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## Endocannabinoid Metabolome of Human Breast Milk

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# ENDOCANNABINOID METABOLOME OF HUMAN BREAST MILK

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by

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## LIST OF ABBREVIATIONS

PUFA	Polyunsaturated Fatty Acid
LCPUFA	Long-Chain Polyunsaturated Fatty Acid
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
ARA	Arachidonic Acid
EC	Endocannabinoids
ECS	Endocannabinoid System
ECM	Endocannabinoid Metabolome
CB1	Cannabinoid Receptor Type 1
CB2	Cannabinoid Receptor Type 2
AEA	Arachidonyl Ethanolamide or Anandamide
PEA	Palmitoyl Ethanolamide
OEA	Oleoyl Ethanolamide
DHEA	Docosahexaenoyl Ethanolamide
EPEA	Eicosapentaenoyl Ethanolamide
EEA	Eicosenoyl Ethanolamide
AG (or 2-AG)	Arachidonoyl Glycerol
PG	Palmitoyl Glycerol
OG	Oleoyl Glycerol
DHG	Docosahexaenoyl Glycerol
EPG	Eicosapenaenoyl Glycerol
EG	Eicosenoyl Glycerol

## ABSTRACT

Human breast milk (HBM) is an extremely complex yet fascinating biofluid tailored to meet an infant's nutritional requirements for development. Amongst the nutrients present in HBM, the long-chain polyunsaturated fatty acids (LCPUFAs) are of high importance due to the pivotal role they play in infant cognitive and visual development, and growth. In addition, the LCPUFAs are precursors to endocannabinoids (EC) which are endogenous lipid mediators. EC exert metabolic responses including appetite and food intake regulation, and they have been identified to play a role in establishing the suckling response of the newborn that is needed to nurse. Thus, we aimed to characterize and quantify the EC present in HBM, termed the EC metabolome (ECM). HBM samples were collected from two different populations, one in Guatemala (n = 26) and the other one in the United States (n = 24). We collected HBM at different lactation stages: transitional (2 weeks postpartum) and mature (4 weeks and 16-24 weeks postpartum) milk. Using liquid chromatography-mass spectrometry analyses, we identified 15 members of the ECM in both lactation stages: arachidonylethanolamine, palmitoylethanolamine, oleoylethanolamine, docosahexaenoylethanolamine, eicoapentaenoylethanolamine, eicosenoylethanolamine, arachidonoylglycerol, palmitoylglycerol, oleoylglycerol, docosahexaenoylglycerol, eicosapentaenoylglycerol, eiconenooylglycerol, arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid. Overall, members in the glycerol group were higher in concentration than those of the ethanolamide group. To date, the mechanisms of action and the role of the ECM in HBM and infant development are not fully understood. Data from the present study provides a foundation to develop future studies to help elucidate how the ECM modulates infant health and development.

## **CHAPTER 1 INTRODUCTION**

Endocannabinoid research has attracted considerable interest because of the physiological functions of these bioactive compounds and their possible therapeutic use in maintaining health.<sup>1</sup> The endocannabinoid metabolome (ECM) consists of different lipid mediators which are derived from fatty acids conjugated with either an ethanolamine or a glycerol group. Regarded as the gold standard, breast milk is a bioactive fluid that has a unique composition to meet infants' needs throughout development. Recent research has demonstrated the presence of these biological signaling molecules, endocannabinoids and endocannabinoid-like compounds, in human breast milk. Endocannabinoids play a role in establishing the suckling response of the newborn by activating the oral-motor musculature needed for milk suckling. The established suckling response in turn supports maternal-infant bonding and maintains infant feeding behavior.

To date, the mechanisms of action and the role of the ECM in breast milk and infant development are not fully understood. The present study has characterized and quantified the ECM in two different populations. The first population assessed corresponds to a cohort from Guatemala, a developing country, whereas the second population corresponds to a cohort in the United States representing a developed country. In this regard, we were able to evaluate how maternal nutritional status can affect the fatty acids and the ECM of breast milk. The present study will set the stage for developing hypotheses for future studies that will help to elucidate how this biological system modulates infant health and development.



## **CHAPTER 2 LITERATURE REVIEW**

### **2.1 Human Milk and Lactation**

Recognized as the gold standard, with its unique composition, breast milk provides the infant with optimal nutrition needed in the minutes following birth and throughout development of the first year. According to Koletzko (2016), “milk can be characterized as an emulsion of milk fat globules in an aqueous liquid.”<sup>2</sup> The American Academy of Pediatrics and the World Health Organization recommend exclusive breastfeeding of infants up to six months of age with continued breastfeeding along with complementary foods for up to two years of age or beyond.<sup>3,4</sup> According to the Center for Disease Control and Prevention,<sup>5</sup> only 22.3% of infants in the United States are exclusively breastfed at six months with 51.8% of infants breastfed at six months and 30.7% at 12 months but supplemented with formula. However, initiation rates for infants ever breastfed are 81.1% which demonstrates that mothers’ intent to breastfeed at least for the first days postpartum (one to two days) while at the hospital after delivery. Globally, only 38% of infants are exclusively breastfed during the first six months of life.<sup>6</sup> The Baby-Friendly Hospital Initiative, which was launched in 1991 and sponsored by the World Health Organization (WHO) and the United Nations Children’s Fund (UNICEF),<sup>7</sup> has helped to improve the national rates for breastfeeding by encouraging and recognizing hospitals and birth centers that offer an optimal level of care for lactation based on the Ten Steps to Successful Breastfeeding (Table 2.1). In addition, the WHO has issued the International Code of Marketing of Breast-Milk Substitutes to provide recommendations on the marketing of breast milk substitutes, feeding bottles, and teats aiming to stop aggressive and inappropriate marketing with the ultimate goal to support breastfeeding.<sup>8</sup>

Table 2.1. WHO/UNICEF Ten Steps to Successful Breastfeeding

- 
1. Have a written breastfeeding policy that is routinely communicated to all health care staff.
  2. Train all health care staff in skills necessary to implement this policy.
  3. Inform all pregnant women about the benefits and management of breastfeeding.
  4. Help mothers initiate breastfeeding within a half-hour of birth.
  5. Show mothers how to breastfeed, and how to maintain lactation even if they should be separated from their infants.
  6. Give newborn infants no food or drink other than breastmilk unless medically indicated.
  7. Practice rooming in - allow mothers and infants to remain together - 24 hours a day.
  8. Encourage breastfeeding on demand.
  9. Give no artificial teats or pacifiers (also called dummies or soothers) to breastfeeding infants.
  10. Foster the establishment of breastfeeding support groups and refer mothers to them on discharge from the hospital or clinic.
- 

### **Lactogenic Hormones**

Two main hormones facilitate and direct lactation: prolactin for milk production and oxytocin for milk ejection. Prolactin is further modulated by pituitary, ovarian, thyroid, adrenal, and pancreatic hormones.<sup>9</sup> Following delivery of the placenta, progesterone levels decrease and prolactin levels increase, stimulating milk synthesis.<sup>10</sup> The regulation of milk production is based on infant demand, with suckling stimulating the release of prolactin in response to nipple manipulation.<sup>9</sup> Oxytocin, which facilitates delivery of the infant by causing the uterus to contract, facilitates milk ejection.<sup>9</sup> Oxytocin is also released in response to sensory pathways when the mother sees, touches, hears, smells or thinks about the infant. On the other hand, release of oxytocin can be blocked by stress and fright.<sup>9,11</sup>

### **Lactating Mammary Gland: Milk Production and Secretion**

The mammary gland meets three specific functions: synthesis, secretion, and delivery of milk to the infant. Mammary development starts as early as during fetal growth, reaching the height of development during the pregnancy-lactation cycle with involution following weaning.<sup>12</sup> Glandular and adipose tissues are the main components of the mammary gland, held together by

connective tissue, called Cooper's ligaments<sup>11</sup> (Figure 2.1). During pregnancy the breast gains approximately 0.75 pounds and becomes firm and full in preparation for lactation.<sup>13</sup> Maternal requirements for energy increase during lactation with the breast using about 25% above the daily energy intake (based on 2,000 kcal/day) to produce milk.<sup>14</sup>

Milk is produced in the alveoli and is released into the ducts. The nipple contains between 15 to 25 ducts that are used for milk delivery/transport.<sup>9</sup> Contraction of myoepithelial cells cause the alveoli to release the milk to be ejected through the nipple.<sup>11,12</sup>

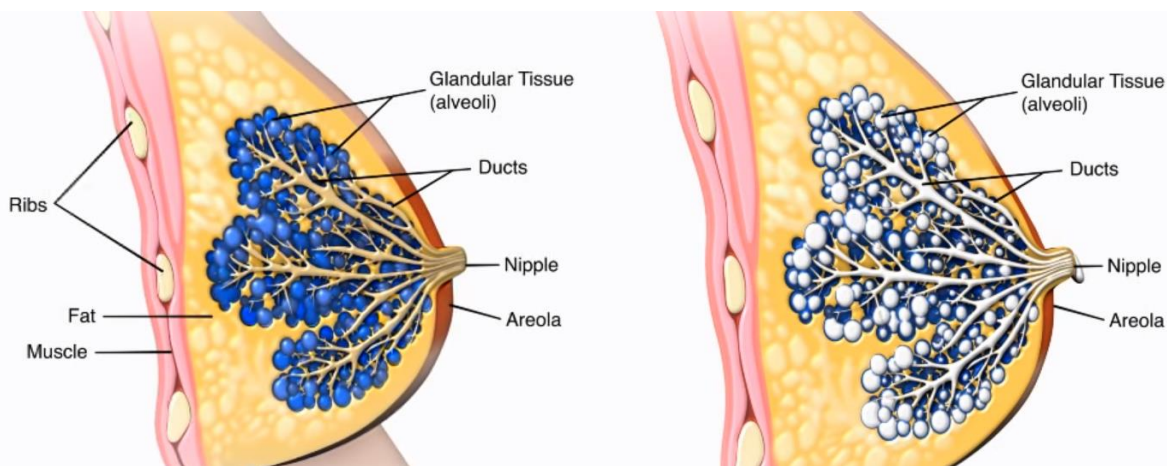


Figure 2.1. Anatomy of the Lactating Breast.<sup>15</sup> Figure on the left side represents a breast during pregnancy in preparation to produce milk. Figure on the right represents a breast that is producing milk postpartum.

To summarize, the “milk ejection reflex” is an interplay between hormones that synthesize and release the milk following a cascade of events initiated by infant suckling which stimulates prolactin and oxytocin release from the pituitary gland, thus stimulating milk synthesis and secretion.<sup>16</sup> Stimulation at the nipple in response to infant suckling causes nerve impulses to stimulate oxytocin release from the posterior pituitary that will act on the mammary gland causing myoepithelial cells to contract to eject milk from the alveoli into the ducts and release it to nurture the infant.<sup>17</sup>

## **Stages of Lactation**

Lactation encompasses three different stages: mammogenesis, lactogenesis, and involution. Mammogenesis refers to the mammary growth and differentiation of alveolar epithelial cells from fetal life through puberty, reaching its complete development following parturition.<sup>18</sup> Lactogenesis refers to the initiation of lactation, milk secretion, until the breast stops producing milk following weaning which is referred to as involution when the mammary gland returns to a nonlactating state.<sup>12</sup>

Lactogenesis is further divided into three stages:

- a. Lactogenesis I: in this stage, mammary tissue undergoes differentiation to change from a nonlactating to a lactating state. During pregnancy, alveolar epithelial cells differentiate into lactocytes that synthesize milk with accumulation of colostrum.<sup>12</sup> High maternal circulating plasma concentrations of progesterone and estrogen halt secretion of milk.<sup>10</sup>
- b. Lactogenesis II: this stage is characterized by the onset of copious milk secretion following parturition.<sup>10</sup> Blood flow increases, accompanied by oxygen and glucose uptake.<sup>9</sup>
- c. Lactogenesis III: this last stage facilitates maintenance of milk production until weaning.<sup>9</sup>

Within a feeding, breast milk is further categorized as foremilk, the initial milk, and hindmilk, the last milk of a feed.<sup>19</sup>

## **Breast Milk Composition**

New mothers are capable of producing milk that meets their infants' needs throughout development. Milk composition changes from the first days following birth to about four weeks postpartum when lactation has been established. The three different stages of human milk following birth are:

- a. Colostrum: this thick yellowish secretion is present for the first three to five days.<sup>12</sup> It is high in protein,<sup>12</sup> immunoglobulins, lactoferrin,<sup>10</sup> oligosaccharides,<sup>20</sup> and immune cells.<sup>21</sup> Colostrum boosts the infant's immunological system and provides protection against infections. Andreas et al. (2015)<sup>22</sup> proposed that the primary role of colostrum in infant health is immunological rather than nutritional.
- b. Transitional: this milk is produced from five days to about two to three weeks (14-21 days) postpartum.<sup>12</sup> The concentration of immunoglobulins, protein, and fat-soluble vitamins decrease while the lactose, fat, water-soluble vitamins, and total caloric content increase compared to colostrum.<sup>16</sup>
- c. Mature: the last stage is from three to four weeks postpartum until weaning occurs.<sup>12</sup>

