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# Enhancing human milk studies: Introducing a less invasive human milk collection technique for the measurement of fatty acids

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## Abstract

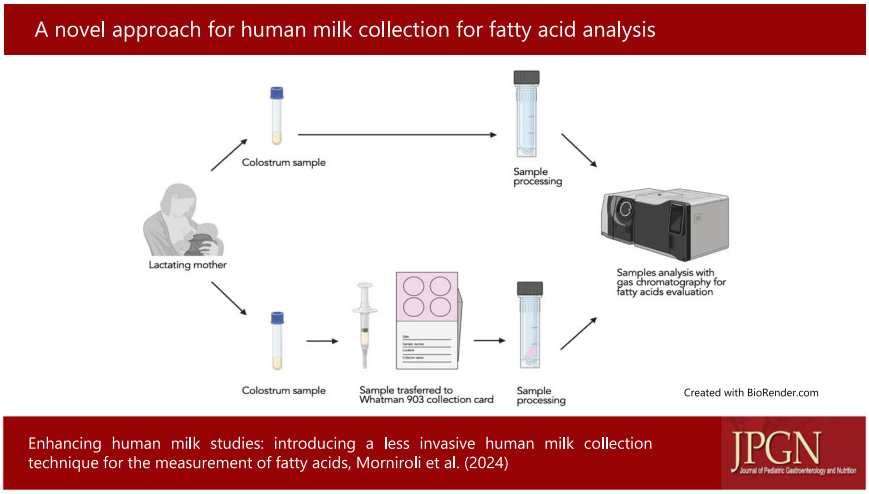
**Objectives:** The fatty acid supply of human milk (HM) contributes to health outcomes. Sampling fresh human milk to analyze its fatty acid content is challenging because of its ever-changing nature. Also, obtaining samples from lactating mothers is challenging. Facilitating HM collection and analysis is therefore an advantage.

**Methods:** We have conducted a study to validate a new method for obtaining HM samples for fatty acid analysis, using biological fluid sample collection pretreated sheets to adsorb drops of milk (Whatman 903 BHT-pretreated biological fluid collection sheet) as an alternative approach to collecting expressed milk. The study population included lactating mothers, enrolled between 24 and 96 h after delivery.

**Results:** A total of 124 breastmilk samples were analyzed using the two distinct approaches. The results of the free milk analysis were comparable to the analysis of adsorbed milk samples. The fatty acid families saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3, and omega-6 had $r^2$ values of 0.93, 0.91, 0.91, 0.86, and 0.90, respectively. Bland-Altman plots showed a high agreement between fresh and adsorbed milk samples for SFA, MUFA, PUFA, omega-3, and omega-6 with a mean bias <2% and 95% limits of agreement within –5% and +5%.

**Conclusions:** The results show no significant differences in fatty acid composition between fresh and adsorbed milk samples, suggesting the new method is equally effective in collecting representative samples for analysis.

Maria Lorella Giannì and Carlo Agostoni share senior authorship.




**KEYWORDS**
breastmilk, colostrum collection, lipid profile, milk samples

## 1 | INTRODUCTION

Breast milk is the natural source of nutrition for infants, providing essential nutrients and bioactive components that promote optimal growth and development.[1,2] Fatty acid (FA) composition is a key component, which playing a relevant role in infant health outcomes.[3]

Accordingly, long-chain polyunsaturated fatty acids, including docosahexaenoic acid (DHA) and arachidonic acid, are critical for neuro-behavioral development in early life.[4,5]

Various methods are used for analyzing the FA composition of human milk samples. Gas chromatography (GC) coupled with flame ionization detection is a widely employed technique due to its accuracy and sensitivity. Other methods include high-performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry-based approaches.[5–7] Sampling fresh human milk presents challenges due to its dynamic nature and variations within feedings. This is particularly true during colostrum collection—the first few days after birth. Obtaining adequate samples from lactating mothers during this critical period can be challenging due to limited supply and difficulties in collection techniques.[8]

Traditionally, human milk samples have been collected by hand expressing milk from lactating mothers: while this method has been widely used in research studies to study FA composition in human milk, it is not without limitations. One key challenge is the collection of colostrum, which is thick and sticky and often very scarce, therefore difficult to extract using hand expression.[9,10] Moreover, breastmilk can be difficult to obtain right after a feeding.

