




Endocannabinoid Metabolome Characterization of Milk from Guatemalan Women Living in the Western Highlands

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ABSTRACT

Background: Recognized as the gold-standard ideal fare, human milk has a unique composition that meets infants' needs throughout development. Endocannabinoids and endocannabinoid-like compounds [endocannabinoid metabolome (ECM)] are endogenous lipid mediators derived from long-chain polyunsaturated fatty acids. Based on animal models, it has been proposed that endocannabinoid arachidonoyl glycerol (AG) plays a role in establishing the suckling response during lactation. In addition, endocannabinoid ethanolamides have been shown to stimulate food intake. The mechanisms of action and the role of the ECM in human milk are not fully understood.

Objectives: The present study aimed to characterize and quantify the ECM in human milk samples from an underserved population in Guatemala.

Methods: Human milk samples were collected from lactating women ($n = 26$) for ECM characterization and quantification. Samples were taken at 3 different time points between 4 and 6 mo of lactation during maternal fasting. Human milk samples were analyzed by liquid chromatography-mass spectrometry. Identified members of the ECM were: arachidonoyl ethanolamide, palmitoyl ethanolamide (PEA), oleoyl ethanolamide, docosahexaenoyl ethanolamide, eicosapentaenoyl ethanolamide, eicosenoyl ethanolamide, AG, palmitoyl glycerol, oleoyl glycerol, docosahexaenoyl glycerol, eicosapentaenoyl glycerol, eicosenoyl glycerol, arachidonic acid (ARA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA).

Results: Overall, concentrations in the ethanolamide group were lower than the glycerols. A time effect was observed for ARA, DHA, EPA, and PEA across the 3 time points ($P \leq 0.05$).

Conclusions: Our study identified the ECM in mature human milk and provides the first report for a population with health disparities within a developing country. The few studies available have been conducted in developed countries. Hypotheses for future studies can be developed based on this study's data to help elucidate specific roles for members of the ECM and how this biological system modulates infant health and development. *Curr Dev Nutr* 2019;3:nzz018.

Introduction

Under conditions of good maternal nutritional health, human milk has been recognized as the ideal food for meeting the growth and development needs of the infant. The lipids of breast milk include the essential fatty acids α -linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6), and the long-chain polyunsaturated fatty acids (LCPUFAs) arachidonic acid (ARA, 20:4n-6) and



Keywords: long-chain polyunsaturated fatty acids, infant health, endocannabinoids, infant feeding, human milk

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Abbreviations used: AEA, arachidonoyl ethanolamide or anandamide; AG, arachidonoyl glycerol; ALA, α -linolenic acid; ARA, arachidonic acid; CB1/2, cannabinoid receptor type 1/2; DHEA, docosahexaenoyl ethanolamide; DHG, docosahexaenoyl glycerol; EC, endocannabinoid; ECM, endocannabinoid metabolome; ECS, endocannabinoid system; EEA, eicosenoyl ethanolamide; EG, eicosenoyl glycerol; EPEA, eicosapentaenoyl ethanolamide; EPG, eicosapentaenoyl glycerol; HM, human milk; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; OEA, oleoyl ethanolamide; OG, oleoyl glycerol; PEA, palmitoyl ethanolamide; PG, palmitoyl glycerol.

DHA (22:6n-3) (1). DHA and ARA are the most abundant LCPUFAs in fetal brain as they are used for structure and function; both accumulate in the infant's brain from gestation continuing after birth (2). ARA is important for growth, whereas DHA plays a key role in the visual and cognitive development of the infant (3-9).

The LCPUFAs have been characterized as precursors of metabolic mediators that include endocannabinoids (ECs) (10, 11). ECs are present in the body, with a high presence of cannabinoid receptor type 1 (CB1) in the central nervous system and CB2 in the periphery (12). CB1 is present in brain areas that are related to motor control, emotional responses, motivated behavior, and energy homeostasis (13). The endocannabinoid system (ECS), comprised of EC, receptors, and enzymes (14), has been shown to modulate physiologic responses in the human body (15). The endocannabinoid metabolome (ECM) has been described as a set of metabolites that include EC and EC-like compounds that interact with the ECS to exert metabolic responses (16, 17). For the purpose of the present study, the ECM includes the following: 1) members of the ethanolamide derivatives—anandamide (AEA), palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA), and eicosenoyl ethanolamide (EEA); 2) members of the glycerol derivatives—arachidonoyl glycerol (AG), palmitoyl glycerol (PG), oleoyl glycerol (OG), docosahexaenoyl glycerol (DHG), eicosapentaenoyl glycerol (EPG), eicosenoyl glycerol (EG); and 3) precursor LCPUFAs—ARA, DHA, and EPA (20:5n-3). The mechanism of actions and the role of the ECM in human milk are not fully understood.

