difference is considered significant at $p<0.05$.

Results and Discussion

Our study showed that cows with HSCC levels in milk tended to have reduced NAb IgG titers in plasma (*p=0.10)* compared with cows with LSCC and MSCC. The immune system responds to infections by naturally initiating the creation and activation of different somatic cells, including macrophages, lymphocytes, and epithelial cells (Pillai et al., 2001; Sordillo *et al.,* 1997). In the current study, plasma IgG titers ranged from 4.79 to 6.42 (figure 2), while plasma IgM titers ranged from 5.87 to 7.65. The effect size (Cohen's d = 0.92) suggests a moderate to large difference in IgG titers between these two groups. The 95% confidence interval for this difference ranged from -3.11 to 0.37, indicating some variability but also suggesting a potential biological relevance in the observed reduction of

IgG titers for MSCC cows. Plasma NAbs titers is a relative level of natural antibodies that present in plasma. The higher the titers, the higher NAbs concentration in plasma present. It seems that there is a decrease in plasma IgG titers when the milk SCC level increases. Our finding is in line with a previous study by Mayasari *et al.,* (2016) which found that cows with high SCC in milk have a low NAbs IgG in plasma.

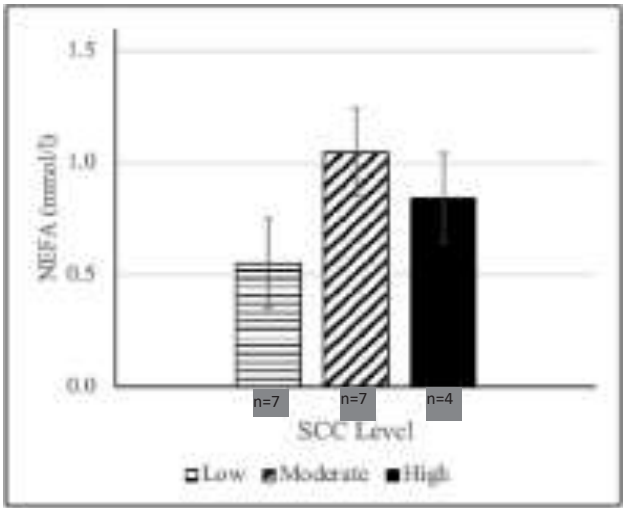

Figure SEQ Figure * ARABIC 1. Plasma NEFA (mmol/l) on different SCC levels of Dairy Cows. Error bars representing standard error of the NEFA mean.

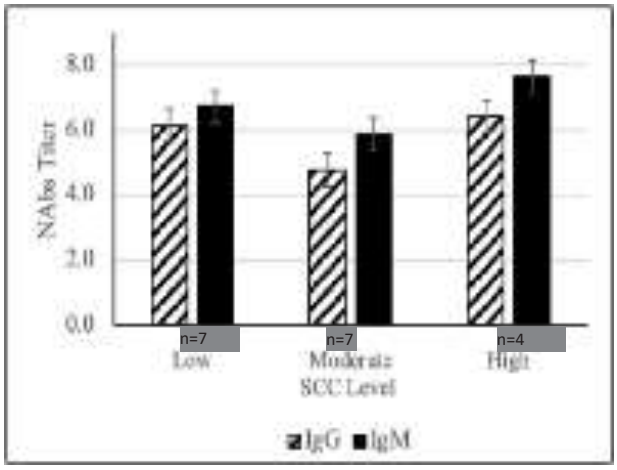

Figure SEQ Figure * ARABIC 2. Plasma natural antibodies (NAbs) titerss (IgG and IgM) on different SCC levels of Dairy Cows. Error bars representing the standard errors of NAbs titers means

Generally, high SCC is considered an indicator of mammary gland inflammation and infection in dairy cows (Rainard *et al.,* 2018). SCC significantly increases after intramammary infections. Infections by major pathogens result in more pronounced and persistent increases in SCC (Kirkeby *et al.,* 2021). Taking into consideration the traditional SCC parameters, the newly developed DSCC provides further details on the status of the infection (Kirkeby *et al.,* 2020; Wall *et al.,* 2018). The increased SCC and DSCC values correspond with different aspects of immune responses, such as increased migration profile of neutrophils, activation of the complement system, and changes in the population of T lymphocytes (Souza *et al.,* 2020; Winther *et al.,* 2023). Therefore, this literature once again reinforces that SCC is high, reflecting not only an inflammation but a complex immune response involving both innate and adaptive immunity.