The "gold standard" method of 24 h collection of full expression of all feeds, is no longer used by research protocols, due to its invasiveness. An ideal method to represent the FA composition of human milk could be represented by the collection of midstream human milk within single, alternate feedings, through 3 days, collecting the whole sample and then picking a sample for analysis. Even in this case, however, the collection method could be demanding for the mother and could interfere with breastfeeding in the first delicate days of life.[11]

Given these premises, we have conducted a study to validate a novel method for obtaining human milk samples to analyze its FA content. This involved adsorbing few drops of milk in pre-treated biological sample collection sheet (Whatman

**What is Known**

- The evaluation of breastmilk's lipid profile has major implications for the health of the dyad.
- Breastmilk collection, especially colostrum, is often difficult due to the small quantities and the difficulty of extraction.

**What is New**

- Milk samples collected via collection sheet are comparable to usual tube milk samples in analyzing the lipid profile of breast milk.
- Collecting breast milk and especially colostrum via a biological fluid collection sheet, and analyzing dried milk spots, could be much easier for mothers and researchers, expanding the possibility of performing large-scale studies.

**TABLE 1** Correlation between free milk and adsorbed milk.

| Fatty acid | $r^2$ | *p*-Value |
|---|---|---|
| 16:0 | 0.938 | <0.000 |
| 16:1n7 | 0.878 | <0.000 |
| 18:0 | 0.909 | <0.000 |
| 18:1n9 | 0.841 | <0.000 |
| 18:1n7 | 0.592 | <0.000 |
| 18:2n6 | 0.934 | <0.000 |
| 18:3n3 | 0.815 | <0.000 |
| 20:3n6 | 0.964 | <0.000 |
| 20:4n6 | 0.940 | <0.000 |
| 20:5n3 | 0.522 | <0.000 |
| 22:0 | 0.791 | <0.000 |
| 22:5n3 | 0.881 | <0.000 |
| 24:0 | 0.736 | <0.000 |
| 22:6n3 | 0.897 | <0.000 |
| 24:1 | 0.880 | <0.000 |
| SFA | 0.936 | <0.000 |
| MUFA | 0.910 | <0.000 |
| PUFA | 0.918 | <0.000 |
| Omega-3 | 0.860 | <0.000 |
| Omega-6 | 0.909 | <0.000 |

Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

903 BHT collection sheet), commonly used to collect and store newborn blood samples for newborn metabolic screening test, as a substitute approach to the conventional way of collecting fresh expressed milk in a tube.

## 2 | METHODS

### 2.1 | Sample collection

The study was conducted at a tertiary neonatology department under the IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, from October 2022 to June 2023. The research protocol followed the tenets of the Declaration of Helsinki and received approval from the Hospital's ethics committee (protocol code 791_2021bis, date of approval 06/07/2021).

The study population comprises women who were admitted to the departments of the Operational Unit of Neonatology and Neonatal Intensive Therapy throughout the recruiting period of the research. Mothers were enrolled during hospitalization within 24–96 h after giving birth.

Mothers who were above the age of 18 and signed a written informed consent were deemed eligible for the study. The research excluded patients who had any of the following characteristics:

limited comprehension of the Italian language; maternal or neonatal conditions that disrupt metabolism and/or the ability to absorb lipids; absence of breastfeeding.

