ANOREXIGENIC AND OREXIGENIC PEPTIDE RESPONSE TO FEEDINGS OF DIFFERENT PROTEIN COMPOSITION IN HEALTHY FORMULA FED INFANTS

by

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ABSTRACT

Rapid weight gain in infancy is a risk factor for later life overweight and obesity. Diet composition is a key factor affecting growth in infancy. Numerous studies show formula fed infants, the majority of whom are fed cow milk formula (CMF), gain more weight than breastfed infants in the first year of life; whereas infants fed extensive protein hydrolysate formula (EHF) have more normative growth, similar to breastfed infants, the gold standard for infant growth. Infants fed EHF have been shown to satiate at a lower volume and earlier than when fed CMF. The mechanism by which EHF leads to earlier satiation at a lower intake volume is unknown, however it is hypothesized the extensively hydrolyzed protein (small peptides and free amino acids) found in EHF formulas may lead to differential responses in the gastrointestinal peptides that play a role in meal termination (satiation).

The overall aim of this study was to determine the effect of formula composition (EHF vs. CMF) on satiation and adiposity peptide response in healthy, formula fed infants. Infants (n=5 males, n=6 females) ages 1-4 months old were recruited from the greater Newark, Delaware area. Subjects completed 2 study visits within 7-days, and received one test formula, EHF or CMF, at each visit in random order. Blood samples were drawn pre-feeding and post-feeding at each visit to assess differences in peptide response by formula type.

Infants were on average 86 days old (95%CI 70.7-101.4) and weighed 5.8kg (95%CI 5.3-6.4). Although the study was underpowered for all outcomes, preliminary

analysis revealed there were no significant differences in the change in concentration of satiation and adiposity peptides by formula type. Further, there were no significant differences in infant feeding dynamics (volume, duration, or rate of feeding) by formula type, although volume and rate of feeding was lower for EHF feeds compared to CMF feeds in 8 of 10 infants. Duration of feeding was inversely correlated with change in PYY concentration, rate of feeding was positively correlated with change in PYY concentration, and time since last feeding was positively correlated with change in PYY and GIP concentrations for CMF but not EHF feeds. Current findings should be interpreted with caution and further analysis with a larger sample size is needed before definitive conclusions may be drawn.

Chapter 1

INTRODUCTION

In the United States, 8.1% of infants have a weight for length at or above the 95th percentile on the Centers of Disease Control and Prevention (CDC) growth charts indicating a weight for length in the obese category.[1] Additionally, the prevalence of overweight and obesity among toddlers, ages 2-5 years, is 14.4% and 8.4% respectively.[2] Increasing evidence suggests there are sensitive periods in the lifespan in which individuals are more prone to long-term effects of environmental factors such as diet.[3,4] Infancy is thought to be one of these sensitive periods. Rapid weight-gain and a higher weight for length during infancy are risk factors for later life overweight and obesity.[5-7] With the overweight and obesity epidemic affecting even infants and toddlers, it beckons the need for obesity prevention at an early age.

A key factor affecting growth during infancy is diet. While exclusive breastfeeding is the gold standard for infant nutrition,[8] by three months of age, approximately 59% of infants are receiving infant formula.[9] By the end of the first year of life, studies have shown formula fed infants have significantly higher weight for length z-scores compared to breastfed infants.[10] Formula fed infants, however, are not a homogenous group. Recently, studies have shown infants feeding extensive protein hydrolysate formula (EHF) grow more normative to breastfed infants while infants fed cow's milk formula (CMF) had accelerated weight gain.[11] Further, several studies have demonstrated that infants randomized to receive EHF consumed less volume to satiation at a feeding compared to infants receiving CMF.[11-13]

The mechanism by which EHF infants satiate at a lower volume during feeding is unclear. It has been hypothesized[11] that the small peptides and free amino acids, abundant in EHF but not CMF, stimulate satiation gut peptide responses more rapidly or to a greater extent, thus resulting in satiation at a lower feeding volume. There is limited research examining satiation gut peptides in response to diets of different macronutrient composition in healthy, term infants

Chapter 2

REVIEW OF THE LITERATURE

2.1 Infant Feeding Practices

2.1.1 Current Feeding Recommendations and Practices

The American Academy of Pediatrics (AAP) and the World Health Organization (WHO) recommend infants be exclusively breastfed for the first six months of life. [14,15] According to the WHO, exclusive breastfeeding is defined as "no other food or drink, not even water, except breast (human) milk (including milk expressed or from a wet nurse) for 6 months of life, but allows the infant to receive ORS, drops and syrups (vitamins, minerals and medicines)." [15] Further, the AAP recommends breastfeeding be continued through the first year of life. [14] In cases where infants are not or cannot be breastfed, infant formula is recommended throughout the first year of life. [14]

Breastfeeding is the gold standard for infant feeding and nutrition. [8] The health benefits of breastfeeding are well documented and range from immunologic benefits for the infant to a reduced risk of breast cancer for the mother. [8,16] According to the Centers for Disease Control and Prevention (CDC) Breastfeeding Report Card 2014, 79.2% of mothers initiate breastfeeding, 49.4% are breastfeeding at 6 months, and 26.7% are breastfeeding at 12 months. [17] These rates reflect any breastfeeding. Exclusive breastfeeding rates however, are approximately 40.7% at 3 months and drop

to 18.8% at 6 months[17]; as such approximately 59% of infants in the United States receive infant formula in part or as a sole source of nutrition by 3 months of age. [9]

A recent study utilized data from the National Health and Nutrition Examination Survey (NHANES) 2003-2012 to determine the percentage of infants (0-12 months) consuming various types of infant formula. [9] Of the infants consuming formula, 68.9% (95% CI 65.1-72.5) consumed a cow milk formula, 11.6% (95% CI 9.6-14.0) consumed a soy-based formula, 6.3% (95% CI 4.9-8.1) consumed a specialty formula (i.e. formulas for pre-term infants, acid reflux, phenylketonuria, or cow milk/soy protein allergy), and 5.4% (95% CI 3.6-7.8) consumed a gentle/lactose-reduced formula. [9] Given that a large proportion of infants in the United States are consuming infant formula, especially cow milk formula (CMF), it is critical to examine how infant formula effects early nutrition and growth.