Table 2.2 indicates the predominant nutrients present in mature breast milk. Milk fat globule membrane proteins (mucins), whey proteins, and caseins comprise the three groups of the protein fraction,<sup>23</sup> with the caseins present in higher concentrations compared to mucins and whey proteins.<sup>22</sup> The predominant proteins are  $\alpha$ -lactalbumin, lactoferrin, and secretory immunoglobulin A (sIgA) which with a wide array of other proteins exert antimicrobial, immunomodulation, and nutrient absorption roles which help in the development of the infant's gastrointestinal tract. Soluble amino acids are present to support growth.<sup>23</sup>

Lactose is the main carbohydrate present in breast milk<sup>16</sup> and the least variable of the macronutrients.<sup>19</sup> Lactose concentrations correspond to the high energy demands of the brain.<sup>22</sup> In mature breast milk, energy estimates range from 65 to 70 kcal/dL.<sup>19</sup> Lipids are the major source of energy in breast milk. Triglycerides account for 98% of the lipid fraction<sup>2</sup> and are the main source of energy in breast milk.<sup>24</sup> Polyunsaturated fatty acids (PUFAs) account for approximately 21% of the total fatty acids present in breast milk.<sup>25</sup> More specifically, for the

long-chain PUFAs (LCPUFAs) estimated levels worldwide are 0.32% for docosahexaenoic acid (DHA, 22:6n3) and 0.47% for arachidonic acid (ARA, 20:4n6) based on the total fatty acids.<sup>26</sup>

Table 2.2. Breast Milk Composition in Mature Milk

Nutrient	Content (%)
Water <sup>a</sup>	88.1
Lactose <sup>b</sup>	6.7 – 7.8
Fat <sup>b</sup>	3.2 – 3.6
Protein <sup>b</sup>	0.9 – 1.2
Other <sup>a</sup>	0.2

<sup>a</sup>Ballard and Morrow, 2013<sup>19</sup>; <sup>b</sup>Lawrence, 1994<sup>16</sup>

Many factors influence milk composition. Maternal factors including age, parity, body mass index, and diet, along with infant factors such as gestational age and birth weight, as well as time of the day and stage of lactation, among others, are known to modify breast milk. Fields et al. (2016)<sup>27</sup> summarized the factors that may influence breast milk composition (Figure 2.2). Even during the same feeding, milk composition changes. For example, within a feeding, the fat content is highly variable, with higher concentrations in the last milk of a feed.<sup>19</sup> The concentration of fatty acids in milk is directly related to maternal diet.<sup>24</sup> Jensen et al. (2000)<sup>28</sup> compared three different sources of DHA supplementation (each providing about 200 mg/day) to lactating women from week two to week eight postpartum. It was determined that DHA content in breast milk increased with dietary supplementation from about 0.24% at baseline to about 0.37% of total fatty acids at the end of the study. In another study by Hawkes et al. (2002),<sup>29</sup> lactating women were provided a supplement with either a placebo, 300 mg or 600 mg of DHA/day from three days to four weeks postpartum. It was found that DHA content of breast milk was increased in relation to DHA intake. Similarly, Sherry et al. (2015)<sup>30</sup> provided lactating women with either a placebo, 200 mg or 400 mg of DHA/day for six weeks. DHA content of breast milk increased over the six weeks of intervention and was directly proportional to the amount of DHA provided in the capsules. DHA content in breast milk is determined by maternal

DHA intake mainly.<sup>31</sup> Fatty acid concentrations in breast milk may even be influenced by dietary consumption of DHA and eicosapentaenoic acid (EPA, 20:5n3) during pregnancy.<sup>32</sup> Therefore, the lipid fraction of breast milk is the most variable nutrient during lactation.<sup>22</sup> Breast milk is dynamic in composition in order to meet infant's needs throughout development.

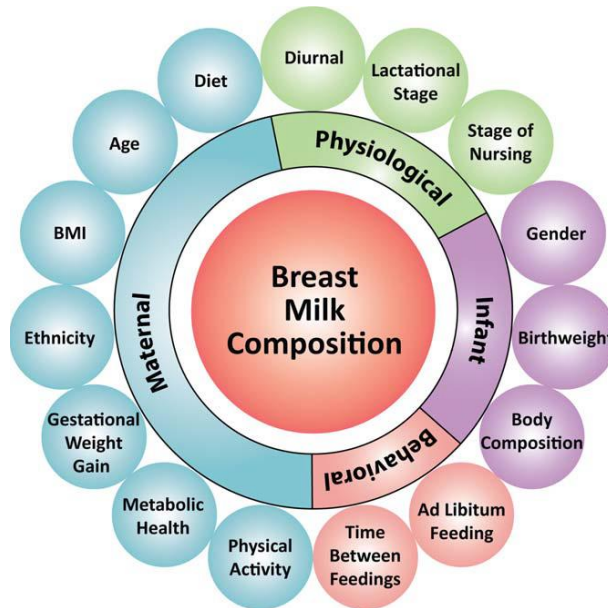


Figure 2.2. Factors Influencing Breast Milk Composition.<sup>27</sup> This figure summarizes the factors that influence breast milk composition in addition to maternal-infant factors, including physiological and behavioral.

## Benefits of Breastfeeding

### a. Infant outcomes:

To what extent the benefits of breastfeeding can be observed depends on the duration and exclusivity versus supplemented breastfeeding. For example, according to Ip et al. (2007)<sup>33</sup> who evaluated the effects of breastfeeding in the short- and long-term for mother and child in developed countries, the incidence of otitis media was 23% lower in infants with any breastfeeding compared to infants fed infant formula only; exclusive breastfeeding for three to six months reduced the risk of otitis media by 50%. Further, reduction in the incidence of gastrointestinal tract infections was associated with *any* breastfeeding. Breastfeeding for four or

more months was associated with reduction in the risk of hospitalization secondary to respiratory diseases by 72%. A meta-analysis conducted by Thompson et al. (2017)<sup>34</sup> showed that breastfeeding for at least two months was associated with protection against sudden infant death syndrome by 40% with an increased protection of 54% when infants were breastfed for four to six months. Obesity rates are lower in breastfed infants suggesting that breastfeeding provides protection against developing childhood obesity later in life.<sup>35</sup> Infants exclusively breastfed for four to six months are at lower risk of developing a nonspecific gastrointestinal infection.<sup>36</sup> Proteins, such as immunoglobulins, lysozyme, lactoferrin, etc., that are present in breast milk provide the infant with immunity and contribute in the development of the intestinal mucosa of the newborn, whereas other types of protein aid in the digestion and uptake of other nutrients in breast milk.<sup>37</sup> Breastfeeding has also been associated with lowering the risk of developing asthma.<sup>38,39</sup>

b. Maternal outcomes

Research has shown that breastfeeding can provide benefits to the mother as well as the child. It can be protective against the development of breast cancer.<sup>40,41</sup> Further, the Women's Health Initiative<sup>42</sup> reported a significant reduction in the incidence of hypertension, hyperlipidemia, cardiovascular disease, and diabetes in women who had a cumulative history of lactation for 12 to 23 months. The development of type 2 diabetes is less likely to occur in mothers who have exclusively breastfed their infants for one-three months.<sup>43</sup> Lactational amenorrhea can act as a natural birth control if breastfeeding exclusively for six months.<sup>44</sup> The role of breastfeeding and postpartum depression is still inconclusive.<sup>33</sup> However, data have shown that multiparous, but not primiparous, women are at reduced risk of having postpartum depression if they breastfed.<sup>45</sup>



## 2.2 Endocannabinoids

Research conducted by Dr. Raphael Mechoulam in the 1960-1970s opened up a new area of research by isolating the main active component of *Cannabis sativa*, commonly known as marijuana, delta-9 tetrahydrocannabinol ( $\Delta$ -9 THC).<sup>46,47</sup> Research in this field has increased considerably over time although many roles, functions, and mechanisms of action remain to be understood. Following that early discovery, endogenous lipid ligands that bind to the same receptors as marijuana, 'endocannabinoids' (EC), were reported.<sup>48,49</sup>

Endocannabinoids are lipophilic molecules derived from LCPUFAs.<sup>50</sup> Two G-protein coupled receptors, cannabinoid receptor type 1 (CB1) and type 2 (CB2), are responsible for the activation/deactivation of the EC.<sup>51</sup> Cannabinoid receptor type 1 is present mainly in the central nervous system and CB2 is expressed predominantly in immune cells.<sup>52</sup>

Arachidonylethanolamide (anandamide, AEA), was the first EC discovered in 1992 and it is the most thoroughly studied to date.<sup>48</sup> Anandamide received its name from the Sanskrit word 'ananda' that means superior bliss.<sup>53</sup> Then, 2-arachidonoylglycerol (2-AG or AG) was identified in 1995.<sup>49</sup> Both AEA and 2-AG are derived from ARA, an n-6 LCPUFA,<sup>54</sup> conjugated with ethanolamine or glycerol, respectively. These molecules bind to CB1 and CB2 receptors, although with different affinities.<sup>55</sup> Anandamide has been found to be a partial agonist of CB1, whereas 2-AG acts as a full agonist for both CB1 and CB2.<sup>56</sup> The main enzymes involved in EC degradation are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL).<sup>57</sup> The endocannabinoid system (ECS) encompasses the EC (AEA and 2-AG), the receptors (CB1 and CB2), and the enzymes involved in their metabolism (MAGL and FAAH). Figure 2.3 shows how the ECS interacts at the cellular level.

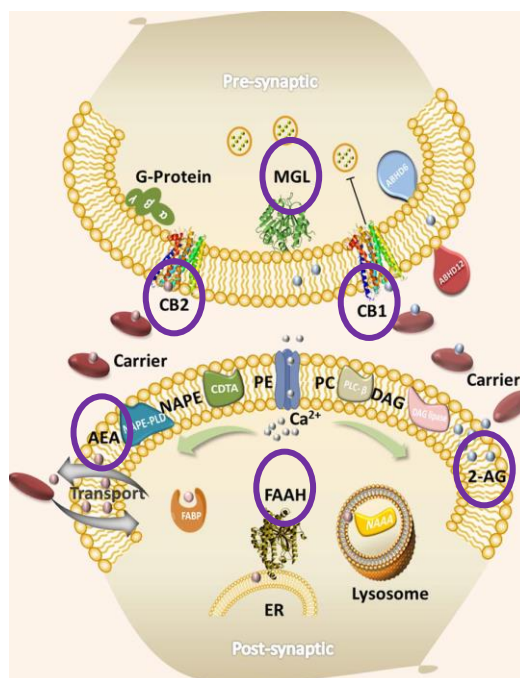


Figure 2.3. The Endocannabinoid System as Drawn by Vemuri and Makriyannis, 2015.<sup>58</sup> This figure represents the interaction of the endocannabinoid system between the post-synaptic and pre-synaptic neuron. Circled members represent the endocannabinoid system as discussed in this chapter: AEA, arachidonylethanolamide; 2-AG, 2-arachidonoylglycerol; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor; MAGL, monoacylglycerol lipase; and FAAH, fatty acid amide hydrolase.

More recently, other endocannabinoid metabolites have been identified. A complete set of 15 metabolites has been termed the ‘endocannabinoid metabolome’ (ECM) (Figure 2.4) which can be defined as a set of endogenous signaling lipid mediators that interact directly or indirectly with the ECS.<sup>59,60</sup> The ECM includes i) six members of the ethanolamide derivatives: AEA, palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA), and eicosenoyl ethanolamide (EEA); ii) six members of the glycerol derivatives: 2-AG, palmitoyl glycerol (PG), oleoyl glycerol (OG), docosahexaenoyl glycerol (DHG), eicosapenaenoyl glycerol (EPG), eicosenoyl glycerol (EG); and iii) three precursor LCPUFAs: ARA, DHA, and EPA. These metabolites are believed to interact with an ‘entourage effect’ meaning that metabolites exert a greater effect to support physiological responses by interacting with each other rather than in isolation or by increasing or



regulation of appetite and food intake.<sup>64</sup> Table 2.3 summarizes some of the physiological roles of members of the ECM.

Endocannabinoids are involved in modulating the inflammatory response through the same pathways as eicosanoids which are also derived from LCPUFAs. Data support that CB1 receptors in the periphery play a role in modulating inflammation related to autoimmune diseases.<sup>66</sup> Omega-3 LCPUFA-derived ethanolamides, DHEA and EPEA, have been shown to inhibit the proliferation of cancer cells.<sup>68</sup> Endocannabinoids also play a role in fertility and reproduction for both males and females. Anandamide has been identified to be present in sperm, and it is believed to play a role in sperm motility and fertilization.<sup>57</sup> On the other hand, in females AEA has been shown to play a role in blastocyst implantation, embryonic survival, and placental development.<sup>65,67</sup>

Table 2.3. Physiological Roles of Members of the Endocannabinoid Metabolome

Metabolite	Physiological role	Reference
AEA	Reproduction Stimulates food intake Mediator of metabolic homeostasis Cognitive performance	Pagotto, et al., 2006 <sup>55</sup> Iannotti et al., 2016 <sup>69</sup>
2-AG	Stimulates food intake Cognitive performance Neuroprotection	Pagotto, et al., 2006 <sup>55</sup> Iannotti et al., 2016 <sup>69</sup> Fride, 2002 <sup>70</sup>
PEA	Anti-inflammatory Anti-nociceptive Neuroprotective Reduces enzymatic breakdown of AEA	Williams et al., 2007 <sup>60</sup> Witkamp, 2016 <sup>66</sup> Iannotti et al., 2016 <sup>69</sup>
OEA	Inhibits EC catabolism Reduces AEA cellular uptake and degradation Regulates food intake Neuroprotective	Williams et al., 2007 <sup>60</sup> Taylor et al., 2010 <sup>67</sup> Iannotti et al., 2016 <sup>69</sup>

(table cont'd.)