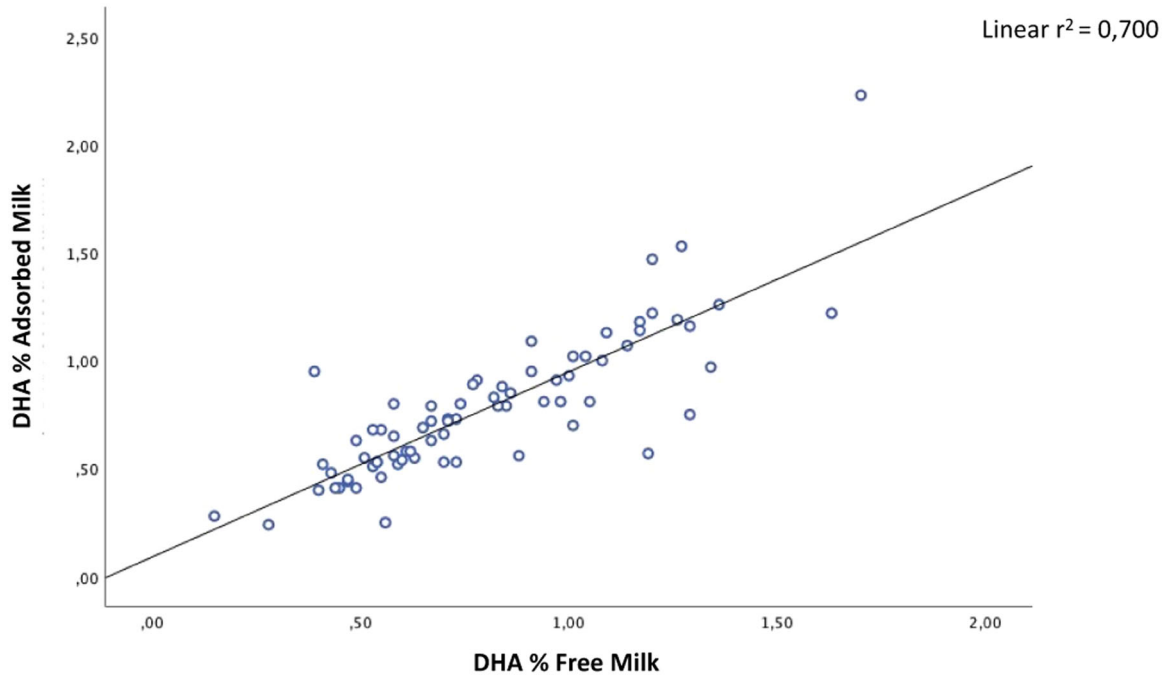



**FIGURE 1** Correlation between DHA% levels in free and adsorbed breastmilk. DHA, docosahexaenoic acid.

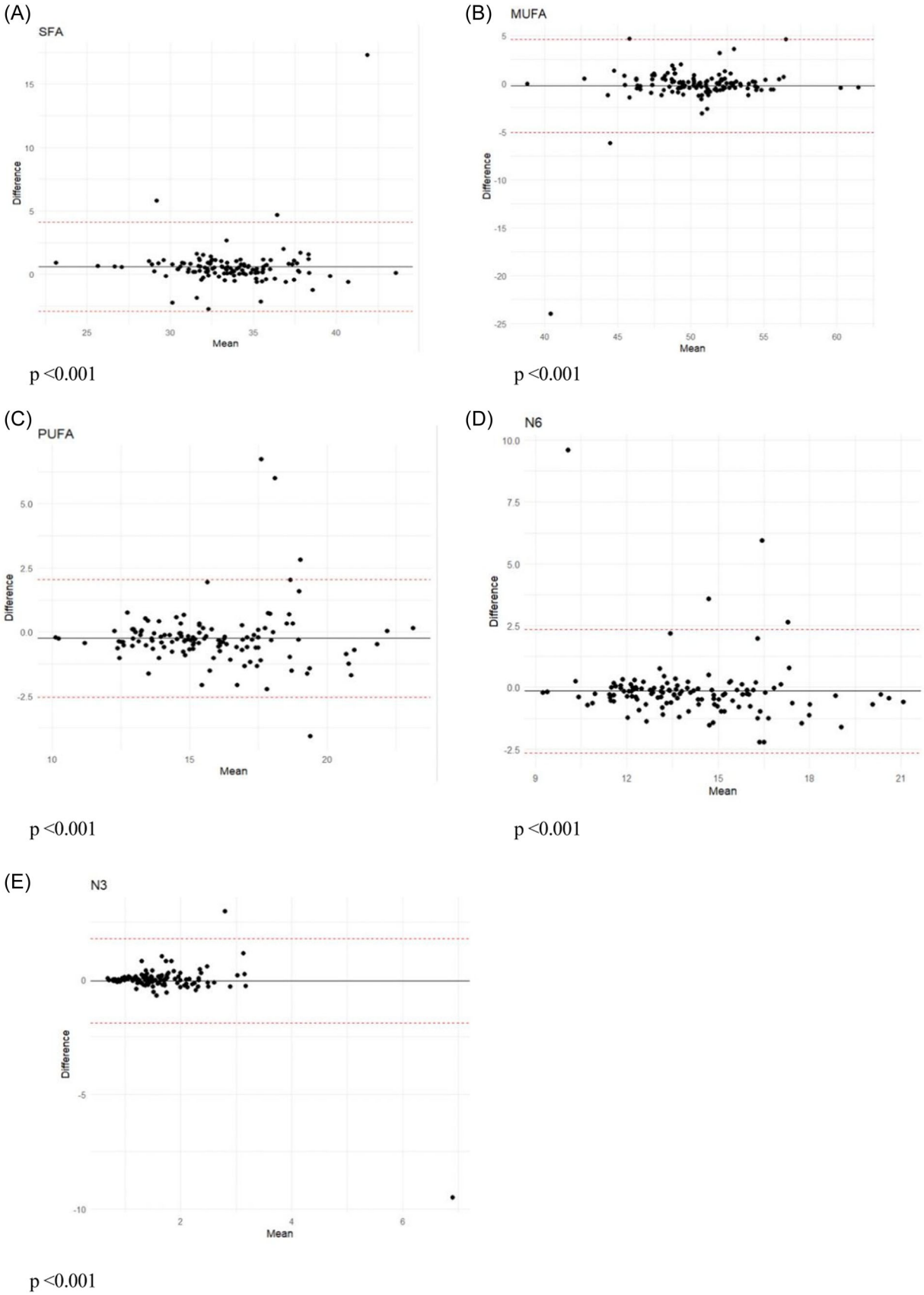



**FIGURE 2** (See caption on next page).

A sample of colostrum was obtained during a time frame of 24–96 h postpartum. Approximately 1 mL of colostrum was obtained by breast hand expression and then kept in a 15 mL sterile tube (free milk). Subsequently, 50 µL were extracted from the tube and transferred to pretreated collection sheets (Whatman 903 collection cards BHT Sigma-Aldrich—adsorbed milk). Since the amount of lipids in breastmilk obtained by hand expression varies according to the degree of breast fullness and the technique/depth of the expression,[11] we preferred to avoid the confounding factor of comparing two different samples expressed one after the other or with even slightly different hand pressure.

Both tubes and cards samples were immediately brought to the laboratory for analysis.

## 2.2 | Sample analysis

An aliquot of 25 µL of fresh milk (free milk) and half circle of protein card server, which corresponds to a volume of 25 µL of adsorbed milk, were transferred into two different vials. Both free and adsorbed milk were then subjected to the following procedure: methylation with 700 µL of HClMe 3N (Sigma Aldrich), incubation for 1 h at 90°C and then refrigeration at 4°C for 10 min. Afterward, 2 mL of KCl solution (Sigma Aldrich) and 400 µL hexane (Sigma-Aldrich) were added. Samples were first vortexed and then centrifuged at 3000 rpm for 10 min. Finally, hexane layer (the upper layer) was collected from each vial and transferred into a GC vial for FAs profile evaluation with gas-chromatograph Shimadzu Nexis