2.2 Diet Composition and Growth

Diet composition in early infancy, specifically macronutrient composition, effects infant growth and body composition. [18] Rapid weight gain in early infancy is associated with a greater risk of later life obesity. [5,6,19,20] Further, studies have shown breastfed and formula fed infants have different growth patterns. [10,21,22] As human milk is the gold standard for infant nutrition, the growth of a breastfed (BF) infant is considered to be the normative and gold standard for infant growth. [8,15] Studies have shown however, that formula fed (FF) infants, the majority of whom are fed cow milk formula grow, differently than their BF counterparts, especially after the first three months of life. [10,21,22] One of the first studies to assess the effect of diet composition on infant growth was published in 1992. This study compared growth of BF (n=46) and FF (n=41) infants from birth to 18 months. [22] Both BF and FF infants grew

similarly between zero and three months, however BF infants gained weight less rapidly than FF infants between three and twelve months resulting in a significant (0.65kg) weight difference at twelve months. Additionally, weight for length z-scores were significantly higher in FF infants from 4-18 months. Finally, no significant differences in length were seen between the BF and FF cohorts.[22] These findings suggest BF infants are leaner than FF infants by the end of the first year old life.

Differences in growth, specifically weight gain, between BF and FF infants is thought to be due in part to differences in the protein content of human milk compared to infant formula.[3,21,23] The protein concentration in human milk is dynamic. It is highest after birth and steadily declines over the course of lactation until approximately six months of age when the protein concentration of human milk tends to stabilize.[24] One study found median protein concentration of human milk to range from 17.3g/L at four days post-partum to 7.7g/L at six months.[24] Additionally, human milk contains high concentrations of free amino acids (FAA) with one study estimating a concentration of 3019.7μm/L.[25] Conversely, cow milk based infant formula (CMF), the most commonly consumed infant formula in the United States, contains a fixed amount of protein typically ranging from 13.0-14.0g/L[26,27]. Further, the FAA concentration in cow milk formula is lower much than human milk.[25] A study of the FAA concentration of seven commercially available infant formulas in Europe found the FAA concentration ranged from 615.5 to 122.4μm/L.[25] The increased total protein content of infant formula, likely above the physiologic needs of the infant, is thought to play a role in the accelerated weight gain of FF infants. This is termed the 'protein hypothesis', and suggests that protein intake above needs, increases the concentration of circulating amino acids, which in turn is believed to

enhance secretion of insulin and insulin-like growth factor 1 (IGF-1), resulting in greater weight gain among FF infants.[3,23] However, emerging research has found not only the amount of total protein, but also the form of protein in infant formula may be important.

Cow milk formula (CMF) is the most commonly consumed infant formula in the United States (consumed by almost 70% of formula fed infants).[9] The protein in CMF comes from non-fat cow milk and is primarily composed of intact casein and whey protein.[3] However, in the United States, approximately 6% of FF infants consume a protein hydrolysate formula (PHF).[9] The major difference between CMF and PHF is the form of the protein.[3] Protein hydrolysate formulas contain hydrolyzed proteins resulting in smaller peptides. These formulas can be either partially hydrolyzed or extensively hydrolyzed. Extensive protein hydrolysate formula (EHF) contains predominately FAA and small peptides with a mass of <1500kDa.[3] Emerging research suggests infants' growth, namely weight gain, is accelerated in infants fed CMF, and more normative in infants fed EHF.[11]

In one study, 56 healthy and predominately formula feeding mother-infant dyads were randomized to receive a CMF (n=32) or EHF (n=24) when infants were 0.5 months of age and continue feeding the assigned formula for the next 7 months.[11] Infant anthropometric measures were collected monthly until 7.5 months. Researchers found infants consuming EHF had significantly lower and more normative weight for length z-scores ($p<0.01$) than CMF fed infants at each time point beginning two months after randomization through the end of the study. Additionally, there were no significant differences in length for age z-scores at any time point between CMF and EHF groups. Finally, the weight for age and weight for length z-scores of the EHF

group remained close to the zero threshold indicating normative growth and weight gain whereas the CMF group was consistently above the zero threshold in weight for age and weight for length z-scores indicating accelerated growth and weight gain. Interestingly, in laboratory feedings that took place at the study site once per month, EHF-fed infants consumed less volume to satiation compared to CMF infants. [11] This study suggests that it is not solely the total protein intake that impacts weight gain, but the form of protein plays a role as well perhaps through its effect on energy intake.

A within-subject designed study by Ventura et al. explored the role of protein form, and more specifically FAA, on formula intake.[12] This study enrolled 30 infants less than four months of age and involved three infant-led feeding sessions. In counter-balanced order, infants were fed three formulas, CMF, EHF, and CMF with added glutamate, one at each feeding session. Glutamate was selected as the amino acid to be added to CMF as it is the most abundant FAA in human milk. [28] At each feeding session caregivers were instructed to feed their infant until the infant signaled satiation (defined as three consecutive signs of the infant being finished with the feeding such as turning their head away, thrusting the bottle out of the mouth with their tongue, etc.). Caregivers and infants remained at the study center until infants signaled hunger again. During the second feeding, all infants were given CMF. Infants consumed a significantly lower volume (mL) of CMF with glutamate ($p<0.02$) and EHF ($p<0.04$) to satiation compared to CMF. [12] This finding suggests the free amino acids found in EHF may promote earlier infant satiation during feedings.