Research by our group has recently demonstrated the presence of these biological signaling molecules, the ECM, in human milk from populations within the United States, representing a developed country. Data from Durham et al. (18) showed a relation between the ECM during pregnancy (measured in plasma) and postpartum (measured in milk), whereas data from Wood et al. (19) showed no differences in the ECM of milk from healthy women compared with those who were diagnosed with gestational diabetes mellitus. Additionally, we have recently characterized the ECM in transitional and mature human milk (20).

Guatemala is located in Central America; it is categorized by the World Bank as a lower middle income country, with 59.3% of its population living in poverty (21). Prevalence of underweight in children <5 y of age is 12.6% (21), and based on the Ministry of Public Health and Social Assistance of Guatemala (22), 46.5% of these children are stunted (low height-for-age), making it the seventh highest rate of stunting in the world (23). Guatemala's indigenous population, comprising 41% of its total population (24), suffer higher disparities in malnutrition compared with mestizos/ladinos, with children and women especially affected (25). For these children, breastfeeding represents the most effective deterrent to effect disease prevention and to avoid nutrient insufficiency. It is well established that undernourished children may be at higher risk of growth restriction, health issues, and impaired cognitive development (26). These data underscore the importance of human milk for meeting the nutritional needs of Guatemala's children and especially the children of the indigenous population. Thus, the present study aimed to characterize and quantify the ECM in human milk samples from an underserved population in Guatemala and provide insights on the composition of human milk to address malnutrition.

Methods

Study design

This was an exploratory study with human milk samples collected between 16 and 24 wk (4-6 mo) postpartum from lactating women living in the western highlands of Quetzaltenango, Guatemala. This was an ancillary study of the parent protocol: 'Short term response of breast milk micronutrient concentrations to a lipid-based nutrient supplement in Guatemalan women' (clinical trial registry number NCT02464111, <https://clinicaltrials.gov>) which was approved by the institutional review boards of the University of California, Davis and the Center for Studies of Sensory Impairment, Aging and Metabolism in Guatemala. For the purpose of this study, only the details pertaining to the milk samples collected for analysis of the ECM are presented.

Subject recruitment

Recruitment of subjects was conducted at the community health center and rural communities in the geographic area of Quetzaltenango, Guatemala. Quetzaltenango is the second most important city in the country and is 125.5 miles (~202 km) away from the capital, Guatemala City. It is located in the western highlands, surrounded by mountains and volcanos. The indigenous population predominates in this area, comprising 60.3% of Quetzaltenango's total population (27).

Potentially eligible lactating women were recruited for participation by direct solicitation among women in the waiting area of the community health center or by distribution of a promotional pamphlet. Women who demonstrated interest in participating in the study were screened for inclusion based on the following criteria: mother 18-40 y of age with a singleton birth; apparently healthy (determined by anthropometrics), with no acute illness (e.g., flu, diarrhea, or mastitis); breastfeeding at 16-24 wk (4-6 mo); willing to stay at the research clinic for breast milk sampling; ≥ 8 breast feeding episodes per day; and only breastfeeding 1 child. Women were contacted to schedule the consenting process (for thorough explanation of the study and for signing of the consent form) and study visits to the research clinic.

Sample collections

Participants were instructed to go to the clinic on 4 separate days ~1 wk apart, each day representing a study visit. During the first visit, participants provided demographic data and anthropometric measures were taken. At 16-24 wk postpartum (visits 2, 3, and 4), fasting mothers were scheduled for milk collection at around 0700. Milk was collected with a manual breast pump (Medela; McHenry) according to the midfeed sampling method (28). Foremilk was collected with a manual pump for 1 min. After 1 min, the foremilk was discarded and a 10-mL aliquot of midfeed milk was collected for analyses. Mothers were instructed to feed their infant on the other breast during the sample collections. Following the milk collection, the mother continued feeding the infant. Samples were immediately divided into aliquots in 2-mL tubes and stored in a cooling container with ice packs, transferred to a -20°C freezer within 2 h, then transferred to a -80°C freezer within 2 wk. Samples were shipped from Guatemala to the Western Human Nutrition Research Center in Davis, CA on dry ice where samples were stored at -80°C. Then, samples were shipped overnight

on dry ice to the Center for Drug Discovery at Northeastern University, Boston, MA and kept at -80°C until analysis.