Metabolite	Physiological role	Reference
PG	Increases 2-AG affinity to CB2	Williams et al., 2007 <sup>60</sup>
DHEA	Anti-inflammatory Inhibits proliferation of prostate and breast cancer cells	Witkamp, 2016 <sup>66</sup> Brown et al., 2010 <sup>68</sup> Brown et al., 2011 <sup>71</sup>
EPEA	Inhibits proliferation of prostate and breast cancer cells	Brown et al., 2010 <sup>68</sup>
EEA, OG, DHG, EPG, EG	Remains to be elucidated	
ECM and its interaction with the ECS	Feeding regulation Energy expenditure Reward mechanisms Regulation of metabolic homeostasis Regulation of pain and inflammation Modulation of immune function  CB1 localization in the body: Brain, adipose tissue, pancreas, gastrointestinal tract, skeletal muscle, heart, liver, lungs, bone, skin, and reproductive system  CB2 localization in the body: Immune system (cells such as monocytes, macrophages, and B- and T-cells)	Iannotti et al., 2016 <sup>69</sup> Silvestri et al., 2013 <sup>72</sup> Mouslechet et al., 2009 <sup>73</sup>

---

Anandamide (AEA), arachidonoyl glycerol (2-AG), palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), palmitoyl glycerol (PG), docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA), eicosenoyl ethanolamide (EEA), oleoyl glycerol (OG), docosahexaenoyl glycerol (DHG), eicosapenaenoyl glycerol (EPG), eicosenoyl glycerol (EG), endocannabinoid metabolome (ECM), endocannabinoid system (ECS), cannabinoid receptor type 1 (CB1), cannabinoid receptor type 2 (CB2)

---

The ECS also modulates motility, inflammation, and secretion in the gastrointestinal tract.<sup>55</sup> Lipid and glucose metabolism are controlled by EC activity, predominantly in the liver and adipose tissue.<sup>72</sup> Blockade of CB1 has been shown to exert anti-obesity effects<sup>64</sup>; thus, CB1 activation by AEA or 2-AG stimulates food intake.<sup>55</sup> Researchers have determined in rodent

models that 2-AG concentrations increase during fasting and return to normal values when animals are being fed to satiation.<sup>74</sup>

Taken together, EC appear to have roles in maintaining homeostasis. The ECS and ECM offer a promising opportunity for treating and modulating physiological and pathological conditions. Despite a wealth of research conducted to date, many roles and mechanisms remain to be elucidated. Animal and clinical research is still needed in order to support any possible therapeutic roles of the ECS.

### **Endocannabinoid Concentrations are affected by Dietary Fatty Acids**

Concentrations of the EC respond to dietary intake of LCPUFAs, recalling that these fatty acids are precursors for EC synthesis. Wood et al. (2010)<sup>75</sup> evaluated the impact of a two-week dietary DHA supplementation on the ECM in murine brain and plasma. The DHA-enriched diet elevated concentrations of n-3 LCPUFAs, DHA and EPA, and their respective derived ECs, DHEA and EPG, in both brain and plasma. Work by Artmann et al. (2008)<sup>76</sup> showed in a rat model that short term consumption of diets resembling human diets specifically modulated tissue levels (brain, liver, and small intestine) of EC. Similarly, Matias et al. (2008)<sup>77</sup> demonstrated that n-3 PUFAs produced a decrease in AEA and 2-AG concentrations while n-6 ARA produced an increase in isolated mouse adipocytes, pointing to dietary PUFAs as modulators of EC concentrations in adipocytes. Berger et al. (2001)<sup>78</sup> demonstrated that AEA, DHEA, and EPEA concentrations in piglets' brain were increased by ARA and DHA in the feed. In another study by Watanabe and Hamazaki (2003),<sup>79</sup> with mice fed an n-3 PUFA deficient or enriched diet, it was found that 2-AG concentrations were modulated by dietary n-3 PUFAs.

### 2.3 Role of Endocannabinoids during Lactation

Members of the ECM have been recently identified in human milk.<sup>80-82</sup> Seminal work by Dr. Esther Fride in the 2000s supported a proposed role of the ECS during lactation.<sup>83</sup> In 2001, Fride and colleagues,<sup>83</sup> after evaluating the effect of a CB1 antagonist administered to newly born mouse pups, concluded that the ECS plays a vital role in the suckling response. Mouse pups were injected with a CB1 antagonist (SR141716A) at either day one or two only or from day two through day eight after birth. As a result, pups stopped suckling after CB1 antagonist administration which resulted in growth inhibition after the first injection and death by day eight. Observations were confirmed when the antagonist effect was minimized by co-administration of  $\Delta^9$ -THC or 2-AG and entourage mediators, PG and linoleoyl glycerol, which delayed pup mortality rates. This CB1 antagonist effect suggested that CB1 and 2-AG activity is needed for growth and survival. A similar study was conducted using a different strain of mice,<sup>84</sup> confirming that CB1 antagonist administration reduced milk intake and survival rates. It was concluded that CB1 receptor-binding activity played a critical role in initiating the suckling response in the newborn mice. Experiments were then undertaken to analyze potential physiological/behavioral mediators by which CB1 antagonist inhibit milk ingestion.<sup>85</sup> Mouse pups that were administered the CB1 antagonist, when manually brought in proximity to the mother's nipple, failed to hold on to the nipple although they opened their mouths and made suckling movements. These data suggested that the CB1 receptor influences the oral-musculature needed to latch on. Taken together, Fride's work<sup>83-85</sup> proposed that 2-AG, which binds to CB1, levels in the pup's brain stimulates the suckling response at birth. Then, 2-AG from maternal milk activates the CB1 receptor in the pup's brain to continually stimulate suckling. When CB1 in the pup's brain was

blocked by an antagonist, pups did not ingest milk which resulted in maternal 2-AG deficit in the pup's brain to activate the CB1 receptor to stimulate milk suckling.

Cannabinoid receptor type 1 is present in brain areas that are related to motor control, emotional responses, motivated behavior, and energy homeostasis.<sup>73</sup> Thus, the role of the ECS in establishing the oral-musculature behavior needed for milk suckling can be understood. In addition, provided that the ECS plays a role in regulating food intake, 2-AG is needed to facilitate infant latching onto the breast and nursing. Although data at this moment are scarce, it is of great interest to evaluate how the ECS may affect infant nursing behavior to ensure that milk is provided to deliver the infant with the countless benefits of breastfeeding, especially growth and development during the first months of life.

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## **CHAPTER 3**

# **ENDOCANNABINOID METABOLOME OF BREAST MILK: A COHORT FROM GUATEMALA**

### **3.1 Introduction**

Under conditions of good nutritional health for the mother, breast milk has been recognized as the ideal food for meeting growth and development of the infant. The lipids of breast milk include the essential fatty acids,  $\alpha$ -linolenic acid (ALA, 18:2n3) and linoleic acid (LA, 18:2n6), and the long-chain polyunsaturated fatty acids (LCPUFAs), arachidonic acid (ARA, 20:4n6) and docosahexaenoic acid (DHA, 22:6n3).<sup>1</sup> Docosahexaenoic acid 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.<sup>2</sup> Worldwide, concentrations in human milk are 0.47% for ARA and 0.32% for DHA, estimated as a weight percentage of total fatty acids. Arachidonic acid is important for growth, whereas DHA plays a key role in visual and cognitive development of the infant.<sup>3-9</sup>

The LCPUFAs have been characterized as precursors of metabolic mediators that include endocannabinoids (EC).<sup>10,11</sup> Endocannabinoids are present in the body with a high presence of cannabinoid receptor type 1 (CB1) in the central nervous system and cannabinoid receptor type 2 (CB2) in the periphery.<sup>12</sup> Cannabinoid receptor type 1 is present in brain areas that are related to motor control, emotional responses, motivated behavior, and energy homeostasis.<sup>13</sup> The endocannabinoid system (ECS), comprised of EC, receptors, and enzymes,<sup>14</sup> has been shown to modulate physiologic responses in the human body.<sup>15</sup> 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.<sup>16,17</sup> For the purpose of the present study, the ECM includes i) members of the ethanolamide derivatives: anandamide (AEA), palmitoyl

ethanolamide (PEA), oleoyl ethanolamide (OEA), docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA), and eicosenoyl ethanolamide (EEA); ii) members of the glycerol derivatives: arachidonoyl glycerol (AG), palmitoyl glycerol (PG), oleoyl glycerol (OG), docosahexaenoyl glycerol (DHG), eicosapenaenoyl glycerol (EPG), eicosenoyl glycerol (EG); and iii) precursor LCPUFAs: ARA, DHA, and eicosapentaenoic acid (EPA, 20:5n3).

Endocannabinoid metabolites, particularly AG which is derived from ARA and binds to CB1 with high affinity, have been demonstrated to play a role in establishing the suckling response in the newborn by activating the oral-motor musculature needed for milk suckling and therefore infant feeding.<sup>18-20</sup> Research conducted by Fride et al. (2001)<sup>18</sup> in animals demonstrated that when a CB1 antagonist (SR141716A) was administered to mouse pups either at day one or two only, or from day two to eight after birth, growth inhibition and death occurred by day eight after mice stopped suckling following the CB1 antagonist administration. Fride (2004)<sup>19</sup> also demonstrated that when mice were administered a CB1 antagonist and were brought into proximity to the mother's nipple, even though mice opened their mouths and made suckling movements, they failed to hold on to the nipple; thus, suggesting that CB1 binding activity influenced the oral-musculature needed to latch on.

Guatemala is located in Central America and it is categorized by the World Bank as a lower middle income country with 59.3% of its population living in poverty.<sup>21</sup> Prevalence of underweight in children less than five years of age is 12.6%,<sup>21</sup> and based on the Ministry of Public Health and Social Assistance of Guatemala (2017),<sup>22</sup> 46.5% of these children are stunted (low height for age), making it the seventh highest rate of stunting in the world.<sup>23</sup> Guatemala's indigenous population, comprising 41% of its total population,<sup>24</sup> suffer higher disparities in nutrition compared to mestizos/ladinos, with its children and women especially affected.<sup>25</sup> For



these children, breastfeeding represents the most effective deterrent for disease prevention and nutrient insufficiency. For the entire Guatemalan population, the exclusive breastfeeding rate for infants under six months of age is 53% and 57% of two year old children continue breastfeeding in addition to consuming foods.<sup>23</sup> It is well established that undernourished children may be at higher risk of growth retardation, health issues, and impaired cognitive development.<sup>26</sup> These data underscore the importance of breast milk for meeting the nutritional needs of Guatemala's children and especially the children of the indigenous population.

Research by our group has recently identified the ECM in human breast milk from a population within the United States.<sup>27-29</sup> The present study aimed to characterize and quantify the ECM in human milk samples from an underserved population in Guatemala.

## **3.2 Materials and Methods**

### **Study Design**

This was an exploratory-longitudinal study with breast milk samples collected between 16 to 24 weeks (4 to 6 months) postpartum from lactating women living in the western highlands of Quetzaltenango, Guatemala. This was an ancillary study from the 'Short term response of breast milk micronutrient concentrations to a lipid based nutrient supplement in Guatemalan women' parent study which was approved by the Institutional Review Boards at the University of California, Davis and the Center for the Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) in Guatemala. For the purpose of this study, only the details pertaining to the breast 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 geographical area of Quetzaltenango, Guatemala. Quetzaltenango is the

second largest city in the country and is 125.5 miles 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.<sup>30</sup>

Lactating women between 16 to 24 weeks postpartum were invited to participate by asking them in person while potential participants were in the waiting room in the community health center or by distributing a flier with information about the study. Women who demonstrated interest in participation in the study were screened for inclusion based on the following criteria:

- Mother 18-40 years of age
- Apparently healthy (determined by anthropometrics), with no acute illness (for example flu, diarrhea or mastitis)
- Breastfeeding at 16-24 weeks (4-6 months)
- Willing to stay at the clinic for breast milk sampling
- 4-6 months of lactation and  $\geq 8$  breast feeding episodes per day (usual frequency  $>20$  times/day)
- Last birth was a singleton birth
- Only breastfeeding one child
- Child was 4-6 months of age

Women were contacted to schedule (i) the consent process (for thorough explanation of the study and for signature of the consent form) and (ii) study visits to the clinic.