The way SCC influences immune status, especially NAb IgG levels, likely involves recruiting immune cells to the site infection in the mammary gland. B-cells, which are responsible for antibody production, may be drawn to the inflamed tissue, focusing antibody production at the local infection site rather than in systemic circulation. It was suggested that immunoglobulins are secreted by B-cells in plasma and locally produced by lymphocytes near the glandular epithelium (Persson *et al.,* 1992). This immunological shift could reduce systemic IgG levels as resources are allocated to support localized immunity in the mammary gland.

In contrast with NAbs IgG, NAbs IgM in plasma were similar among treatments. This result supports the theory that there is a difference in the mode of action, physiochemical, and biological properties between IgG and IgM (Nirula *et al.,* 2011). The higher IgM titers observed in cows with high SCC levels, albeit in contrast to previous studies associating elevated IgM with reduced mastitis likelihood (Mayasari *et al.,* 2016; Thompson-Crispi *et al.,* 2013), suggest an adaptive immune response where IgM levels are sustained for systemic protection while IgG is redirected to the infected mammary tissue. This aligns with Boes *et al.,* (2000), who reported that natural antibodies in mammalian plasma are primarily of the IgM isotype.

No significant differences on milk yield and feed intake in cows with different level of SCC in milk (P>0.05, Table 2). In our study, the milk yield ranged from 13.44 to 16.25 kg/head/day, and the feed intake ranged from 40.05 to 46.78

kg/head/day (Table 2). The effect size (Cohen's d = 0.17) indicates a small difference in yield, with a mean difference of -0.72 kg/day between groups. The 95% confidence interval for this difference ranged from -5.66 to 4.23, spanning a broad range and suggesting considerable variability with no clear biological relevance. Cows with a HSCC in milk had 2-3 kg/head/day more milk yield compared with MSCC or LSCC group (16.25 vs 13.44 vs 14.15 kg/head/day, respectively). The milk yield in this study was similar to that of an earlier study in a tropical area (Singh *et al.,* 2020), with a range of 13.44 to 16.25 kg/head/day, even though the feed intake was higher compared to the earlier study. While higher SCC is typically associated with infection and reduced productivity, several factors could explain this unexpected trend. One possible explanation is that cows in the HSCC group may have a high genetic potential for milk production, leading to increased metabolic demands and potential immune compromise, which could contribute to elevated SCC (Heringstad *et al.,* 2006). High-producing cows may also be susceptible to subclinical mastitis or other stress-related conditions that elevate SCC without visibly reducing milk yield. Furthermore, cows with a HSCC had a lower feed intake compared with those with and MSCC and LSCC (40.05 vs 46.78 vs 43.77 kg/head/day, respectively), although the differences was not significant.

No relationship was found in our study between postpartum NEFA concentration and different levels of SCC ($p>0.05$, Table 2), in agreement with previous reports (Nicola *et al.,* 2022; Schwegler *et al.,* 2013). The effect size (Cohen's d = -0.56) indicates a moderate effect, suggesting that SCC level is meaningfully associated with NEFA levels. The 95% confidence interval for the difference was broad, ranging from -0.62 to 1.62, although it leaned toward higher NEFA in the MSCC group, suggesting some biological relevance. The absence of association could also be linked with the delay between NEFA concentration evaluation and the identification of inframammary infection. Other study found high levels of NEFA before lambing predispose to several periparturient health disorders, such as clinical mastitis (Karagiannis *et al.,* 2014).

Our study revealed that plasma NEFA concentrations ranged from 0.55 to 1.05 mmol/L, consistent with the findings of Mäntysaari et al. (2019). NEFA plays a crucial role in understanding energy balance in dairy cows, especially during early lactation when cows often experience negative energy balance (NEB) due to the high energy demands of milk production. Elevated NEFA levels generally indicate that cows are mobilizing body fat reserves to meet these energy demands, a process that can affect both metabolic health and immune function (Chen *et al.,* 2015; van Knegsel *et al.,* 2014).