2.3 Energy Intake, Satiation, and Mechanisms that Regulate Intake

Energy intake is regulated via complex endocrine signaling pathways that relay signals between the central nervous system (CNS) and peripheral organs such as the

gastrointestinal tract.[29-31] Hormones and peptides produced by the gastrointestinal tract (gut) signal, among other feelings, satiation and satiety. Satiation peptides are secreted in response to food intake and ultimately bring the meal or eating occasion to a close. Satiety peptides prevent ingestion of more food between meals, or until hunger signals its time to eat again.[29-31]

Gut peptides and hormones act along to the gut-brain axis, communicating with the brain to control energy intake.[29-31] These gut peptides can be categorized as either satiation or adiposity peptides, and satiation peptides can be further dichotomized as either orexigenic (appetite stimulating) or anorexigenic (appetite diminishing).[30,31] Additionally, gut peptides can be deemed short or long-term signals depending on whether they act on a meal-to-meal basis or on a longer-term basis.[29]

2.3.1 Orexigenic Satiation Gut Peptides

All of the satiation peptides are anorexigenic (meaning they cause loss of appetite) except ghrelin.[29-31] Ghrelin is orexigenic in nature and therefore stimulates appetite. Ghrelin is predominately produced in the stomach, but can be produced to a lesser extent in the small intestine. Circulating concentrations of ghrelin peak just before a meal and then fall during the postprandial period.[29,30]

2.3.2 Anorexigenic Satiation Gut Peptides

Glucagon-like peptide 1 (GLP-1) is a short-term, anorexigenic biomarker of satiation that is synthesized in the ileum in response to the presence of food.[29,30] GLP-1 exists in two major forms, the active form ($GLP\text{-}1_{7\text{-}36\ amide}$) and the inactive form ($GLP\text{-}1_{9\text{-}36\ amide}$). Active GLP-1 stimulates the beta cells of the pancreas to release insulin thereby lowering blood glucose concentrations. Circulating concentrations of

GLP-1 rise in anticipation of a meal, usually peak shortly after a meal, and fall during the post-prandial period. Presence of GLP-1 promotes reduced food intake and is thought to delay gastric emptying. [29,30]

Gastric inhibitory peptide (GIP) is a short-term, anorexigenic satiation biomarker that is produced by the enteroendocrine K cells of the small intestine. [32] Similarly to GLP-1, GIP also stimulates beta cells of the pancreas to release insulin. GIP concentrations rise in response to a meal and generally peak 15-30 minutes after the start of the meal before returning to fasting concentrations.[32]

Peptide YY (PYY) is an anorexigenic biomarker of satiation and is secreted by the L-cells of the colon in response to food. [29,30] PYY exists in two major isoforms, PYY_{3-36} and PYY_{1-36}, where PYY_{3-36} is the major isoform. PYY concentrations are low before a meal and peak shortly after a meal commences. However, unlike GLP-1 or GIP, circulating concentrations of PYY remain elevated for 1-2 hours after a meal.[29,30]

2.3.3 Adiposity Peptides

Leptin is a hormone produced by adipocytes, and circulating leptin is positively correlated with total body fat. [29,30,33] Once released from adipocytes, leptin is able to cross the blood brain barrier (BBB) in order to communicate information about energy or fat stores to the brain. [30] With respect to energy balance, leptin is not thought to be a short-term satiation peptide, meaning its concentrations do not vary greatly in response to meals. Rather, leptin is thought to play a role in longer-term appetite regulation, and given its relationship with fat stores, leptin is considered an adiposity peptide. [29-31] Plasma leptin concentrations decrease in response to early (>24hr) starvation and increase in response to overfeeding after about 24 hours. [29] In

cases of positive or negative energy balance, leptin is negatively correlated with appetite and food intake.[29,30,33]

2.3.4 Interconnection of Satiation and Adiposity Gut Peptides

The gut peptides work in unison to send signals to the brain to help determine how much is eaten and when eating occasions end. [31] However, efficacy of satiation peptides can depend on adiposity peptides. [31] For example, when individuals are on a diet or have been food restricted, circulating leptin concentrations are low, and therefore the adiposity peptide response is reduced. As a consequence, there is lower sensitivity to satiation peptides, resulting in an increased food intake to satiation. Conversely, increased leptin due to overeating or weight gain increases sensitivity to satiation peptides resulting in decreased food intake. [30,31]

2.3.5 Studies of Satiation and Adiposity Gut Peptides in Infants in Response to Feeding

Very few studies have explored the effect of infant feeding on satiation and adiposity peptide response. A study of GIP concentrations in response to feeding evaluated 158 healthy pre-term neonates to explore the response of gastrointestinal hormones to a feed. [34] Infants were grouped into four groups based on age; 1-4 days, 5-7 days, 9-17 days, and 18-42 days. Infants were fed human milk via nasogastric (NG) tube for five minutes. Venous blood samples were taken either before feeding or at 30, 60, or 120 minutes post-feeding. With respect to GIP, researchers found there was no significant rise in GIP after a feeding in infants in the first three groups (ages 1-17 days). However, there was a sharp and significant rise in GIP in infants' ages 18-42 days, peaking approximately 30 minutes post feeding, perhaps suggesting that infant peptide responses become stronger as infants age closer to term.[34]

A second study in infants conducted by Padidela et al., enrolled 22 infants and aimed to measure basal and post-feed GLP-1 concentrations.[35] Infants, aged four to ten days, were fed 60-70mL of a standard cow milk formula and blood samples were taken before feeding began (time zero), and 20 minutes and 60 minutes after feeding began. The mean infant GLP-1 concentration at time zero was 79.1pmol/L, 156.6pmol/L at 20 minutes post-feed and 121.5pmol/L at 60 minutes post-feed.[35] These results demonstrate that GLP-1 rises in response to a feeding and peak concentrations occur <20 minutes after a feeding.