### **Sample collections**

Participants were instructed to go to the clinic on four days approximately one week apart, each day representing a study visit. During the first visit, participants provided

demographic data and anthropometric measures were taken. At 16-24 weeks postpartum (visits two, three, and four), fasting mothers were scheduled for breast milk collections between 6 and 7 am. Women were encouraged to feed the infant from one breast while the researcher collected a sample from the opposite breast. Milk collections were made with a manual breast pump (Medela, McHenry, IL) by using the mid-feed sampling method in which the mother was instructed to feed her infant for one minute before the milk sample was collected. Following a one-minute collection, the mother continued feeding the infant.<sup>31</sup> Samples were immediately aliquoted in 2 mL tubes and stored in a cooling container with ice packs, transferred to a -20 °C freezer within 2 hours, then transferred to a -80 °C freezer within 2 weeks. 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**

Breast milk samples were analyzed by liquid chromatography-mass spectrometry (LC-MS) with a state of the art methodology established at the Center for Drug Discovery at Northeastern University, Boston, MA. Milk samples were thawed in a 37 °C water bath and vortexed at medium speed for 10 s at room temperature. Protein precipitation was carried out with chilled acetonitrile and PBS (pH 7.4) and the addition of an internal standard mixture, followed by centrifugation (14,000 × g, 5 min, 4°C). The resulting supernatant was diluted with four volumes of 5% phosphoric acid followed by solid phase extraction using OASIS HLB reverse-phase chromatography cartridges (Waters Corp., Mildford, MA) which were previously rinsed with methanol and water prior to loading the diluted samples. Loaded cartridges were washed with 40% aqueous methanol prior to eluting 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, San Jose, CA) with an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington, DE) at the front end was used for identification and quantification. Separation of analytes was carried out using an Agilent 2.1x50 mm, 5 µm Zorbax SB-CN column<sup>16,32</sup> with gradient elution using 10 mM ammonium acetate (pH 7.3) and methanol (flow rate, 0.5 ml/min). 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 breast milk were calculated. Each sample was analyzed in triplicate and concentrations were averaged.

### **Statistical analyses**

Statistical analyses were performed using SAS by SAS Institute, Inc., version 9.4 (Cary, NC). The level of significance was set at  $\leq 0.05$ . Descriptive statistics (mean, standard deviation, and range) were used for numeric variables. Repeated measures analysis of variance using 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.

### **3.3 Results**

Twenty-six maternal-infant dyads were recruited to participate in this study. Table 3.1 shows participant characteristics. On average, mothers had a body mass index of  $26.3 \pm 4.2$

kg/m<sup>2</sup> and a mid-upper-arm circumference of 28.0 ± 3.1 cm. Infants were 4.5 ± 0.5 months old, weighed 7.0 ± 1.1 lbs., and the majority (65%, n = 17) had a normal height to length ratio.

Table 3.1. Maternal-Infant Characteristics (n = 26)

Characteristics	Mean or % (frequency)	SD
Maternal BMI (kg/m <sup>2</sup> )	26.3	4.2
Maternal MUAC (cm)	28.0	3.1
Maternal literacy (literate)	88.5 (23)	
Marital status (married)	100 (26)	
Infant age at start (months)	4.5	0.5
Infant weight (lbs)	7.0	1.1
Infant gender		
Girls	50 (13)	
Boys	50 (13)	
Infant HAZ	-1.33	1.32
Normal height/length	65.4 (17)	
Moderately stunted	19.2 (5)	
Severely stunted	15.4 (4)	

BMI, body mass index; MUAC, mid-upper-arm circumference; HAZ, length-for-age Z-score

Table 3.2 shows the average concentration of the members in the ECM. Standard curves for each member were linear and had regression values  $\geq 0.99$ . Overall, ECM concentrations in the glycerols were between 2,000 to 4,850 times higher than the concentrations for the ethanolamides. For some ECM members, the range was highly variable. Arachidonic acid was the principal LCPUFA with 70.8%, followed by DHA and EPA with 21.8% and 7.4%, respectively. Palmitoyl ethanolamide 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%. The samples did not contain the full aliquot needed to detect ethanolamides and EPG with confidence. EPEA was not detected in most of 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 than as a reference in comparisons to other data/results.

There was a time effect across the three collection days of sampling for the concentrations of PEA and the LCPUFAs ARA, DHA, and EPA ( $p \leq 0.05$ ) (Table 3.2). 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 3.1). Arachidonic acid and DHA concentrations did not correlate with AEA and DHEA, respectively. A small correlation 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.

Table 3.2. Endocannabinoid Metabolome of Breast Milk

Metabolite	Mean $\pm$ SD ng/mL	Range	$p$ value <sup>1</sup>
<b>Fatty Acids</b>			
ARA	1672.87 $\pm$ 1119.42	176.69 – 6187.31	<b>0.0034</b>
DHA	514.13 $\pm$ 404.02	41.38 – 2549.15	<b>0.0052</b>
EPA	173.50 $\pm$ 216.19	14.18 – 1625.74	<b>0.0234</b>
<b>Ethanolamides</b>			
AEA <sup>2</sup>	0.05 $\pm$ 0.03	0.01 – 0.14	0.7997
PEA <sup>3</sup>	12.39 $\pm$ 26.15	0.27 – 143.43	<b>&lt;.0001</b>
OEA	1.06 $\pm$ 0.81	0.14 – 4.80	0.3441
DHEA <sup>2</sup>	0.07 $\pm$ 0.07	0.03 – 0.48	0.5072
EPEA <sup>2</sup>	0.08 $\pm$ 0.16	-0.03 – 0.56	NA
EEA <sup>2</sup>	0.02 $\pm$ 0.01	0.00 – 0.06	0.7177
<b>Glycerol esters</b>			
AG	135.00 $\pm$ 95.51	6.45 – 472.71	0.5457
PG <sup>3</sup>	26730.13 $\pm$ 18583.17	2189.00 – 95047.28	0.1132
OG	4395.75 $\pm$ 3088.07	418.69 – 14720.33	0.9231
DHG	259.05 $\pm$ 221.31	17.56 – 1350.77	0.0761
EPG	14.02 $\pm$ 13.14	0.68 – 74.38	0.1268
EG	97.00 $\pm$ 68.35	10.25 – 351.94	0.6450

<sup>1</sup> $p$  value represents the effect of time across the three time points. Significant difference marked in bold. <sup>2</sup>Some values were below the standard curve. <sup>3</sup>Some values were above the standard curve. NA, not analyzed (not enough values to calculate). ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AEA, Anandamide; PEA, palmitoyl ethanolamide; OEA, oleoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide; EPEA, eicosapentaenoyl ethanolamide; EEA, eicosenoyl ethanolamide; AG, arachidonoyl glycerol; PG, palmitoyl glycerol; OG, oleoyl glycerol; DHG, docosahexaenoyl glycerol; EPG, eicosapenaenoyl glycerol; EG, eicosenoyl glycerol.

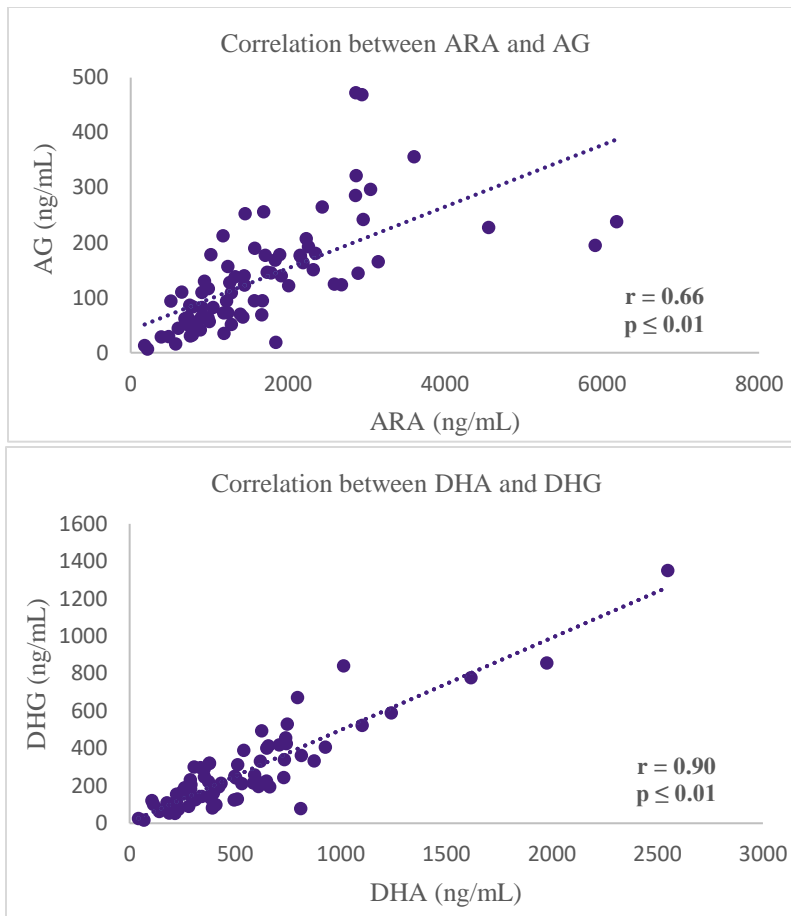


Figure 3.1. Correlations between the precursor fatty acid and its derived endocannabinoid. Figure at the top shows the correlation between arachidonic acid (ARA) and anandamide (AEA). Figure at the bottom shows the correlation between docosahexaenoic acid (DHA) and docosahexaenoyl glycerol (DHG).

### 3.4 Discussion

Our study provides the first report for the ECM in human breast milk from a developing country. Other studies have reported on members of the ECM, mainly AEA and other ethanolamides, in human breast milk but these studies have focused on populations from the United States,<sup>27,29,33</sup> Sweden,<sup>34</sup> Israel,<sup>18</sup> or the United Kingdom.<sup>35</sup> As Guatemala has the seventh highest rate of stunting in the world,<sup>25</sup> breast milk, the ‘liquid gold’, becomes a very important source of nutrients for infants and children and provides a means for addressing infant malnutrition. A study by Beusekom et al., (2013)<sup>36</sup> with a similar population in Quetzaltenango,

determined that 58% of women were either exclusively or predominantly breastfeeding their infants at six months of life.

In the current study, we evaluated the presence of ECM members in mature (16-24 weeks) milk to characterize the metabolites present in breast milk and to quantify them. Our results for AEA, EEA, and PG are very similar to those presented by Lammi-Keefe et al. (2017)<sup>27</sup> for breast milk at 10 weeks postpartum from a cohort in Baton Rouge, Louisiana; however they differ for the rest of the ECM members. For both populations, Lammi-Keefe et al. and the present study, PEA, PG, and ARA were the main metabolites in the ethanolamide, glycerol, and fatty acid groups, respectively. In the current study, AG was present in higher concentrations in breast milk than that for AEA (2,700 times higher), which is similar to the finding of Fride et al. (2001)<sup>18</sup> who reported that AG was 5,800 times higher than AEA in mature milk. This finding may support the importance of AG for establishing the suckling response in the newborn<sup>18,20,37,38</sup> as breast milk will be a continuous exogenous source of AG for the infant's brain. In the current study, glycerol metabolites tended to be present in higher concentrations than ethanolamides as previously reported by Fride et al. (2001)<sup>18</sup> and Lammi-Keefe et al. (2017)<sup>27</sup> for breast milk. 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 by the action of enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase.<sup>39</sup> Affinities of anabolic and catabolic enzymes for the EC likely differ for each metabolite. For example, FAAH is the principal enzyme for metabolizing AEA although it can also act on AG albeit at a different rate.<sup>40</sup> In addition, the presence of *entourage metabolites*<sup>41</sup> (e.g. PG, OG, OEA, DHG, EPEA, etc.) can interfere with enzyme activity as 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.<sup>16</sup> Any of these factors would presumably impact the activity of one over another. Entourage metabolites<sup>41</sup> (PEA, OEA, DHEA, EPEA, EEA, PG, OG, DHG, EPG, and EG) in breast milk of our population could support AG's function. At this time there is insufficient evidence to speculate about the synthesis and degradation pathways for each group of metabolites, ethanolamides versus glycerols. A limited number of studies have reported EC concentrations in human breast milk, all with different study limitations including a limited number of study subjects to variation in lactation stage when samples were collected. Nonetheless, all of these studies<sup>18,27,29,33-35</sup> support the presence of different EC and EC-like components in breast milk.

In a global survey of the n-3 fatty acids EPA and DHA conducted by Stark et al. (2016),<sup>42</sup> it was determined that Guatemala had a relative low n-3 status (< 4% DHA+EPA of total fatty acids in erythrocytes) compared to other countries worldwide. In a study conducted by Solomons et al. (2015)<sup>43</sup> in the Pacific Coast area of Guatemala, it was determined that women and children had a DHA status of 3.09% and 3.49%, respectively. Additionally, in a study in the geographical area of Quetzaltenango, Bermudez et al. (2010)<sup>44</sup> reported that children were consuming limited amounts of DHA (32 mg/day) and food sources of DHA included eggs, chicken, fish, and shrimp. These data provide an overview of the diet in the population included in this study. Considering the important role of DHA for pregnancy and development outcomes,<sup>45</sup> including the roles as a precursor for EC metabolites, there is a need to assess dietary intake of fatty acids. In the current study, LCPUFAs ARA, DHA, and EPA were present in breast milk. For a similar population in Quetzaltenango, N. Solomons, MD (email communication, April 2018) has reported the amounts of DHA+EPA in breast milk to be 0.23% of the total fatty acids, an amount lower than the average worldwide of 0.32% for DHA.<sup>46</sup> Although dietary data were not available

for the current study, it might be speculated that dietary sources contributed to the high range of concentrations found for most of the ECM members. As maternal diet is a source for EC precursors, i.e. fatty acids, it is of great importance that these women consume enough fatty acids, especially the LCPUFAs, to ensure adequate quantities in breast milk to meet infant demands. There was a time effect for the three samples collected in the current study for ARA, DHA, EPA, and PEA. Breast milk fatty acids are directly affected by diet,<sup>47,48</sup> which suggest a direct effect of diet on the LCPUFAs and PEA concentrations between collection dates.