GC-2030 (Shimadzu) equipped with a 30 m fused silica capillary column FAMEWAX Restek (Restek). The GC results were elaborated using Lab Solution 5.97 SP1 software (Shimadzu). Consistently with existing literature, single FA were expressed as relative percentage of total FAs. We also proceeded to calculate the percentage of main FA subgroups: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3, and omega-6.

## 2.3 | Statistical analysis

The statistical analysis was performed with the IBM SPSS program, version 25.0 (SPSS Inc, IBM Company). Data were correlated by two tails Pearson bivariate analysis a $p$-values $< 0.05$ were considered statistically significant. To further explore the mean difference between methods, limits of agreement, and bias across the range we performed a Bland-Altman analysis for five subgroups of FA (SFA, MUFA, PUFA, and omega-3 and -6) attracting the most interest in nutritional research.

# 3 | RESULTS

A total of 124 breastmilk samples were collected with the two different methods.

To validate the collection method for biological samples using pretreated biological sample collection sheet (Whatman 903 BHT—adsorbed milk) while comparing it with the traditional method (free milk), we assessed the correlation between the percentage concentration of each FA analyzed, both for the free milk samples and the adsorbed samples (Table 1).

Pearson bivariate analysis showed that the results of the free milk analysis is not different from the analysis obtained with adsorbed milk samples. All FA families, SFA; MUFA; PUFA; omega-3; and omega-6 showed a $r^2$ value of 0.93, 0.91, 0.91, 0.86, and 0.90 respectively. The highest correlation regarded 20:3n6 (0.96) while the lowest regarded 20:5n3 (0.52).