2.3.6 Studies of Satiation and Adiposity Gut Peptides in Children in Response to Feeding

A study of obese (n=34) and normal weight (n=20) children aimed to evaluate fasting and post-prandial concentrations of leptin and ghrelin, and to examine the relationship between both leptin and ghrelin concentration with adiposity and insulin resistance in prepurbetal obese children.[36] Anthropometric measures were taken to determine body mass index (BMI) and classify children as either obese, a BMI greater than the 97th percentile for age and sex, or normal weight, a BMI between the 25th and 75th percentiles for age and sex. Baseline fasting blood samples were collected from all children to assess fasting concentrations of leptin and ghrelin. Next, all children were fed a standardized breakfast containing 438 kcal, 9.8g of protein (8.9% kcal), 15.5g of fat (31.8% kcal), and 64.8g of carbohydrates (59.1% kcal). Blood samples were drawn again one, two, and three hours after breakfast to assess changes in leptin and ghrelin in response to food. Plasma leptin concentrations were significantly higher in obese children compared to normal weight children at all time points ($p<0.001$). Ghrelin was similar between obese and normal weight children at baseline

(fasting) however the post-prandial time course between groups was significantly different (p=0.012). Ghrelin concentrations one and two hours after the meal were significantly lower than fasting in both obese and normal weight children. However, 3 hours post-breakfast, obese children had higher ghrelin concentrations than normal weight children (p=0.046) and similar to fasting (p=0.439). Comparatively, normal weight children showed plasma ghrelin concentrations still significantly lower than fasting at 3 hours ($p<0.001$) [36], suggesting obese children may have decreased time between meals before ghrelin concentrations peak and stimulate hunger/eating again.

Lomenick et al. studied the differences in ghrelin and PYY secretion after consuming a high carbohydrate, high protein, and high fat meal in normal weight (n=13) and obese (n=19) children. [37] In this study there were a total of three study visits, and subjects received the high carbohydrate, high protein, or high fat meals in random order. Subjects arrived to the lab 8:00am, at least 8-hours fasted, upon which a blood sample was collected. Then, subjects were provided one of the three macronutrient breakfasts, and blood samples were collected at 8:30am, 9:00am, 10:00am, 11:00am, and 12:00pm. Blood samples were analyzed for total ghrelin and PYY concentrations. Fasting ghrelin concentrations were significantly higher in normal weight children compared to obese children, but there were no differences in fasting PYY concentrations. In response to the high-protein meal, ghrelin concentrations decreased significantly from 8:00-11:00am (p=0.0001) in normal weight children, and did not increase between 11:00am and 12:00pm (p=0.26). In obese children, ghrelin decreased between 8:00am and 12:00pm (p=0.0001). The area under the curve for ghrelin was significantly lower in normal weight subjects and obese subjects, 61% and 28% respectively. PYY concentrations in normal weight

children increased from 8:00am to its peak at 12:00pm (p=0.0001), and in obese children PYY increased significantly to a peak at 10:00am (p=0.0001) with no significant decline between 10:00am and 12:00pm (p=0.26). There was no significant difference in the PYY area under the curve in normal weight versus obese children. Following the high carbohydrate and high fat meals, ghrelin reached its minimum between 8:00am and 9:00am, however following the high protein meal it appears ghrelin reached a minimum between 10:00am and 11:00am in normal weight children and between 11:00am and 12:00pm in obese children. In obese children, PYY was significantly higher in response to the protein meal, compared to carbohydrate ($p<0.0001$) and fat meals ($p=0.007$). [37] This study demonstrates ghrelin and PYY concentrations change in response to meal intake, and that both body weight status (normal weight vs. obese) and macronutrient composition of the meal influence concentrations of the peptides at fasting and after a meal.

2.3.7 Studies of Satiation and Adiposity Gut Peptides in Adults in Response to Feeding

A randomized crossover study of 39 overweight men (n=19) and women (n=20) ages 18-60 years aimed to examine the effects of whey protein (WP), pea protein hydrolysate (PPH), a combination of WP and PPH, or a control of milk protein (MP) on postprandial changes in ghrelin, GLP-1, and PYY. [38] Subjects completed four trials, one for each protein source, and arrived to each trial following an overnight fast. At time zero, subjects drank an isometric volume of each protein shake (WP, PPH, WP+PPH, or MP) and 150mL of water. Blood samples were drawn fasting (time zero) and 30, 60, 90, and 120 minutes after time zero. PYY concentrations at 30 minutes were significantly higher after consuming WP+PPH compared to WP, PPH,

or MP alone ($p<0.05$). At 60 minutes, GLP-1 concentrations after consuming MP were significantly higher than consuming WP or PPH ($p<0.05$). Additionally, at 120 minutes, GLP-1 concentrations after consuming MP were significantly higher than after consuming PPH ($p<0.05$). Finally, at 120 minutes, ghrelin concentrations were significantly lower after consuming WP+PPH compared to MP ($p<0.05$). GLP-1 had the highest concentrations the first time the blood was drawn, 30 minutes after consuming the shake, for all protein types, and decreased there after through 120 minutes. PYY reached its highest concentrations between the first and second blood draws (30 and 60 minutes), and decreased thereafter through 120 minutes. Ghrelin reached a minimum concentration before the first blood draw (30 minutes) for MP, between the first and second blood draws (30-60 minutes) for PPH and WP+PPH, and between the third and fourth blood draw (60-90 minutes) for WP. Finally, between 90 and 120 minutes, while not statistically significant, ghrelin concentrations rose in subjects who consumed WP or MP, but remained relatively stable in those who consumed WP+PPH or PPH.[38] This study suggests the composition of the feeding, specifically protein composition, can differentially affect concentrations of satiation peptides.