Differences in time for the LCPUFAs but not for most of the EC and EC-like metabolites may be explained, at least in part, by binding affinity, degradation, and synthesis rates and how these are utilized by the infant.

There was a positive correlation between ARA and AG and also DHA and DHG in the breast milk for this 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 breast milk at higher concentrations than ethanolamides as shown in our results and other reports.<sup>18,27,34</sup> In addition to its role in suckling behavior, AG is also involved in stimulating food intake. A study by Kirkham et al. (2002)<sup>49</sup> shown 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 infant's cognitive development.

Long-chain PUFAs play a critical role in infant development and AG has a documented role in infant feeding behavior,<sup>37</sup> underscoring the interest and importance of understanding this biological system, the ECM in human milk. Breast milk can be influenced by maternal nutrition status, thus modulating infant health. For populations with health disparities and economic

disadvantages, understanding breast milk and its biological components can provide insight to 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 ECM relevance in health and as a possible intervention for infants with health disparities.

### **3.5 Limitations**

The current study had limitations. The study sample was not necessarily representative of the Guatemalan population. Participants were recruited from an underserved population in the highlands of Quetzaltenango that is documented to have limited access to a diet that meets nutrient requirements. Some breast milk samples were collected in the field where a freezer was not available. Therefore, these samples had to be stored in a cooling bag with ice packs until taken to the clinic to be stored at -20 °C until transferred to a -80 °C freezer for long term storage. 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 breast milk.

### **3.6 Conclusion**

Our study identified members of the ECM in mature breast milk and provided, to the best of our knowledge, the first report for a population with health disparities within a developing country. These data indicate that EC and EC-like components may play a role in infants' feeding behaviors which, in turn, influence infant growth and development. Our finding of differences of some metabolites over time warrants further investigation with regards to maternal diet. The

role(s) of each member in the ECM in infant development have not yet been established and this also warrants further investigation.

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## CHAPTER 4

# ENDOCANNABINOID METABOLOME OF BREAST MILK: A COHORT FROM BATON ROUGE, LOUISIANA

### 4.1 Introduction

According to the Center for Disease Control and Prevention (2016),<sup>1</sup> almost 61% of infants in Louisiana are ever breastfeed, with 31.2% breastfeeding at six months, 13.3% breastfeeding at 12 months, and only 11.8% meeting the global recommendation to breastfeed exclusively for six months.<sup>2,3</sup> The recommendation for exclusive breastfeeding during the first months following delivery is based in part on the knowledge that breast milk provides the infant with nutrients that meet his requirements during development. These beneficial nutrients include the long-chain polyunsaturated fatty acids (LCPUFAs), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (ARA, 20:4n6), that play a pivotal role in cognitive and retinal development and growth of the infant.<sup>4</sup> These nutrients are transferred to the infant across the placenta during pregnancy and through breast milk after birth.

It has been shown that LCPUFAs are precursors to endocannabinoids (EC) which are endogenous lipid mediators that bind to the same receptors as *Cannabis sativa* (marijuana).<sup>5</sup> Endocannabinoids have been shown to play a pivotal role in appetite and food intake<sup>6</sup> by activating cannabinoid receptor 1 (CB1) which is predominant in the central nervous system.<sup>7</sup> Cannabinoid receptor 1 is activated by two different EC, arachidonylethanolamide (anandamide, AEA) and arachidonoyl glycerol (2-AG or AG), both derived from n-6 ARA. In particular, for infant feeding behavior, 2-AG has been demonstrated to play a role in establishing the suckling response of the neonate when nursing.<sup>8</sup> Evidence in mouse pups suggest that CB1 activation by 2-AG is needed to establish the suckling response by activating the oral-motor musculature behavior needed for milk suckling.<sup>8-10</sup> Establishment of this role for 2-AG was



demonstrated after administration of a CB1 antagonist (SR141716A) to mouse pups which resulted in growth inhibition and even death by day eight after birth.<sup>8</sup>

Recent work has indicated that EC and EC-like compounds (collectively referred to as the endocannabinoid metabolome, ECM) are present in human breast milk.<sup>11,12</sup> Endocannabinoid-like compounds, referred to as entourage metabolites,<sup>13</sup> may support the activity and physiologic responses of the EC system by interacting with AEA and AG, their enzymes, or their receptors. These entourage metabolites exert cannabimimetic effects (similar pharmacological effects to those of cannabis).<sup>14</sup> The ECM encompasses 15 metabolites identified to date: i) ethanolamide derivatives: AEA, palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA), and eicosenoyl ethanolamide (EEA); ii) glycerol derivatives: 2-AG, palmitoyl glycerol (PG), oleoyl glycerol (OG), docosahexaenoyl glycerol (DHG), eicosapentaenoyl glycerol (EPG), eicosenoyl glycerol (EG); and iii) precursor LCPUFAs: ARA, DHA, and eicosapentaenoic acid (EPA, 20:5n3). There is limited information regarding the ECM of human breast milk and its role in infant development. Thus, in the present study, we characterized and quantified the ECM in human breast milk in transitional and mature milk and evaluated if the concentrations of these metabolites changed over time.

## **4.2 Materials and Methods**

### **Study Design**

This research project was an exploratory-longitudinal study to evaluate if there was a difference in the ECM of transitional milk (two weeks postpartum) and the ECM of mature milk (four weeks postpartum).

## **Subject Recruitment**

Pregnant women from the greater Baton Rouge, Louisiana area who were planning to breastfeed for a minimum of four weeks were invited to participate in this study. Recruitment was based on intent to breastfeed. Subjects were invited to participate before delivery through private physicians' offices and hospital prenatal clinics or by posting flyers describing the study around the community. Women who demonstrated interest in participation in the study were contacted to explain the study and for pre-screening based on the inclusion criteria:

- Maternal age of 18-40 years at the time of delivery
- Full term delivery ( $\geq 37$  gestational weeks)
- Singleton birth
- Plan to breastfeed for at least 4 weeks
- Willing to provide a breast milk sample (complete breast emptying from one breast) during the morning (6-10 am)
- Have not been breastfeeding or pregnant in the previous year

Before delivery, women were contacted again to schedule the consent process (thorough explanation of the study and for signature of the consent form). The exclusion criteria were discussed at the time of consent:

- Any tobacco use during lactation
- Alcohol consumption ( $>1$  drink per week)
- Presumed or confirmed congenital birth defects

Materials provided to the subjects for the study included two breast milk storage bags, instruction on how to collect the breast milk sample, and a schedule card for visits. These were

provided the same day that the consent was obtained. The Louisiana State University Agricultural Center Institutional Review Board approved the study.

### **Sample collection**

Participants provided written consent and filled out a health history questionnaire that included questions about previous and current pregnancies, pregravid body mass index (BMI), and prior lactation experience. Details regarding infant birth weight and length were completed following the infant's birth. In addition, participants provided information about education and socioeconomic status. This information was confirmed by their health care providers.

Breast milk samples:

Samples were collected at two and four weeks postpartum at the participants' homes. Participants were asked to provide a breast milk expression from a single breast (emptying a full mammary gland by collecting all the milk from that breast)<sup>15</sup> by using an electric breast pump. In preparation for milk collection, participants fasted for at least two hours and collections were made between 6 and 10 am. The sample was stored under refrigeration at the participant's house (for a maximum of 24 hours) in the breast milk storage bag provided by the researcher. Samples were transported on ice to the laboratory where the milk was warmed in a 37 °C water bath, manually gently swirled to mix, and ~15 mL aliquots were made in small glass vials with Teflon-lined caps and stored at -80 °C until analyses. Information including the breast pump brand used, exclusive breastfeeding, and use of formula for supplementation were also recorded. Samples were shipped overnight on dry ice to the Center for Drug Discovery, Northeastern University, Boston, MA and kept at -80 °C until analysis.

## Sample analysis

The breast milk samples were analyzed by liquid chromatography-mass spectrometry (LC-MS) with a state-of-the-art methodology established at the Center for Drug Discovery at Northeastern University, Boston, MA. Milk samples were thawed in a 37 °C water bath and vortexed at medium speed for 10 s at room temperature. Protein precipitation was carried out with chilled acetonitrile and PBS (pH 7.4) and the addition of an internal standard mixture, followed by centrifugation (14,000 × g, 5 min, 4°C). The resulting supernatant was diluted with four volumes of 5% phosphoric acid followed by solid phase extraction using OASIS HLB reverse-phase chromatography cartridges (Waters Corp., Milford, MA) which were previously rinsed with methanol and water prior to loading the diluted samples. Loaded cartridges were washed with 40% aqueous methanol prior to eluting 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, San Jose, CA) with an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington, DE) at the front end was used for identification and quantification. Separation of analytes was carried out using an Agilent 2.1x50 mm, 5 µm Zorbax SB-CN column<sup>16,17</sup> with gradient elution using 10 mM ammonium acetate (pH 7.3) and methanol (flow rate, 0.5 ml/min). 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 ( $\mu\text{g/mL}$ ) of breast milk were calculated. Each sample was analyzed in triplicate and concentrations were averaged.

### **Statistical analyses**

Statistical analyses were performed using SAS by SAS Institute, Inc., version 9.4 (Cary, NC). The level of significance was set at  $\leq 0.05$ . Descriptive statistics (mean, standard deviation, and range) were used for numeric variables. Repeated measures analysis of variance using proc mixed was used to assess the effect of time across the two different time points on the concentrations of members of the ECM.

### **4.3 Results**

One hundred thirty-one potential participants were invited to participate in the study from which 31 consented to participate. Seven women dropped out during the study; thus, data from 24 participants was included in the study. Table 4.1 provides the participants' characteristics. Lactating women in the study were between 18 and 39 years old. Only seven participants (29%) indicated having a previous breastfeeding experience. Rates for infants in this study being exclusively breastfed were 83% ( $n = 20$ ) and 67% ( $n = 16$ ) at two and four weeks, respectively.

Table 4.2 shows the constituents of the ECM at two weeks (transitional milk) and four weeks (mature milk) postpartum. Standard curves for each metabolite were linear and had regression values  $\geq 0.99$ , except for PG which was 0.98. Extraction efficiencies were greater than 80%, except for OG which was greater than 78%. The main metabolite present in the fatty acids group was ARA accounting for more than 60% of that fraction. In the ethanolamide group, OEA accounted for more than 50% of that portion, and PG in the glycerol group accounted for more than 90%. Eicosenoyl ethanolamide and EPG were present in the lowest concentrations in the ethanolamide and glycerol groups, respectively.

Table 4.1. Maternal-Infant Characteristics (n = 24)

Characteristic	Mean $\pm$ SD or % (frequency)	Range
<b>Maternal Characteristics</b>		
Age (y)	30.5 $\pm$ 5.0	18.0 – 39.0
Pre-pregnancy BMI (kg/m <sup>2</sup> )	28.0 $\pm$ 5.8	19.6 – 41.6
Race		
White	71 (17)	
Black	17 (4)	
Hispanic	8 (2)	
Asian	4 (1)	
Gestational age at delivery (weeks)	39.2 $\pm$ 1.3	37.0 – 41.0
Previous breastfeeding experience		
Yes	29 (7)	
No	71 (17)	
Education		
Some high school	4 (1)	
High school	4 (1)	
Some college	21 (5)	
4-year post-high school	25 (6)	
Post-graduate	46 (11)	
Marital Status		
Single	21 (5)	
Married	79 (19)	
WIC participation		
Yes	13 (3)	
No	88 (21)	
<b>Infant characteristics</b>		
Sex		
Girls	33 (8)	
Boys	67 (16)	
Mode of delivery		
Vaginal	75 (18)	
Cesarean	25 (6)	
Birth weight (lbs)	7.4 $\pm$ 0.8	5.5 – 9.2
Feeding type		
Exclusively breastfed	83 (20)	67 (16)
Supplemented with formula	17 (4)	33 (8)
BMI, body mass index; WIC, Woman, Infant, and Children Special Supplemental Nutrition Program		

For some samples, EPEA and EEA were below the standard curve and PG was above although values were close to the curve for the later. Therefore, those results should be interpreted with caution. Only DHEA demonstrated a time effect ( $p \leq 0.05$ ) across the two

different time points postpartum [transitional (two weeks) versus mature (four weeks) milk] with higher concentrations in transitional milk. Overall, breast milk glycerol group concentrations were higher than those of the ethanolamides. Glycerol concentrations were 15,564 and 56,052 times higher in transitional and mature milk, respectively than the ethanolamide concentrations.

Table 4.2. Endocannabinoid Metabolome of Human Breast Milk

Metabolite	Transitional milk <sup>1</sup>	Mature milk <sup>1</sup>	<i>p</i> value <sup>2</sup>
<b>Fatty Acids</b>			
ARA	2818.96 ± 2785.29	7030.33 ± 16674.49	0.2451
DHA	2031.17 ± 2332.63	2384.71 ± 5224.73	0.7569
EPA	381.49 ± 646.23	1362.93 ± 4475.69	0.2979
<b>Ethanolamides</b>			
AEA	0.15 ± 0.23	0.08 ± 0.04	0.1772
PEA	0.90 ± 0.48	0.74 ± 0.40	0.1095
OEA	1.48 ± 1.18	1.12 ± 0.47	0.0841
DHEA	0.11 ± 0.07	0.07 ± 0.03	<b>0.0022</b>
EPEA <sup>3</sup>	0.07 ± 0.14	0.11 ± 0.20	0.5184
EEA <sup>3</sup>	0.03 ± 0.03	0.03 ± 0.01	0.2382
<b>Glycerol esters</b>			
AG	166.85 ± 177.85	312.11 ± 575.36	0.2550
PG <sup>4</sup>	37477.67 ± 35745.95	110091.70 ± 261103.75	0.1905
OG	4059.33 ± 3511.83	7719.96 ± 10885.00	0.1225
DHG	673.50 ± 970.05	866.30 ± 1839.67	0.6352
EPG	24.70 ± 37.74	61.99 ± 138.34	0.2161
EG	242.48 ± 327.00	899.37 ± 2445.21	0.2078

All data are presented in ng/ml and are mean ± SD. <sup>1</sup>Two and four weeks postpartum.