Figure 1 provides a comprehensive analysis of the correlation observed between colostrum DHA in free milk and adsorbed milk samples. Bland-Altman plots showed a high agreement between fresh and adsorbed milk samples for SFA; MUFA; PUFA; omega-3 and omega-6 with a mean bias <2% and 95% limits of agreement within –5% and +5% (Figure 2). All $p$-values are <0.001 (the regression line graphs, and calculated $p$-values are available as Supporting Information).

# 4 | DISCUSSION

Studying the FA content in human milk provides valuable insights into maternal nutrition, infant health, and overall well-being of the dyad. Our innovative approach could allow for easy collection of human milk samples by simply dabbing the breast or nipple area onto the pretreated card. The adsorbed milk on the collection cards can then be stored or transported easily without any loss of sample. In addition, even only few drops of breastmilk could suffice to saturate the card.

The results and findings reveal that there are no significant differences in terms of FA composition between fresh samples and adsorbed milk samples,

**FIGURE 2** Bland-Altman plots show agreement between fresh and adsorbed milk samples for FA subgroups of SFA (A); MUFA (B); PUFA (C); omega-3 (D); and omega-6 (E). Black solid line is the mean bias. Dashed red lines point out 95% limits of agreement. All $p$-values are <0.001 as indicated in each panel. The regression line graphs, and calculated $p$-values, are available as supplementary material. FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

indicating that the new method is just as effective in collecting representative samples for analysis.

This new method offers other methodological advantages over traditional approaches. First, it reduces sample losses and spills as compared to direct expression of fresh samples. Additionally, it reduces the storage space required, allowing for easier collection and storage of many samples for large population studies. This noninvasive technique could also minimize invasiveness as it eliminates the need for pumping or even manual expression (if collected after a feed) which may cause discomfort to lactating mothers.

Expressing milk by hand is a time-consuming technique that requires patience and determination. In addition, some women may not receive appropriate help or direction in learning effective hand expression strategies, resulting in frustration and hopelessness.[12]

Lactating mothers may be more willing to participate and provide multiple samples when they do not have to undergo discomfort or inconvenience associated with traditional collection methods.

Although in fact it was not directly tested in our study, which is indeed a limitation, the results of the correlation between the two methods, are promising in the possibility of exploring this direct collection method in future studies.

In fact, the adsorbed milk technique allows the card to be imbibed by simply placing it on the nipple either at the moment of the first milk ejection reflex, to obtain foremilk, or immediately after a feeding to collect hindmilk.

Analyzing the FA profile of breast milk with a quick and noninvasive method is crucial for several reasons. First, it provides valuable information about the nutritional adequacy of breast milk, ensuring that infants receive essential FAs necessary for their growth and development.[13] Furthermore, analyzing the FA profile has implications beyond immediate nutrient needs. Research suggests that specific FAs are involved in various biological processes related to immune function, inflammation regulation, and cognitive development.[14–16] Therefore, understanding the complete picture of breast milk's FA composition allows researchers and healthcare professionals to explore potential long-term health implications for infants.

Maternal diet is one influential factor that can affect the FA content in breast milk. Consuming foods rich in omega-3 FAs, such as fish or flaxseed oil, can increase DHA levels in breast milk.[17] Therefore, having a less invasive methods of human milk collection could rapidly provide valuable information regarding the FA profile of the mother's milk and allow for maternal dietary interventions by balancing the dietary lipids.

The implications of this new efficient method for research and clinical practice could be vast. In research settings, it opens new possibilities for studying maternal nutrition, infant health outcomes, and overall breastfeeding patterns on a larger scale due to increased sample collection rates and easy storage. Future studies should also focus on the impact of direct collection of breastmilk, by dabbing the collection sheet directly on the nipple. Further research should focus on improving the methodology by studying the possibility, as for other biological samples such as dried blood spot,[18] of storing the collection card at room temperature for long periods, as is already done for newborn metabolic screening.[19] If this were to be confirmed, it would open the possibility of sample remote collection in longitudinal studies or delayed analysis over time.

## 5 | CONCLUSION

By overcoming the limitations associated with traditional methods through noninvasive adsorbed milk collection cards, researchers gain access to larger sample sizes and more representative samples. This method has implications for research studies on maternal nutrition and infant health. Further improvements in the methodology could allow an easier, remote collection, paving the way for personalized nutrition recommendations based on individual variations in maternal milk FA composition. This could lead to tailored interventions aimed at improving infant health outcomes and reducing the risk of long-term health conditions.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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