Sample analysis

Human milk samples were analyzed by LC-MS with a methodology established at the Center for Drug Discovery at Northeastern University, Boston, MA. This methodology has been described in a previous study from our laboratory (20). Briefly, protein precipitation was carried out and an internal standard mixture was added. The resulting supernatant was diluted followed by solid-phase extraction with OASIS HLB reverse-phase chromatography cartridges (Waters Corp.) to elute the absorbed lipids with acetonitrile. The acetonitrile fraction was evaporated to dryness under nitrogen, reconstituted in ethanol, vortexed and sonicated, and centrifuged prior to LC-MS analysis. The autosampler was kept at 4°C to prevent analyte degradation. A TSQ Quantum Ultra triple-quadrupole mass spectrometer (Thermo Electron) with an Agilent 1100 liquid chromatograph (Agilent Technologies) at the front end was used for identification and quantification. Elution of fatty acids was achieved while the mass spectrometer was in negative ionization mode, followed by a change in the mass spectrometer to positive ionization mode for elution of ethanolamine and glycerol esters. Eluted peaks were ionized via atmospheric pressure chemical ionization in multiple reaction monitoring mode. Deuterated internal standards were used to derive a standard curve for each analyte, and concentrations (ng/mL) of human milk were calculated. Each sample was analyzed in triplicate and concentrations were averaged.

Statistical analyses

Statistical analyses were performed with SAS version 9.4 software (SAS Institute Inc.). The level of significance was set at ≤ 0.05 . Descriptive statistics were used for numeric variables. Repeated-measures ANOVA with SAS “proc mixed” was used to assess the effect of time on the concentrations of members of the ECM. The relationship between the parent fatty acid and its respective derived EC was assessed by calculating Pearson correlation coefficients.

Results

Twenty-six maternal-infant dyads were recruited to participate in this study. Table 1 shows participant characteristics (mean \pm SD). On average, mothers had a BMI of 26.3 ± 4.2 kg/m² and a mid-upper-arm circumference of 28.0 ± 3.1 cm. Infants were 4.5 ± 0.5 mo old, weighed 7.0 ± 1.1 lb (3.2 ± 0.5 kg), and the majority (65%, $n = 17$) had a normal height-for-age ratio.

Table 2 shows the concentrations of the members of the ECM. Standard curves for each member were linear and had regression values ≥ 0.99 . Overall, ECM concentrations in the glycerols were between 2000 and 4850 times higher than the concentrations for the ethanolamides. For some ECM members, the range was highly variable. ARA was the principal LCPUFA with 70.8%, followed by DHA and EPA with 21.8% and 7.4%, respectively. PEA was the main metabolite in the ethanolamide group, comprising 90.6%, followed by OEA with 7.7%, and AEA, DHEA, EPEA, and EEA comprising the remaining 1.7%. For the glycerol group, PG comprised 84.5%, OG 13.9%, and AG, DHG, EPG, and EG the remaining 1.6%. EPEA was not detected in most of

TABLE 1 Maternal-Infant Characteristics ($n = 26$)¹

Characteristics	Mean \pm SD or % (frequency)
Maternal BMI, kg/m ²	26.3 \pm 4.2
Maternal MUAC, cm	28.0 \pm 3.1
Maternal literacy (literate)	88.5 (23)
Marital status (married)	100 (26)
Infant age at start, mo	4.5 \pm 0.5
Infant weight, lb	7.0 \pm 1.1
Infant gender (girls)	50 (13)
Infant HAZ	-1.33 \pm 1.32
Normal height-for-age	65.4 (17)
Moderately stunted	19.2 (5)
Severely stunted	15.4 (4)

¹HAZ, height-for-age z score; MUAC, mid-upper-arm circumference.

the samples. For some samples, PEA and PG were above the standard curve. However, these values were close to the curve and it could be assumed that they did not saturate the detector. Therefore, those results should be interpreted with caution and used more as a guide as this was an exploratory study to determine which ECM members were present in human milk.

There was a time effect across the 3 collection days of sampling for the concentrations of PEA and the LCPUFAs ARA, DHA, and EPA ($P \leq 0.05$) (Table 2). Concentrations were higher on the third day of collection for PEA and on the second day for ARA, DHA, and EPA. There was a significant correlation between the parent fatty acid and its derived EC in the glycerol group for ARA and AG ($r = 0.66$, $P \leq 0.01$) and DHA and DHG ($r = 0.90$, $P \leq 0.01$) (Figure 1). ARA and DHA concentrations did not correlate with AEA and DHEA, respectively. A small correlation/modest association was found for AEA and AG ($r = 0.32$, $P \leq 0.01$). For EPA, there were no correlations for its derived EC: EPEA or EPG.