<sup>2</sup>*p* value represents the effect of time across the two time points. Significant difference marked in bold. <sup>3</sup>Some values were below the standard curve. <sup>4</sup>Some values were above the standard curve.

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AEA, Anandamide; PEA, palmitoyl ethanolamide; OEA, oleoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide; EPEA, eicosapentaenoyl ethanolamide; EEA, eicosenoyl ethanolamide; AG, arachidonoyl glycerol; PG, palmitoyl glycerol; OG, oleoyl glycerol; DHG, docosahexaenoyl glycerol; EPG, eicosapenaenoyl glycerol; EG, eicosenoyl glycerol.

Combining the two time points together to evaluate relationships, it was observed that there were significant correlations between the precursor LCPUFA and its derived EC for ARA and AG ( $r = 0.88$ ,  $p \leq 0.01$ ), DHA and DHEA ( $r = 0.69$ ,  $p \leq 0.01$ ), DHA and DHG ( $r = 0.95$ ,  $p \leq 0.01$ ), EPA and EPEA ( $r = 0.80$ ,  $p \leq 0.01$ ), and EPA and EPG ( $r = 0.91$ ,  $p \leq 0.01$ ). No

correlations were observed between ARA and AEA. Docosahexaenoyl ethanolamide correlated with DHG ( $r = 0.61$ ,  $p \leq 0.01$ ) and EPEA with EPG ( $r = 0.84$ ,  $p \leq 0.01$ ).

#### **4.4 Discussion**

To the best of our knowledge, this study is the first to characterize the ECM in transitional and mature human milk and to explore differences in the ECM concentrations at these two stages of breast milk production. The mechanisms of action and the roles of the ECM in both breast milk and for infant development are not fully described/understood. Understanding breast milk and its bioactive components (i.e. ECM) provides an opportunity to underline the importance of breast milk for infant nourishment and development.

In this exploratory study, we have characterized the ECM of transitional and mature milk. Our results showed that only DHEA, a derivative of n-3 DHA conjugated with ethanolamine, was different across the two different time points ( $p \leq 0.05$ ) with higher concentrations in transitional milk. Research evaluating the role of the endocannabinoid system (ECS) in infant feeding behavior has been focused on the activation of CB1 when binding to AG, which in turn activates the oral-motor musculature needed for milk suckling.<sup>8</sup> However, DHEA has also been shown to be an agonist to CB1.<sup>18</sup> Although the role of DHEA in food intake has not been studied, it may be hypothesized that by binding to the same receptor as AG, DHEA exerts some of the same activities. In addition, as DHA plays a key role in infant cognitive development<sup>19,20</sup> and is a precursor to DHEA, it is plausible that DHEA also supports brain development. Moreover, the development of the hippocampus, a brain area related to learning and memory, has been shown to be supported by DHEA.<sup>21</sup> Although the fatty acids and glycerol-derivatives were higher and the ethanolamide-derivatives were lower in mature milk, there were no significant differences for the other members of the ECM across transitional and mature milk.



Perhaps the analysis of colostrum may indicate different results. Colostrum is present for the first three to five days after birth<sup>22</sup> and its main function is to provide immunological protection to the newborn.<sup>23</sup> We hypothesize that concentrations of the ECM would be higher in colostrum than in mature milk attributable to a higher demand when a newborn is adapting to feed at the breast. As a result, ECM concentrations would be higher at the beginning of postnatal life and would decrease over time when the infant has established the feeding behavior needed for milk suckling.

Scarce data are available for a comparison with our current results. However, the earliest study by Fride et al. (2001)<sup>8</sup> that established a role for the ECS in mouse pup suckling and growth, also analyzed milk from various sources including human milk. Even though the study by Fride et al. (2001) did not specify the number of milk samples analyzed, our results follow the same pattern in demonstrating that PG is present in human breast milk in higher concentrations than AG. Furthermore, a study by Di Marzo et al. (1998)<sup>24</sup> reported 330 ng/ml of AG in mature human breast milk, a concentration very similar to our result of 312.11 ng/ml, and indicated that AG is found in human breast milk in higher concentrations than AEA which is also demonstrated in our present results. Similarly, a study by Schuel et al. (2002)<sup>25</sup> in which ethanolamides were analyzed in human fluids, including mature milk, demonstrated that OEA was present in higher concentrations than PEA, and PEA in higher concentrations than AEA, as also shown in our results. In addition, our results are in line with those presented by Lammi-Keefe et al. (2017)<sup>11</sup> who studied a population similar to the current population and analyzed the same members of the ECM that were investigated in the current study. Our results follow the same pattern as Lammi-Keefe et al. (2017) in terms of the proportion of each member within each group: fatty acids, ethanolamides, and glycerols. In summary, to the best of our knowledge, there are only a few

studies available for a comparison to the findings of our current study that support the presence of EC and EC-like metabolites in human breast milk.

In the current study, we have taken a step to evaluate correlations between the precursor LCPUFAs and their ethanolamide- and glycerol-derivatives. There were significant correlations ( $p \leq 0.01$ ) for DHA-DHEA-DHG, EPA-EPEA-EPG, and ARA-AG but not AEA. The most robust correlations were for the precursor LCPUFA and its derived glycerol metabolites: ARA-AG ( $r = 0.88$ ), DHA-DHG ( $r = 0.95$ ), and EPA-EPG ( $r = 0.91$ ). The strong correlations between the precursor LCPUFA with its glycerol- but not its ethanolamide-derivatives, may support a more important role for the glycerols (AG, PG, OG, DHG, EPG, and EG) in establishing the suckling response of the newborn by modulating motor development and behavior. In addition, the presence of entourage metabolites (PEA, OEA, DHEA, EPEA, EEA, PG, OG, DHG, EPG, and EG), which exhibit cannabimimetic responses,<sup>13</sup> may enhance the activity of the two most thoroughly studied EC, AG and AEA. For example, PG has been shown to increase AG affinity to CB2 by acting as a lipid signaling mediator<sup>17</sup>; and PEA and OEA reduce enzymatic breakdown, cellular uptake, and degradation of AEA. These entourage metabolites may interfere with enzymatic activity as they can also act as substrates for catabolic and anabolic enzymes. In addition, the presence of these lipid mediators may prevent EC activation or deactivation. All of these interactions may also explain our finding that n-3 LCPUFA derivatives, both ethanolamides and glycerols, correlated with each other [DHEA-DHG ( $r = 0.61$ ,  $p \leq 0.01$ ) and EPEA-EPG ( $r = 0.84$ ,  $p \leq 0.01$ )]. The associations among the n-3 LCPUFA derivatives, but not for the n-6 derivatives (AEA and AG), leads to the speculation that they support the role of DHA in infant cognitive development, although their roles have yet been fully elucidated.

To date the mechanisms of action regarding how the ECM as a whole interacts with the ECS and its role in infant feeding behavior, and therefore infant development and growth, are still poorly understood. Our results provide evidence that there are metabolites similar to the previously described EC,<sup>5</sup> i.e. AEA and AG, present in human breast milk. With an understanding of the role of the ECM and its interactions with the ECS in human breast milk and the infants' brain, potential interventions could be developed for infants with difficulties latching on and for preterm infants who could be aided by the countless benefits of breast milk to ensure continued development outside the womb. While this is out of the scope of this study, it merits further exploration.

#### **4.5 Limitations**

This study was limited by its small sample size (n = 24). Having a relatively small group of participants did not allow for further explorations between the concentrations for some of the ECM members and demographic data such as BMI and race, for example. However, this study provides an opportunity to develop hypotheses for future studies to evaluate how the ECM of breast milk may be modulated on the basis of maternal and/or infant factors.

#### **4.6 Conclusion**

Our study provides evidence that EC and EC-like metabolites are present in human milk. The findings in this study not only support the role of AG in establishing the suckling response of the newborn by activating oral-motor musculature needed for milk suckling, but also suggest that other bioactive constituents in breast milk may also play a role in infant health and development. In addition, knowing that EC-like metabolites are present in breast milk, future studies can be developed to elucidate specific roles for each member of the ECM.

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## **CHAPTER 5**

# **MATERNAL DIETARY FATTY ACIDS AND THEIR RELATIONSHIP TO DERIVED ENDOCANNABINOIDS IN BREAST MILK**

### **5.1 Introduction**

Breast milk provides the infant with nutrients needed for development, beginning immediately after birth and continuing into infancy. With respect to these nutrients, of high importance are the long-chain polyunsaturated fatty acids (LCPUFAs), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (ARA, 20:4n6), which play a pivotal role in infants' cognitive and retinal development and growth.<sup>1,2</sup> It is established that maternal diet influences breast milk composition, especially for the LCPUFA concentrations.<sup>3,4</sup> Furthermore, the lipid fraction is the most variable nutrient during lactation.<sup>5</sup> Within a feed, the fat content is higher in the hind milk as compared to the fore milk.<sup>6</sup> During the stages of lactation, the fat content varies from 2% in colostrum, 2.9% in transitional milk, and 3.6% in mature milk.<sup>7</sup> Worldwide, the DHA content of breast milk is reported to be 0.32% and 0.47% for ARA of the total fatty acids.<sup>8</sup>

During the first six months of lactation, it is recommended that women increase their energy intake by 500 kcal/day and by 400 kcal/day from months six through 12.<sup>9</sup> Further, lactating women are advised more recently to consume at least 200 mg/day of DHA.<sup>10</sup> In addition, to help meet the requirement for DHA, the Dietary Guidelines for Americans suggest that lactating women consume at least 8 and up to 12 ounces of a variety of seafood low in methyl mercury per week which provide an average of 250 mg/day of eicosapentaenoic acid (EPA, 20:5n3) and DHA combined.<sup>11</sup>

Endocannabinoids (EC) are lipid mediators derived from the LCPUFAs.<sup>12</sup> The chemical structure of EC resembles that of the precursor fatty acid conjugated with either ethanolamine or

glycerol.<sup>12</sup> Seminal work by Fride et al. (2001)<sup>13</sup> identified the presence of the main EC, arachidonylethanolamide (AEA) and arachidonoylglycerol (AG) that are both derived from n-6 ARA, in breast milk from different species including human milk. Very recently, in addition to AEA and AG, EC-like metabolites have been identified in human breast milk.<sup>14,15</sup> These EC-like metabolites include docosahexaenoyl ethanolamide (DHEA) and docosahexaenoyl glycerol (DHG) which are derived from n-3 DHA; and eicosapentaenoyl ethanolamide (EPEA) and eicosapenaenoyl glycerol (EPG) that are derived from n-3 EPA.

Appetite and food intake have been shown to be influenced by the EC.<sup>16</sup> In a study using a mouse model, Fride et al. (2001)<sup>13</sup> determined that AG is needed to activate the oral-motor musculature needed for milk suckling. On the basis of evidence in the mouse model and of relevance to infant nutrition, research has shown that EC may influence infant feeding behavior and that breast milk is an exogenous source of EC for the infant.<sup>13,17</sup> There is evidence that circulating and tissue levels of EC metabolites are modulated by dietary intake of precursor fatty acids.<sup>18-20</sup> Nonetheless, the relationship of maternal LCPUFA intake to EC concentrations in breast milk has not been previously explored. Therefore, the aim of the present study was to assess maternal intake of LCPUFAs and their relationship to concentrations of derived EC and EC-like metabolites in breast milk.

## **5.2 Materials and Methods**

### **Study Design**

This research project was an exploratory study to assess the relationship between dietary LCPUFAs and derived EC in breast milk. Dietary intake was collected at two, three, and four weeks postpartum and breast milk samples were collected at two and four weeks postpartum.

## Subject Recruitment

Lactating women from the greater Baton Rouge, Louisiana area who were planning to breastfeed for a minimum of four weeks were invited to participate in this study. Recruitment was based on intent to breastfeed. Subjects were invited to participate before delivery through private physicians' offices and hospital prenatal clinics in the greater Baton Rouge area or by posting flyers describing the study around the community. Women who demonstrated interest in participation in the study were contacted to explain the study and for pre-screening based on the inclusion criteria:

- Maternal age of 18-40 years at the time of delivery
- Full term delivery ( $\geq 37$  gestational weeks)
- Singleton birth
- Plan to breastfeed for at least 4 weeks
- Willing to provide a breast milk sample (complete breast emptying from one breast) during the morning (6-10 am)
- Have not been breastfeeding or pregnant in the previous year

Before delivery, women were contacted again to schedule the consent process (explanation of the study and signature of the consent form). The exclusion criteria were discussed at the time of consent:

- Any tobacco use during lactation
- Alcohol consumption ( $>1$  drink per week)
- Presumed or confirmed congenital birth defects

Materials provided to the subjects for the study included two breast milk storage bags, instructions on how to collect the breast milk samples, a schedule card for study visits, and food



amounts booklet (for calculating portion sizes of foods consumed). These were provided the same day that the informed consent process was conducted. The study was approved by the Louisiana State University Agricultural Center Institutional Review Board.