Another study, by Karl et al., aimed to determine the combined effects of eating rate (ER) and energy density (ED) on appetite and energy intake measured during consecutive meals, and to examine effects on postprandial pancreatic and gut hormone responses.[39] Subjects were healthy, non-obese men (n=12) and women (n=8) ages 18-55 years. All subjects completed four study visits on non-consecutive days with no more than two visits per week. Subjects arrived 12-hours fasted upon which fasting blood samples was collected. After the blood draw, subjects were given

either a low or high energy density breakfast at a prescribed eating rate, either 20g/min or 80g/min, where the order in which subjects completed the four trials was random. Blood samples were drawn 15, 30, 45, 60, 90, 120, and 180 minutes after the first bite of breakfast was taken. Fasting concentrations of GLP-1, PYY, and ghrelin did not differ across the four trials. The area under the curve for GLP-1 and PYY was significantly higher for the fast rate ($p \leq 0.05$) and high energy density ($p \leq 0.05$) trials. At meal completion, GLP-1 concentrations were higher after the slow rate meal ($p=0.05$) and high energy density meal ($p=0.05$). Eating rate had a significant effect on peak GLP-1 ($p \leq 0.05$) concentrations and minimum active ghrelin concentrations ($p \leq 0.05$) with the faster eating rate resulting in higher and lower points, respectively. Finally, PYY concentration at 180 minutes was significantly higher during the higher energy density trials ($p \leq 0.05$). [39] More studies are needed, however this study suggests that both eating rate and energy density have an effect on satiation gut peptides.

2.3.8 Eating Rate as a Factor Influencing Satiation Gut Peptides

Diet composition, specifically macronutrient composition, effects satiation and adiposity peptides in response to a meal. Research suggests eating rate may also have an effect on satiation peptide response. Seventeen healthy, adult males were enrolled in a study examining the differences in the postprandial responses of ghrelin, PYY, and GLP-1 when identical meals were consumed at two eating rates (fast and slow). [40] The test meal was 300mL of ice cream (675 kcal, 59% kcal from fat, 33% kcal from carbohydrates, and 8% kcal from protein). Subjects consumed the meal at a fast rate (five minutes) and a slow rate (30 minutes), and blood samples were collected at baseline and every 30 minutes after the meal began through 210 minutes. The area

under the curve was significantly greater following the 30-minute meal compared to the five-minute meal for GLP-1 ($p=0.001$) and PYY ($p=0.004$). PYY concentrations were significantly higher following the 30-minute meal at 90-150 minutes. Finally, GLP-1 concentrations were significantly higher 60-210 minutes following the 30-minute meal compared to the five-minute meal.[40] These findings suggest eating more slowly may result in a greater anorexigenic peptide response.

Another study compared post-prandial responses to GLP-1 and PYY at two eating rates, slow and fast, between obese adolescents (n=9) and obese adults (n=9).[41] Subjects arrived to the study center following an overnight 12-hour fast. Fasting blood samples were collected before the meal (time zero) and then every 30 minutes through 210 minutes. The test meal, 10kcal/kg of ice cream (59% kcal from fat, 33% kcal from carbohydrates, and 8% kcal from protein), was consumed fast (five minutes) or slow (30 minutes). Concentrations of GLP-1 and PYY were significantly higher compared to baseline from 60 through 210 minutes ($p<0.05$). Additionally, concentrations of GLP-1 and PYY were significantly higher during the slow eating rate compared to the fast eating rate in obese adolescents ($p<0.05$), but these results were not seen in obese adults.[41] These findings suggest a slow eating rate yields a more pronounced GLP-1 and PYY response compared to a fast eating rate. Further, significant anorexigenic peptide responses were only seen in obese adolescents, suggesting perhaps the anorexigenic peptide response may be disrupted in obese adults.

2.4 Literature Review Summary

Results from these studies demonstrate concentrations of ghrelin, leptin, GLP-1, GIP, and PYY change in response to feeding. Moreover, the change in each

peptide, from pre- to post-feeding concentrations, appears to be affected by a variety of factors including macronutrient composition of the diet, weight status, and eating rate. Very few studies have been conducted in infants to examine satiation and adiposity gut peptide response to infant feeding. Further, no studies to date in term infants have accounted for the effect of infant diet composition, specifically the type of formula (EHF vs. CMF), on satiation and adiposity gut peptide response, and its subsequent effect on intake.

Chapter 3

AIMS

The overall aim of this proposal is to study the effect of formula composition (EHF vs. CMF) on gut peptide response in healthy, formula fed infants. Relationships between volume of infant feeding (mL), duration of infant feeding (min), rate of infant feeding (mL/min), and change in concentration of each gut peptide will be explored in a 2x2 cross-over design study where infants are fed a test feeding of either CMF or EHF.

3.1 Specific Aims

The **primary aim**:

Specific Aim 1: Assess pre- and post-prandial concentrations of anorexigenic (GLP-1, GIP, PYY) and orexigenic (ghrelin) gastrointestinal peptides in response to feedings that differ in protein composition (EHF vs. CMF) in 1-4 month-old infants. Because EHF has been shown to transit the gastrointestinal tract at a faster rate than intact protein formula, [42] we hypothesize the increase in post-prandial concentrations of anorexigenic peptides will be greater, and the decrease in post-prandial concentration of the orexigenic peptide will be lower, when fed EHF versus CMF.

The **secondary aims** are:

Specific Aim 2: Explore relationships among dynamics of feeding (volume (mL), duration (min), and rate (mL/minute)), with change in anorexigenic peptide concentrations in response to feeding formulas that differ in protein composition (EHF

vs. CMF). Consistent with previous findings, [11,12] we hypothesize infants will consume a lower volume of the EHF feed versus CMF feed. Because a slower feeding rate has been shown to result in more pronounced anorexigenic gut peptide response and lower weight of food intake, [40] we hypothesize that rate of infant feeding will be inversely associated with change in anorexigenic peptide concentration.