Discussion

Our study provides the first report describing the ECM in human milk from a developing country. Other studies have reported on members of the ECM, mainly AEA and other ethanolamides, in human milk, but these studies have focused on populations from the United States (18–20, 29), Sweden (30), Israel (31), or the United Kingdom (32). As Guatemala has the seventh highest rate of stunting in the world (25), human milk, the “liquid gold,” is a very important source of nutrients for infants and children and provides a means for addressing infant malnutrition. A study by van Beusekom et al. (33) of a similar population in Quetzaltenango determined that 58% of women were either exclusively or predominantly breastfeeding their infants at 6 mo of age.

In the current study, we evaluated the presence of ECM members in milk at 16–24 wk to characterize the metabolites present in human milk and to quantify them. Our results for AEA, EEA, and PG are very similar to previous reports by our group (18, 19) for breast milk at 10 wk postpartum from a cohort in Baton Rouge, LA; however they differ for the other ECM members. For both populations, i.e. our previous reports and the present study, PEA, PG, and ARA were the

TABLE 2 Endocannabinoid Metabolome of Human Milk (n = 26)¹

Metabolite	Time point			P value ²
	1	2	3	
Fatty acids				
ARA	1431.89 ± 169.80	2111.88 ± 279.00	1474.86 ± 173.70	0.0034
DHA	408.04 ± 45.01	664.20 ± 110.77	470.14 ± 60.10	0.0052
EPA	134.00 ± 23.07	251.55 ± 62.33	134.95 ± 27.46	0.0234
Ethanolamides				
AEA ³	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.80
PEA ³	0.82 ± 0.06	5.02 ± 2.89	31.33 ± 7.13	<0.0001
OEA	0.94 ± 0.12	1.21 ± 0.21	1.02 ± 0.14	0.34
DHEA ³	0.09 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.51
EPEA ³	0.30 ± 0.26	0.02 ± 0.02	0.04 ± 0.01	NA
EEA ³	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.72
Glycerol esters				
AG	124.49 ± 18.06	146.28 ± 14.14	134.24 ± 23.34	0.54
PG ⁴	24,638.76 ± 3644.94	31,319.78 ± 3836.68	24,231.83 ± 3411.15	0.11
OG	4440.54 ± 734.99	4512.50 ± 448.58	4234.21 ± 621.74	0.92
DHG	214.94 ± 31.73	318.08 ± 55.55	244.14 ± 38.51	0.08
EPG	10.47 ± 1.55	15.16 ± 1.96	15.00 ± 3.66	0.13
EG	96.36 ± 15.03	103.69 ± 9.72	90.96 ± 15.15	0.64

¹Data are presented in ng/mL and are mean ± SEM. AEA, arachidonyl ethanolamide or anandamide; AG, arachidonoyl glycerol; ARA, arachidonic acid; DHA, docosahexaenoic acid; DHEA, docosahexaenoyl ethanolamide; DHG, docosahexaenoyl glycerol; EEA, eicosenoyl ethanolamide; EG, eicosenoyl glycerol; EPA, eicosapentaenoic acid; EPEA, eicosapentaenoyl ethanolamide; EPG, eicosapentaenoyl glycerol; NA, not analyzed (not enough values to calculate); OEA, oleoyl ethanolamide; OG, oleoyl glycerol; PEA, palmitoyl ethanolamide; PG, palmitoyl glycerol.

²P value represents the effect of time across the 3 time points. Significant differences are marked in bold.

³Some values were below the standard curve.

⁴Some values were above the standard curve.

main metabolites in the ethanolamide, glycerol, and fatty acid groups, respectively. In the current study, glycerol metabolites tended to be present in higher concentrations than ethanolamides in human milk as previously reported by Fride et al. (31), Wood et al. (19), and our recent report from a cohort in the United States (20). Each report shows different concentrations, but they all follow the same distribution pattern.

Although both AG and AEA, and also DHG and DHEA, are derived from ARA and DHA, respectively, synthesis/degradation rates may differ between those two groups presumably due to the action of enzymes such as fatty acid amide hydrolase and monoacylglycerol lipase (34). The affinities of anabolic and catabolic enzymes for the EC likely differ for each metabolite. For example, fatty acid amide hydrolase

is the principal enzyme for metabolizing AEA, although it can also act on AG albeit at a different rate (35). In addition, the presence of “entourage metabolites” (36) (PG, OG, OEA, DHG, EPEA, etc.) can interfere with enzyme activity because some of these metabolites can act as substrate for the same enzymes. Alternatively, metabolites may inhibit uptake and degradation of EC or increase receptor affinity for either CB1 or CB2 (16). Any of these factors would presumably affect the activity of one over another. There is currently insufficient evidence to speculate about the synthesis and degradation pathways for each group of metabolites, i.e., ethanolamides compared with glycerols. A few studies have reported EC concentrations in human milk, all with various study limitations, including limited numbers of study subjects and variation in the lactation stage at which samples were collected.