### **Sample collections**

After providing written consent, participants were asked to fill out a health history questionnaire that included questions about previous/current pregnancy, pregravid body mass index (BMI), prior lactation experience, and infant birth weight and length which were completed following infant birth. In addition, participants provided information about education and socioeconomic status. This information was confirmed by the women's health care providers.

#### **1. Dietary intake**

Twenty-four hour dietary recalls were conducted during weeks two, three, and four weeks postpartum via telephone interview. A multi-pass interview approach was used in a standardized fashion to collect the recalls. The Nutrition Data System for Research (NDSR) software version 2017 (Minneapolis, MN, USA) was used to analyze the nutrient content of the foods/beverages reported. The tool also provided a dietary supplement assessment.

#### **2. Breast milk samples**

Samples were collected at two and four weeks postpartum in the participants' homes. The women were asked to provide a breast milk expression from a single breast (emptying a full mammary gland by collecting all the milk from that breast)<sup>21</sup> using an electric breast pump. Before collecting the milk, participants were asked to fast for at least two hours and to collect the milk between 6 and 10 am. The sample was stored under refrigeration at the participant's house (for a maximum of 24 hours) in the breast milk storage bag provided by the researcher. Samples

were transported on ice to the laboratory where the milk was warmed in a 37 °C water bath, manually gently swirled to mix, and ~15 mL aliquots were made in small glass vials with Teflon-lined caps and stored at -80 °C until analysis. Information including the breast pump brand used, exclusive breastfeeding, and use of formula for supplementation were also recorded. Samples were shipped overnight on dry ice to the Center for Drug Discovery, Northeastern University, Boston, MA and kept at -80°C until analysis.

### **Sample analyses**

#### 1. Dietary intake

To perform a 24-h dietary recall, individuals were asked to recount all food, beverages and nutritional supplements consumed the day before the interview. Individuals were provided a food amounts booklet to assist them in assessing the portion sizes of food and beverages they had consumed. The period being recalled consisted of the 24 hours which went from midnight to midnight from the previous day. Nutrient data for the three dietary recalls performed were analyzed and averaged.

#### 2. Breast milk samples

Breast milk samples were analyzed by liquid chromatography-mass spectrometry (LC-MS) with a state of the art methodology established at the Center for Drug Discovery at Northeastern University, Boston, MA. Milk samples were thawed in a 37 °C water bath and vortexed at medium speed for 10 s at room temperature. Protein precipitation was carried out with chilled acetonitrile and PBS (pH 7.4) and the addition of an internal standard mixture, followed by centrifugation (14,000 × g, 5 min, 4°C). The resulting supernatant was diluted with four volumes of 5% phosphoric acid followed by solid phase extraction using OASIS HLB reverse-phase chromatography cartridges (Waters Corp., Mildford, MA) which were previously

rinsed with methanol and water prior to loading the diluted samples. Loaded cartridges were washed with 40% aqueous methanol prior to eluting 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, San Jose, CA) with an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington, DE) at the front end was used for identification and quantification. Separation of analytes was carried out using an Agilent 2.1x50 mm, 5 µm Zorbax SB-CN column<sup>22,23</sup> with gradient elution using 10 mM ammonium acetate (pH 7.3) and methanol (flow rate, 0.5 ml/min). 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 breast milk were calculated. Each sample was analyzed in triplicate and concentrations were averaged.

### **Statistical analyses**

Statistical analyses were performed using SAS by SAS Institute, Inc., version 9.4 (Cary, NC). The level of significance was set at  $\leq 0.05$ . Descriptive statistics (mean, standard deviation, and range) were used for numeric variables. Pearson correlations were used to evaluate the relationship between the parent fatty acid and its derived EC and EC-like compounds.

### 5.3 Results

One hundred thirty-one potential participants were invited to the study from which 31 consented to participate. Seven women dropped out during the study, so the final number of participants was 24. Table 5.1 provides participants' characteristics. Lactating women in the study were between 18 and 39 years old. Seventy-five percent (n = 18) of infants were delivered vaginally and a third (n = 8) of infants were girls. The rates of infants being exclusively breastfed were 83% (n = 20) at 2 weeks and 67% (n = 16) at 4 weeks postpartum.

Table 5.1. Maternal-Infant Characteristics (n = 24)

Characteristic	Mean $\pm$ SD or % (frequency)	Range
<b>Maternal Characteristics</b>		
Age (y)	30.5 $\pm$ 5.0	18.0 – 39.0
Pre-pregnancy BMI (kg/m <sup>2</sup> )	28.0 $\pm$ 5.8	19.6 – 41.6
Race		
White	71 (17)	
Black	17 (4)	
Hispanic	8 (2)	
Asian	4 (1)	
Gestational age at delivery (weeks)	39.2 $\pm$ 1.3	37.0 – 41.0
Previous breastfeeding experience		
Yes	29 (7)	
No	71 (17)	
Education		
Some high school	4 (1)	
High school	4 (1)	
Some college	21 (5)	
4-year post-high school	25 (6)	
Post-graduate	46 (11)	
Marital Status		
Single	21 (5)	
Married	79 (19)	
WIC participation		
Yes	13 (3)	
No	88 (21)	
Infant characteristics		
Sex		
Girls	33 (8)	
Boys	67 (16)	
(table cont'd.)		

Characteristic	Mean $\pm$ SD or % (frequency)	Range
Mode of delivery		
Vaginal	75 (18)	
Cesarean	25 (6)	
Birth weight (lbs)	7.4 $\pm$ 0.8	5.5 – 9.2
Feeding type	2 weeks	4 weeks
Exclusively breastfed	83 (20)	67 (16)
Supplemented with formula	17 (4)	33 (8)
BMI, body mass index; WIC, Woman, Infant, and Children Special Supplemental Nutrition Program		

Table 5.2 presents the daily dietary intake of participants. Dietary intake of DHA was  $98 \pm 162$  mg and EPA was  $49 \pm 91$  mg; the combined DHA+EPA was  $147 \pm 251$  mg. The mean for these two LCPUFAs was inflated because two participants were consuming a high amount in relation to the majority of the other participants. One participant was consuming 186 mg EPA and 377 mg DHA and the other participant was consuming 428 mg EPA and 751 mg DHA; amounts surpassing the recommendation of consuming *at least* 200 mg of DHA or 250 mg of EPA+DHA per day. These amounts were achieved in part by consuming salmon, a very well know source of EPA+DHA which provides 1,200-2,400 mg/4 ounces,<sup>24</sup> in addition to other food sources of DHA. The mean value without including these two participants was  $26 \pm 27$  mg of EPA and  $56 \pm 52$  mg of DHA per day. The amount of linoleic acid (LA) consumed ( $17 \pm 5$  g) was 10 times higher than that of  $\alpha$ -linolenic acid (ALA) ( $1.7 \pm 0.7$  g).

Only 17% (n = 4) of women in the study were meeting the recommended DHA intake of at least 200 mg/day. When adding supplemental DHA, the number of women meeting the recommendation increased to 38% (n = 9) (Table 5.3). Only 42% of the study subjects (n = 10), were consuming a supplement containing between 100 to 350 mg of DHA.

Table 5.2. Daily Dietary Intake by Lactating Women (n = 24)

Nutrient	Average daily intake (mean ± SD)	Range (Min – Max)
Energy (kcal)	2,013 ± 514	1,057 – 3,188
Protein (g)	76 ± 25	34 – 118
Carbohydrate (g)	245 ± 60	129 – 342
Total fat (g)	85 ± 30	39 – 164
Saturated fat (g)	28 ± 13	12 – 67
Polyunsaturated fat (g)	19 ± 6	8 – 31
Linoleic acid (g) – LA	17 ± 5	7 – 28
α-Linolenic acid (g) – ALA	1.7 ± 0.7	0.4 – 3.0
Arachidonic acid (mg) – ARA	155 ± 73	36 – 310
Eicosapentaenoic acid (mg) – EPA	49 ± 91	1.3 – 428
Docosahexaenoic acid (mg) – DHA	98 ± 162	1.7 – 751
n-6/n-3**	10 ± 4	6 – 23

\*\* n-6/n-3 = (LA + AA) / (ALA + EPA + DHA)

Table 5.3. Consumption of DHA by Lactating Women (n = 24)

DHA Source	< 200 mg DHA/day mean ± SD (n)	≥ 200 mg DHA/day mean ± SD (n)
Dietary DHA	45 ± 38 (20)	364 ± 280 (4)
Dietary + Supplemental DHA	61 ± 59 (15)	381 ± 216 (9)

Lactating women are advised to consume at least 200 mg/day of DHA<sup>10</sup>  
DHA, docosahexaenoic acid.

Table 5.4 shows the concentrations of the precursor LCPUFA (DHA, EPA, and ARA) and their derived EC metabolites (DHEA, DHG, EPEA, EPG, AEA, and AG) in breast milk. Arachidonic acid comprised 61% of the LCPUFA fraction followed by DHA and EPA comprising 28% and 11%, respectively. For each group, the glycerol-derivatives were present in higher concentrations than those of the ethanolamide-derivatives.

Table 5.4. Fatty Acids and its Derived EC and EC-like Metabolites in Breast Milk (n = 24)

Metabolite	Mean $\pm$ SD ng/mL	Range (Min – Max)
n-3 DHA	2159.87 $\pm$ 3076.02	130.50 – 14172.50
DHEA	0.09 $\pm$ 0.05	0.04 – 0.22
DHG	752.85 $\pm$ 1123.40	46.35 – 4674.00
n-3 EPA	844.16 $\pm$ 2268.62	13.81 – 10835.50
EPEA*	0.09 $\pm$ 0.12	0.01 – 0.47
EPG	42.07 $\pm$ 71.87	0.84 – 296.10
n-6 ARA	4712.24 $\pm$ 8281.88	433.00 – 37150.00
AEA	0.11 $\pm$ 0.12	0.04 – 0.60
AG	233.31 $\pm$ 299.11	16.20 – 1106.00

Data are presented by showing the precursor fatty acid followed by its derived endocannabinoid metabolites.

\*Some values were below the standard curve.

DHA, docosahexaenoic acid; DHEA, docosahexaenoyl ethanolamide; DHG, docosahexaenoyl glycerol; EPA, eicosapentaenoic acid; EPEA, eicosapentaenoyl ethanolamide; EPG, eicosapenaenoyl glycerol; ARA, arachidonic acid; AEA, Anandamide; AG, arachidonoyl glycerol.

To evaluate the relationships between the dietary LCPUFAs and the concentrations in breast milk for the fatty acids and their derived EC, we performed log transformation of the data. Table 5.5 shows significant correlations for DHA and EPA only. There was a positive correlation between dietary DHA and the DHA ( $r = 0.50$ ,  $p \leq 0.05$ ), DHEA ( $r = 0.66$ ,  $p \leq 0.05$ ), and DHG ( $r = 0.46$ ,  $p \leq 0.05$ ) in the breast milk. Dietary EPA was only correlated to the EPA in breast milk ( $r = 0.41$ ,  $p \leq 0.05$ ), but not with the derived EC. No correlations were found between dietary ARA and the fatty acid and EC concentrations in the breast milk.

Table 5.5. Significant Correlations between Dietary Long-Chain Fatty Acids and the Concentrations of Fatty Acids and its Derived EC Metabolites in Breast Milk (n = 24)

Metabolite	Pearson correlation coefficient	<i>p</i> value
DHAd* – DHA	0.50	0.0159
DHAd* – DHEA	0.66	0.0005
DHAd* – DHG	0.46	0.0226
EPAd* – EPA	0.41	0.0454

\*From diet.

DHA, docosahexaenoic acid; DHEA, docosahexaenoyl ethanolamide; DHG, docosahexaenoyl glycerol; EPA, eicosapentaenoic acid.

## 5.4 Discussion

This study, to the best of our knowledge, is the first report evaluating the relationships between maternal dietary intake of LCPUFAs and EC and EC-like metabolites in human breast milk. Provided that the EC may play a role in infant feeding behavior,<sup>17</sup> maternal intake of LCPUFAs during lactation is important to ensure that breast milk is supplying the infant not only with sufficient quantities for adequate development, but also to provide precursor fatty acids for EC metabolism. On average, lactating women in the present study were not consuming adequate amounts of DHA (98 mg/day) to meet the dietary recommendation to consume at least 200 mg of DHA per day during lactation.<sup>10</sup> That is, lactating women in this study were only receiving 49% of the recommended amount. The majority of women (83%, n = 20) were *not* meeting that recommendation. When amounts from supplementation were included, the percentage only decreased by 21%, meaning that 62% (n = 15) of women still failed to meet the DHA recommendation. This observation with American women is in contrast to a study conducted with Canadian women where it was concluded that women who consumed a supplement with DHA were 11 times more likely to meet the DHA recommendation for lactating women.<sup>25</sup> However, in this Canadian study women were consuming 186 mg/day of DHA from the diet (major food sources were seafood, fish, and seaweed products), an amount 1.9 times higher than the amount consumed by women in our study. When considering EPA+DHA combined, women in this population were only consuming 147 mg/day which is below the amount recommended by the Dietary Guidelines for Americans<sup>11</sup> that suggest consuming an average of 250 mg/day of EPA+DHA. Unfortunately, our results are in line with the last report by the National Health and Nutrition Examination Survey 2013-2014<sup>26</sup> which indicated that women of childbearing ages 20-39 years old in the United States are only consuming 25 mg of EPA and 55 mg of DHA daily.