High NEFA concentrations can serve as biomarkers for NEB and are associated with an increased the risk of metabolic disorders, such as ketosis and fatty liver, which are common in

Table 2. Effects of milk SCC levels on plasma IgG titer, plasma IgM titer, and plasma NEFA.

Parameter	SCC Levels[1]			Pooled SEM	p-value	Significance[2]
	LSCC (n=7)	MSCC (n=7)	HSCC (n=4)			
Performance						
Milk Yield (kg/head/day)	14.15	13.44	16.25	0.91	0.5	NS
Feed Intake (kg/head/day)	43.77	46.78	40.05	2.09	0.5	NS
Plasma metabolite						
NEFA (mmol/l)	0.55	1.05	0.84	0.19	0.6	NS
Plasma natural antibodies						
IgG titers	6.16	6.42	4.79	0.34	0.1	NS
IgM titers	6.73	5.87	7.65	0.45	0.3	NS

[1]SCC Levels:
LSCC Low: SCC<160,000 cells/ml
MSCC Moderate: SCC 160,000 - 400,000 cells/ml
HSCC High: SCC>400,000 cells/ml
[2]NS: Non-significant

high-producing dairy cows postpartum. Adewuyi *et al.,* (2005) and Ospina *et al.,* (2010) identified plasma NEFA concentrations above 0.6 mmol/L as indicative of severe NEB and a predictor of health problems in early lactation. In our study, NEFA levels across SCC groups did not differ significantly (P>0.05), which may suggest that cows in all groups were able to maintain a relatively stable energy balance. However, cows with lower SCC tended to exhibitlightly lower NEFA levels, suggesting a potentially better energy status and a reduced need for body fat mobilization. Normal NEFA concentrations for cows in positive energy balance are estimated to be less than 0.2 mmol/L (Adewuyi *et al.,* 2005). Earlier studies reported that plasma NEFA concentration above 0.4 mmol/L was associated with NEB risk (Oetzel, 2004), while other study reported that cows with high lipo-mobilization had plasma NEFA above 0.5 mmol/L (González *et al.,* 2011).

Apart from energy status, earlier studies reported that NEFA concentrations were influenced by several factors, such as feeding frequency and handling protocol (Brickner *et al.,* 2007). Blum et al. (2000) reported that cows fed twice a day had greater NEFA concentrations than cows fed six times a day, especially when on a high-concentrate diet. In the current study, we fed cows twice daily, and the milking protocol was similar for all treatments. We found that cows with a LSCC had lower plasma NEFA values than those in other treatments (a 0.5 mmol/L difference), although the difference was not statistically significant. The cows in this study were most likely not in NEB due to their similar milk yield and feed intake.

While this study contributes essential findings, certain limitations must be considered. The modest sample size restricts the generalizability of the results, indicating that future studies with larger groups may yield more conclusive insights. Conducted in a specific regional context with distinct dietary and environmental factors, this study's outcomes may vary under different climates or feeding regimes. Research in diverse geographical locations and with controlled dietary conditions could clarify the influence of these variables on NEFA, SCC, and immune responses. Additionally, since only a single dairy breed was examined, exploring multiple breeds in future studies may highlight how genetic and physiological differences shape responses to SCC and NEFA levels. Addressing these limitations would help enhance our understanding of dairy cow health across various environmental and management systems.

Conclusion

In conclusion, the milk SCC level was tended to show an association with NAb IgG in plasma, which is important information for assessing immune status during disease prediagnosis. The absence of relationship among milk production, feed intake, and plasma NEFA concentration, as well as the level of SCC in milk was influenced by many factors, such as sampling timing, health status, energy status, and immunogenetic competence of the cows. However, the effect sizes found in this study may suggest that moderate SCC levels may have underlying impacts on immune function and metabolic status. Nevertheless, another study with a larger number of cows is needed to confirm this finding, along with other plasma metabolite parameters to provide more comprehensive results.

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