Chapter 4

METHODS

4.1 Subjects

Mothers and their infants from the greater Newark, Delaware area were recruited to participate in this study. Institutional Review Board (IRB) approved advertisements were distributed via newspapers, flyers, Special Supplemental Nutrition Program for Women Infants and Children (WIC) clinics, expectant mother support groups, primary care medical practices, and child care centers. To be eligible to participate in the study, the inclusion criteria specified infants be: healthy, term (≥37 and ≤42 week gestation at birth), singleton, appropriate for gestational age infant, between ≥30 days and ≤120 days old at enrollment where the infant's date of birth equals day zero, primarily receiving a standard (intact protein) cow milk infant formula, have no allergies to cow milk formula, and have never received an EHF (Nutramigen, Alimentum, Pregestimil or PurAmine). Exclusion criteria were: infants who had major congenital malformations (i.e. cleft palate, extremity malformation) or genetic disorders, infants who had suspected or documented systemic or congenital infections (e.g., human immunodeficiency virus, cytomegalovirus), infants who had evidence of significant cardiac, respiratory, endocrine, hematologic, gastrointestinal, or other systemic diseases, and infant who were receiving any prescription medication. A total of 12 formula fed infants were screened for this study. Of the 12 infants screened, 11 formula fed infants completed the study.

4.2 Research Design

This study, and all supporting documents (informed consent, study protocol, data collection forms, and subject recruitment fliers), were approved by the Institutional Review Board (IRB) at the University of Delaware before commencing any study activities. Mothers with eligible infants contacted study personnel at the Energy Balance and Nutrition Lab (EBNL) at the University of Delaware. Initial screening was conducted via telephone to assess interest and eligibility. Assuming eligibility criteria were met, mother-infant dyads were enrolled in the study.

This study required two, three-hour visits to the EBNL within seven days of one another. Subjects scheduled both visits via telephone with study personnel after screening and eligibility were complete. Formula feeding mother-infant dyads were instructed to bring a clean, empty bottle from home. Study formula included two isocaloric formulas, a CMF, Enfamil[27] (Mead Johnson Nutrition, Evansville, IN), and an EHF, Nutramigen[43] (Mead Johnson Nutrition, Evansville, IN) (**Table 1**). Formulas were provided at the study visits. Formula fed infants received both formulas, one at each visit, in random order.

4.3 Study Visit Procedures

Prior to the Visit: Mothers were emailed a blank copy of the informed consent document, a map of the building where study visits occurred, parking directions, and contact information for study personnel.

Visit 1: Upon arrival to the lab, the informed consent document was reviewed verbally with mothers, and mothers were encouraged to ask any questions about the study. Once the informed consent was signed, study visit procedures commenced as follows **(Figure 1)**:

- Inclusion/exclusion criteria were completed
- Topical numbing cream was placed on the outside of the infant's heel to allow ample time for numbing to occur
- Formula-fed infants were randomized to the order in which they received the test formulas
- Infant length, weight, and head circumference were measured
- Obtained pre-feeding blood sample via heel stick two minutes prior to feeding
- Bottles were prepared with the appropriate test formula and then weighed to the nearest 0.1 gram
- Observed and videotaped infant feeding
- Weighed bottle after feeding to the nearest 0.1 gram
- Weighed infant after feeding
- Obtained post-feeding blood sample via heel stick five minutes after feeding
- Completed demographic, medication, general interview, and infant feeding history questionnaires
- Mothers signed subject payment verification form

Visit 2: Upon arrival to the lab, topical numbing cream was placed on infant's heels, and then study visit procedures commenced as follows **(Figure 1)**:

- Infant length, weight, and head circumference were measured
- Obtained pre-feeding blood sample via heel stick two minutes prior to feeding

- Bottles were prepared with the appropriate test formula and then weighed to the nearest 0.1 gram
- Observed and videotaped infant feeding
- Weighed bottle after feeding to the nearest 0.1 gram
- Weighed infant after feeding
- Obtained post-feeding blood sample via heel stick five minutes after feeding
- Completed medication, infant feeding, and baby eating questionnaires
- Mothers signed subject payment verification form

4.4 Demographic Measures

Demographic data, including but not limited to maternal and paternal race/ethnicity, education level, and income were collected at visit one via demographic and general interview forms (**Appendix C**).

4.5 Infant Anthropometric Measures

Infant weight was measured on an electronic scale (SECA) accurate to 0.001 kg.[44] Infant recumbent length was measured with a measuring rod attachment (SECA) for the electronic scale accurate to 0.1cm.[45] Infant head circumference was measured with a non-elastic tape measure accurate to 0.1cm. Anthropometric z-scores were calculated using the World Health Organization (WHO) Multicenter Growth Reference Standards.[46] Weight for age, length for age, and weight for length z-scores were calculated for each infant.

4.6 Infant Feeding Measures

At each study visit, two six-ounce bottles of the assigned test formula (EHF or CMF) were prepared. Mothers fed their infants ad libitum until the infant exhibited three consecutive signs of satiation such as waving arms, leaning away, pushing the bottle away, or falling asleep.[47] Bottles were weighed to the nearest 0.1g before feeding and after feeding. Volume of infant feeding (mL) was determined by subtracting the post-feeding weight of the bottle from the pre-feeding weight of the bottle. Then, the amount consumed in grams was divided by 1.03, resulting in the total volume of intake in milliliters. Duration of infant feeding (min) was determined from time feeding started to time feeding stopped. From these measures, rate was determined as volume (mL) divided by duration (min).