On the other hand, for the essential fatty acids LA and ALA, amounts consumed (17 and 1.7 g, respectively) exceeded the adequate intake recommended by the Institute of Medicine which is 13 g/d for LA and 1.3 g/d for ALA.<sup>9</sup> The n-6/n-3 ratio was 10:1 which is typical of the American diet that is characterized by a higher consumption of n-6 than n-3 fatty acids.<sup>26</sup>

Our data showed a positive correlation between dietary DHA and EPA and the amounts of these LCPUFAs present in the breast milk. Our results are in agreement with previous research that found that LCPUFAs in breast milk reflect maternal intake and/or supplementation. In a study by Jensen and colleagues (2005),<sup>27</sup> lactating women who were supplemented during four months with 200 mg DHA/day demonstrated higher levels of DHA in their breast milk compared to the control group who consumed a placebo. Another study by Sherry et al. (2015)<sup>28</sup> supplemented lactating women with a placebo, a low-dose DHA (200 mg/day), or a high-dose DHA (400 mg/day). After six weeks of supplementation, both DHA doses increased the DHA content of the breast milk by 50% and 123% (low and high dose, respectively) from baseline. Eicosapentaenoic acid was also increased over time, although no significant differences were found among the intervention groups.

This study is the first to explore the relationships between dietary LCPUFAs and the EC in breast milk. Our data indicates that dietary DHA is positively correlated with its derived EC, DHEA and DHG only. For dietary EPA and ARA, no correlations were found with its derived EC (EPEA-EPG and AEA-AG, respectively) in the breast milk. Research to date has been focused on evaluating circulating and tissue levels (e.g. brain, liver, and small intestine) in response to dietary interventions in animal models. In a study by Berger et al. (2001),<sup>29</sup> piglets were fed diets with and without ARA and DHA in similar quantities to those found in porcine milk during 18 days. Levels of the ehanolamide-derivatives from ARA and DHA were evaluated

in the piglet's brain. For piglets consuming the diet containing ARA and DHA, brain levels of the ethanolamide-derivatives (AEA, DHEA, and EPEA) were increased. Similarly, Artmann et al. (2008)<sup>19</sup> demonstrated in a rat model that short term consumption (one week) of diets resembling human diets modulate LCPUFA ethanolamide-derivatives levels in brain, liver, and small intestine. In another study using mice, Wood et al. (2010)<sup>18</sup> evaluated the effect of a two-week DHA-diet (containing DHA and EPA from fish oil) on the EC levels in brain and plasma. After supplementation, the DHEA significantly increased in mouse's brain and plasma but DHG did not change. Eicosapentaenoyl glycerol was also increased in both tissues. Likewise, the DHA-diet significantly increased the ratios of DHEA/AEA and DHG/AG.

Research data<sup>18,19,29</sup> have shown that dietary LCPUFAs can modulate tissue levels of bioactive compounds (i.e. EC and EC-like metabolites). Our results showing that DHA ethanolamide-derivatives in breast milk are associated with dietary DHA permit to hypothesize that maternal dietary interventions providing LCPUFAs could modulate EC levels in breast milk. Research studies can be designed based on our results to confirm this hypothesis. It could be also speculated that supplementation with LCPUFAs during lactation will elicit changes in the EC concentrations in the breast milk, not only for the DHA-derivatives but also for the other EC-like metabolites in both glycerol and ethanolamide groups. In the present study, it could be possible that EPA acted as a substrate for synthesis of DHA, followed by DHEA and DHG synthesis rather than as a substrate for EPEA and EPG synthesis. This could have prevented us from finding a significant correlation between the dietary EPA and derived EC in the breast milk. Biosynthesis rates in the mammary gland may differ for each EC group, ethanolamides and glycerols. Our results indicated a stronger correlation between dietary DHA and DHEA ( $r = 0.66$ ) than dietary DHA and DHG ( $r = 0.46$ ). These observations suggest that there is a

preference for DHEA synthesis rather than for DHG or that the presence of other entourage metabolites (data not shown), which exert cannabimimetic effects,<sup>30</sup> may interfere with the activity of one over another presumably by acting as substrates for enzymatic activity, reducing cellular uptake and/or degradation, or by affecting binding activity to the receptors.<sup>12,31</sup>

## **5.5 Limitations**

The study had limitations. Dietary data were collected using 24-h dietary recalls and this could have prevented us from identifying dietary patterns regarding consumption of food sources that provide LCPUFAs. These three days of intake may not have captured typical consumption of these nutrients. We recommend that future studies include food frequency questionnaires, with specific fish types included, in addition to 24-h dietary recalls to better estimate DHA dietary intakes.

## **5.6 Conclusion**

Our study explored the relationships between dietary LCPUFAs and the EC metabolites in breast milk. We have provided evidenced that i) lactating women are not achieving recommended amounts of DHA, and ii) dietary DHA is associated with levels of its derived EC in breast milk. Although the roles of the different EC metabolites have not been fully elucidated, we have demonstrated that they are present in the breast milk and are associated with dietary precursors. These observations lead to proposed questions. Are these EC metabolites present in breast milk to support infant development? Do dietary LCPUFAs modulate the EC tone in breast milk? Could DHA-derivatives be more susceptible to changes in the diet than their counterparts (EPA- or ARA-derivatives)? These queries remain to be explored.

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## **CHAPTER 6**

### **SUMMARY AND CONCLUSIONS**

The present study has demonstrated that endocannabinoids and endocannabinoid-constituents are present in human breast milk. Using a state of the art lipidomic methodology of analysis we have identified and quantified fifteen constituents of the endocannabinoid metabolome that includes three long-chain polyunsaturated fatty acids. Our data sets the ground for designing future studies to help elucidate not only the mechanisms by which endocannabinoids are synthesized and utilized in the mammary gland, but also which role endocannabinoids play in infant health and development. In the two different populations studied, both with different dietary patterns, endocannabinoids have been identified in the breast milk. Considering this observation, we propose that a research study can be developed to evaluate if the levels of endocannabinoids in the breast milk respond to maternal dietary supplementation of long-chain fatty acids. Although we conducted the studies at different lactation stages (1/2-1 month and 4-6 months of lactation), both studies showed that different endocannabinoid metabolites are present in the breast milk regardless of the stage. However, we hypothesize that colostrum may have increased levels of endocannabinoids provided that they may play a role in establishing the suckling response of the newborn. Taking the three studies presented in this umbrella study, we have been able to raise questions regarding the dynamics of the endocannabinoid metabolome of human breast milk that can be used to determine the future directions of this field of study.

## APPENDIX A. STUDY FLIER



# ARE YOU PLANNING TO BREASTFEED YOUR BABY?

If you answered yes, you may be eligible to participate in a research study to evaluate natural substances in breast milk that help the baby feed at the breast.

- ✓ Qualified participants will receive \$75 at the end of the study
- ✓ Your participation will help us, and other nutrition scientists, to understand the role of these biological substances in the quality of breast milk for infant health and development



**You may be eligible if you:**

**Are 18 - 40 years old**

**Have a singleton birth**

**Deliver at full term (≥ 37 gestational weeks)**

**Plan to breastfeed for at least 4 weeks**

**Are willing to provide a breast milk sample when your baby is 2 and 4 weeks old**

**Have not been breastfeeding or pregnant in the previous year**

### **CONTACT US!**

**(225) 993-1234  
(text or call)**

**agaita1@lsu.edu**

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# APPENDIX B. INSTITUTIONAL REVIEW BOARD APPROVAL UC DAVIS

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SACRAMENTO, CALIFORNIA 95817

March 16, 2017

Juliana Haber  
Phone: 925-548-2993  
Email: jahaber@ucdavis.edu

Dear Ms. Haber:

On March 16, 2017 the UC Davis Social & Behavioral Committee C reviewed the following protocol:

Type of Review:	Continuing Review/Progress Report
Title:	Short Term Response of Breast Milk Micronutrient Concentrations to a Lipid Based Nutrient Supplement in Guatemalan Women
Investigator:	Haber, Juliana
IRB ID:	723323-5
Funding:	UCGHI GloCal Health Fellowship, UCD Blum Center for Developing Economics, Fogarty International, NIH
Grant ID and Title:	5R25TW009343-03 GloCal Health Fellowship
IND, IDE or HDE:	None
Documents Submitted:	* Continuing Review/Progress Report - HRP-212 - FORM - Continuing Review.pdf

The IRB approved protocol from **March 16, 2017 to March 15, 2018** inclusive.

Risk Determination:	Minimal Risk
Category:	Expedited [B]

This Assurance, on file with the Department of Health and Human Services, covers this activity.

FWA No: 00004567  
Expiration Date: December 22, 2020  
IORG: 0000251

**Before January 29, 2018 or within 25 business days of study closure, whichever is earlier, you are to submit a completed "FORM: Continuing Review (HRP-212)" and required attachments to request continuing approval or closure.**

If continuing review approval is not granted before the expiration date of **March 15, 2018** approval of this protocol expires on that date.

- 1 -

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In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,



Cynthia M. Gates, J.D., R.N., C.I.P.  
Director, Institutional Review Board Administration  
University of California, Davis  
2921 Stockton Blvd., Suite 1400  
Sacramento, CA 95817  
916-703-9154



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## APPENDIX C. INSTITUTIONAL REVIEW BOARD APPROVAL LSU AGCENTER



LSU AgCenter Correspondence

August 15, 2017

To: Dr. Carol Lammi-Keefe

From: Michael Keenan, Chair LSU AgCenter IRB

Cc: Adriana Gaitan

Re: Protocol H17-01

Your protocol "Endocannabinoid Metabolome of Breast Milk" has been approved for one year with expiration date of August 15, 2018. Approximately 30 days before the expiration date, send me an e-mail if you wish to renew.

# APPENDIX D. PERMISSION TO USE COPYRIGHT MATERIAL



College of Agriculture  
School of Nutrition and Food Sciences

June 6<sup>th</sup>, 2018

David Fields, PhD  
Department of Pediatrics, Section of Endocrinology and Diabetes  
University of Oklahoma Health Sciences Center  
Oklahoma City, OK 73104

Dear Dr. Fields:

I am completing a doctoral dissertation at Louisiana State University entitled "Endocannabinoid Metabolome of Human Breast Human." I would like your permission to reprint the following material in my dissertation:

Figure 1 "Maternal, physiological, and behavioral factors that impact breast milk composition" that is shown on: Fields DA, Schneider CR, Paveia G. A narrative review of the associations between six bioactive components in breast milk and infant adiposity. *Obesity*. 2016;24(6):1213-1221.

The image will be included in the literature review of my dissertation (displayed in color) to help explain the different factors that may influence the composition of breast milk.

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To grant this permission, please sign where indicated below and return it to me. Please contact me should you have any questions or need additional information. Thank you very much.

Sincerely,

  
Adriana Gaitan  
PhD Candidate  
203 Knapp Hall  
School of Nutrition and Food Sciences  
Louisiana State University  
Baton Rouge, LA 70803

Louisiana State University  
100 Knapp Hall, 203 Knapp Hall  
Baton Rouge, LA 70803

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College of Agriculture  
School of Nutrition and Food Sciences

June 6<sup>th</sup>, 2018

Alexandros Makriyannis, PhD  
Center for Drug Discovery  
116 Mugar Life Science  
Northeastern University  
Boston, MA 02115

Dear Dr. Makriyannis:

I am completing a doctoral dissertation at Louisiana State University entitled "Endocannabinoid Metabolome of Human Breast Human." I would like your permission to reprint the following material in my dissertation:

Figure 1 "The endocannabinoid signaling system" that is shown on: Vemuri VK, Makriyannis A. Medicinal chemistry of cannabinoids. *Clinical Pharmacology & Therapeutics*. 2015;97(8):553-558.

The image will be included in the literature review of my dissertation (displayed in color) to help explain the endocannabinoid system and the mechanism of action.

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To grant this permission, please sign where indicated below and return it to me. Please contact me should you have any questions or need additional information. Thank you very much.

Sincerely,

  
Adriana Gaitan  
PhD Candidate  
203 Knapp Hall  
School of Nutrition and Food Sciences  
Louisiana State University  
Baton Rouge, LA 70803

Louisiana State University  
100 Knapp Hall, 203 Knapp Hall  
Baton Rouge, LA 70803

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## **VITA**

Adriana Virginia Gaitán was born in Guatemala City, Guatemala. She received her Bachelor of Science degree in Food Science in 2007 from the Escuela Agrícola Panamericana, Zamorano in Honduras. Adriana was working in the food science industry during five years prior to start graduate school. She received her Master and Doctorate degrees with a concentration in human nutrition at Louisiana State University in 2015 and 2018, respectively. Adriana is a member of the Zamorano Agricultural Society at LSU, the American Oil Chemists' Society (AOCS), the American Society for Nutrition (ASN), the Institute of Food Technologists (IFT), and the International Society for Research in Human Milk and Lactation (ISRHML).