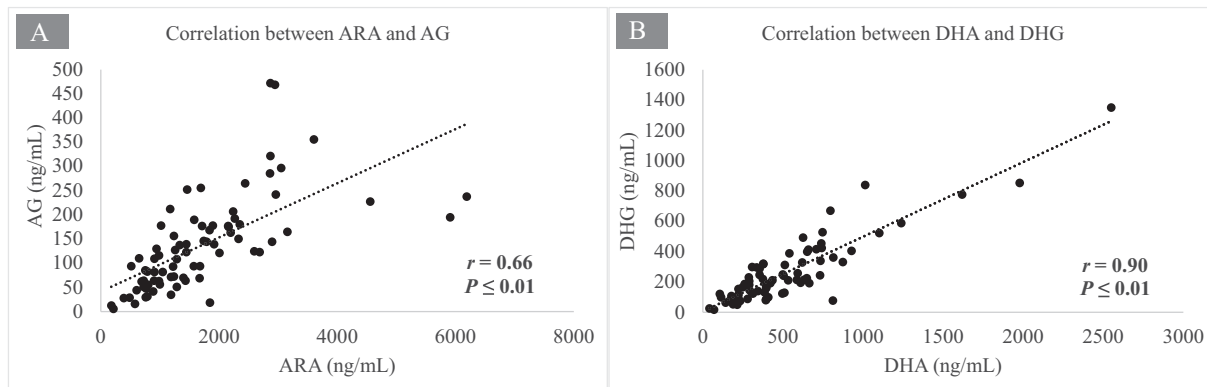


FIGURE 1 Correlations between the precursor fatty acid and its derived endocannabinoid. (A) The correlation between ARA and AG (excluding the 3 highest points, r = 0.77). (B) The correlation between DHA and DHG (excluding the 3 highest points, r = 0.79). ARA, arachidonic acid; AG, arachidonoyl glycerol; DHA, docosahexaenoic acid; DHG, docosahexaenoyl glycerol; EPA, eicosapentaenoic acid.

Nonetheless, all of these studies (18–20, 29–32) support the presence of different EC and EC-like compounds in human milk.

There was a positive correlation between ARA and AG and also DHA and DHG in the milk of this current population. These correlations between the parent fatty acid and its derived glycerol—but not its derived ethanolamide—metabolites may be explained by the fact that glycerols are present in human milk at higher concentrations than ethanolamides. Although specific roles in maternal and infant health for the EC and EC-like compounds have not been described to date, AG has been suggested to play a role in infant feeding behavior (37) and has been shown to be involved in stimulating food intake. A study by Kirkham et al. (38) showed that AG levels increased in areas of the brain related to motivation to eat when fasting, exerting a stimulation to eat. The role of DHG remains to be elucidated. It is plausible, however, that DHA derivatives (i.e., DHG and DHEA) sustain an infant's cognitive development. LCPUFAs play a critical role in infant development underscoring the interest and importance of understanding this biological system, the ECM, in human milk.

For populations with health disparities and economic disadvantages, understanding breast milk and its biological components can provide insight into understanding and addressing stunting rates, especially during the first months of age, which are likely to have an impact later in life. Data are limited at this time. The current study opens the door to develop research hypotheses in this field to support the relevance of ECM to health and as a possible intervention for infants with health disparities.

Our data add to the wealth of research around human milk composition and provide insights into novel bioactive compounds that may play a role in infant health. Nonetheless, the current study had limitations. The study sample was not necessarily representative of the Guatemalan population. Participants were recruited from a convenience sample in an underserved population in the highlands of Quetzaltenango that is documented to have limited access to a diet that meets nutrient requirements compared with affluent populations within the country. The aliquot size was less than the amount needed to detect ethanolamides and EPG concentrations with confidence; thus ethanolamides, especially EPEA, were not detected as originally planned. The data from this study should be interpreted with caution and considered preliminary findings that provide one of the first reports on the ECM of human milk.

In conclusion, our study identified members of the ECM in mature human milk and provided, to the best of our knowledge, the first report for a population with health disparities within a developing country. These data shows the presence of 15 members of the ECM in human milk, with a higher concentration of ARA in the fatty acids, PEA in the ethanolamides, and PG in the glycerols. Our finding of differences of some metabolites over time warrants further investigation with regards to maternal diet. The roles of each member in the ECM in infant development have not yet been established, and this also warrants further investigation.

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