4.7 Biochemical Measures

Blood Sample Collection: Infant blood samples were drawn twice at each study visit, once pre-feeding (two minutes prior to feeding) and once-post feeding (five minutes after feeding ended to allow infants time for the feeding to settle before positioning for the heel stick). Infants' heels were numbed with topical numbing cream (Emla, Fougera Pharmaceuticals, NY), and then cleaned using iodine swab sticks (Dynarex, NY). Trained personnel completed the heel stick using TenderFoot Newborn devices (Accriva Diagnostics, CA).[48] Blood was collected drop-wise via capillarity tube into a micro-collection tube containing EDTA, 5.0μL of dipeptidyl peptidase IV (Millipore, MA), and 25.0μL of aprotinin (Sigma-Aldrich, MO).[49,50] Approximate volume at each heel stick was 1.0mL.

Biologic Sample Processing and Analysis: After blood was collected, blood was immediately stored on ice until the conclusion of the study visit, at which time

blood was centrifuged for 15 minutes at 4,400 rpm. Plasma was separated and stored at -80°C until it was ready to be assayed.

A human metabolic hormone magnetic bead panel - metabolism multiplex assay (Millipore, Germany) was used to determine the concentration of ghrelin, leptin, GLP-1, GIP and PYY. All samples were run in duplicate and the mean of the duplicates was calculated.

4.8 Data Analysis and Statistics

Variables of interest in this analysis are the independent variables: formula type (EHF vs. CMF) and the dependent variables: volume of infant feeding (mL), duration of infant feeding (min), rate of infant feeding (mL/min) and the pre- and post-feeding concentrations of ghrelin, leptin, GLP-1, GIP, and PYY.

Descriptive statistics on infant characteristics (age, sex, anthropometrics, etc.) and maternal characteristics (age, anthropometrics, education, and income level) are described using minimums, maximums, and means (95% CI) if normally distributed, or as median (IQR) if skewed.

Specific Aim 1: Assess pre- and post-prandial concentrations of anorexigenic (GLP-1, GIP, PYY) and orexigenic (ghrelin) gastrointestinal peptides in response to feedings that differ in protein composition (EHF vs. CMF) in 1-4 months old infants. To assess specific aim 1: summary statistics including minimum, maximum, frequency, mean, and 95% CI were determined for the change in each gut peptide. For each gut peptide, a paired samples t-test was used to test for a difference in the change in concentration, by formula type (EHF vs. CMF).

Specific Aim 2: Explore relationships among dynamics of feeding (volume, duration, and rate (mL/minute)), with change in anorexigenic peptide

concentrations in response to feeding formulas that differ in protein composition (EHF vs. CMF). To assess specific aim 2: summary statistics including minimum, maximum, frequency, mean, and 95% CI were determined for volume of feeding, duration of feeding, and rate of feeding, for each formula type. Pearson or Spearman correlation coefficients, depending on normality of the variables, were used to determine associations between the change in concentration of each anorexigenic peptide and the feeding dynamic variables, by formula type (EHF vs. CMF).

Chapter 5

RESULTS

5.1 Normality and Distributions of Variables

All continuous variables were tested for normality using the Shapiro-Wilk W test. The null hypothesis of the Shapiro-Wilk W test is data come from a normal distribution. Therefore, $p<0.05$ indicates non-normal data.

For infant characteristics and anthropometric measures, the variables age (days), weight (kg), length (cm), head circumference (cm), weight for age z-score, length for age z-score, and weight for length z-score were normally distributed ($p>0.05$). Therefore, means and 95% confidence intervals (95% CI) are presented for these variables. For maternal characteristics and anthropometric measures, the variables maternal age (years) and maternal BMI (kg/m^2) were normally distributed ($p>0.05$). Therefore, means (95% CI) are presented for these variables.

For infant feeding dynamics and infant feeding timing, the variables volume of infant feeding (mL), duration of infant feeding (min), total duration of infant feeding (min), rate of infant feeding (mL/min), time since last feeding (min), and time elapsed from the end of the test feeding to the post-feeding blood draw (min) were normally distributed ($p>0.05$). Therefore means (95% CI) are presented for these variables. The variable time elapsed from pre-feeding blood draw to start of test feeding was not normally distributed ($p<0.05$). Therefore, median (IQR) is presented for this variable.

For the satiation and adiposity gut peptides, distributions for the pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP, and PYY were examined by visit. The

pre-feeding concentration of each gut peptide by visit number was normally distributed ($p>0.05$) except ghrelin concentration at visit one and GIP concentration at visit two ($p<0.05$). Distributions of the change in concentration, pre-feeding subtracted from post-feeding, for each peptide were also examined. The variables change in leptin concentration, change in GLP-1 concentration, change in GIP concentration, and change in PYY concentration were normally distributed ($p>0.05$). The variable change in ghrelin was not normally distributed ($p<0.05$). Finally, the relative difference, between CMF and EHF, was calculated for ghrelin, leptin, GLP-1, GIP, and PYY. The distributions of the relative differences for ghrelin, leptin, GLP-1, GIP, and PYY were normally distributed ($p>0.05$).

5.2 Completion of Study Visit Testing

Eleven healthy, formula fed infants were enrolled in the study. All 11 infants participated in both visit one and visit two. Of the 11 infants, one infant regurgitated one of the formula meals, discontinuing the feeding component of the testing session. A second infant refused to consume the EHF formula therefore the post-feeding blood sample was not collected for one of the two visits. For both of the above infants, the pre-feeding blood samples were not included in the analysis as there was no corresponding post-feeding sample.

5.3 Infant and Maternal Demographic and Anthropometric Characteristics

Infant demographic and anthropometric characteristics are summarized in **Table 2A**. Eleven healthy, formula fed infants (n= 5 males and n=6 females) participated in the study. Six infants were white/Caucasian and five infants were black/African American. Mean infant age at visit one was 86 days (95% CI 70.7-

101.4 days). Mean infant weight at visit one was 5.8kg (95% CI 5.3-6.4 kg), and mean infant length at visit one was 58.5cm (95%CI 56.4-60.5). Comparatively, at visit two the mean infant age was 91.1 days (95% CI 75.0-107.2) and mean infant weight was 6.0kg (95% CI 5.5-6.6). Finally, weight for age, length for age, and weight for length growth z-scores were generated using the WHO Multicenter Growth Reference Standard.[46] The mean weight for age z-score was -0.1 (95% CI -0.8-0.6), the mean length for age z-score was -0.7 (95% CI -1.6-0.1), and the mean weight for length z-score was 0.7 (95% CI 0.0-1.5). Weight for length z-scores for all 11 infants fell within ± 2 standard deviations.

Maternal demographic and anthropometric characteristics are summarized in **Table A.2b**. Mothers were, on average, 27.6 years old (95% CI 23.2-32.0). The mean maternal body mass index (BMI) was 32.3kg/m^2 (95% CI 29.8-35.1) with 27.2% (n=3) of mothers having a BMI in the overweight category, ≥25.0 kg/m^2 but <30.0 kg/m^2, and 72.7% (n=8) in the obese category, ≥30.0 kg/m^2. No mothers had a BMI in the normal weight category. Six mothers self-identified their race/ethnicity as white/Caucasian and five identified as black/African American. The majority of mothers were co-habitating (45.4%), 36.3% were married, and 18.1% were single. Additionally, 45.4% of mothers had a high school education or below, and 36.3% had a family total annual income under $10,000.

5.4 Infant Feeding Dynamics

Data on volume of feeding (mL), duration of feeding (min), total duration (duration of infant feeding + time elapsed from end of feeding to post feeding heel stick), rate of feeding (mL/min), and time elapsed since last feeding (min) was normally distributed ($p>0.05$ for all variables). Data on feeding dynamics by formula

type are shown in **Table A.3a**. The mean volume of intake was 147.2mL (95% CI 104.9-189.4) and 128.7mL (95% CI 83.0-174.4) for CMF and EHF feeds, respectively. This difference in volume was not statistically significantly different (paired samples t-test, p=0.1189). Upon examination at the individual level, of the ten infants who completed both feeds, eight consumed a lower volume (mL) of EHF compared to CMF (**Figure B.2a**). The mean duration of infant feeding during CMF feeds was 16.5 minutes (95% CI 10.2-22.7) compared to 21.1 minutes (95% CI 13.1-29.0) during EHF feeds, but this difference was not statistically significantly different (paired samples t-test, p=0.1063). The total duration from the start of the lab test feeding to the post-feeding heel stick was 29.7 minutes (95% CI 23.1-36.4) for CMF feeds and 29.4 minutes (95% CI 21.3-37.4) for EHF feeds. The difference in total duration was not statistically significant (paired samples t-test, p=0.1658). Finally, with respect to feeding rate, while not statistically significant (paired samples t-test, p=0.0959), there was a trend for a slower rate of feeding during EHF feeds, 7.2 mL/min (95% CI 3.8-10.7), compared to CMF feeds, 11.3 mL/min (95% CI 6.8-15.8). At the individual level, of the ten infants who complete both feeds, eight had a slower rate of feeding during the EHF feed compared to the CMF feed (**Figure B.2b**). To determine if the probability of eight of ten infants (0.8) having a lower volume of intake (mL) and a slower rate of feeding (mL/min) during EHF feeds was significantly different than chance (0.5), a Likelihood ratio test was used. The lower volume of intake and slower rate of feeding observed in eight of the ten infants does not appear to be due to chance (p=0.0496).

5.5 Time Elapsed Since Last Feeding

The mean time since last feeding for all visits was 177.5 minutes (95% CI 153.5-201.4). Time since last feeding was also explored by feeding type, EHF vs. CMF, to ensure there were no differences (**Table A.3a**). The mean time since last feeding was 163 minutes (95% CI 119.0-208.0) and 191.5 minutes (95% CI 167.2-215.8) for EHF and CMF feedings, respectively; this difference was not statistically significant (paired samples t-test, p=0.0999).

5.6 Timing of Pre- and Post-Feeding Heel Sticks

The median (IQR) time elapsed from the pre-feeding heel stick to the start of feeding, for all feeds, was 6.0 minutes (IQR 5.0-9.0). This variable was also explored by formula type. The median (IQR) time from the pre-feeding heel stick to the start of feeding was 5.0 minutes (IQR 5.0-7.0) for EHF feedings and 7.5 minutes (IQR 4.5-9.5) for CMF feedings. There was no significant difference in the time elapsed from the pre-feeding heel stick to the start of feeding by formula type (Wilcoxon Signed Rank test, p=0.6641) (**Table A.3a**).

The mean (95% CI) time elapsed from the end of the test feeding to the post-feeding blood draw was 9.2 minutes (95% CI 7.5-10.9). By formula type, the median (IQR) time elapsed from the end of the test feeding to the post-feeding blood draw for EHF feedings was 10.0 minutes (IQR 5.0-11.0) and was 10.0 minutes (IQR 6.0-13.0) for CMF feedings. There was no significant difference in the time elapsed from the end of the test feeding to the post-feeding blood draw by formula type (Wilcoxon Signed Rank test, p=0.1641) (**Table A.3a**).

5.7 Satiation and Adiposity Gut Peptides

5.7.1 Baseline (Pre-Feeding) Concentrations of Satiation and Adiposity Peptides by Visit

Concentrations of satiation and adiposity gut peptides were assessed before feeding at both study visits. Pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP, and PYY are summarized in **Table A.3b.** A Wilcoxon Signed Rank test (ghrelin and GIP) or paired samples t-test (leptin, GLP-1, and PYY), depending on the distribution, were conducted to determine if there were group-level differences in pre-feeding ghrelin, leptin, GLP-1, GIP and PYY concentrations at visit one versus visit two. There were no significant differences in pre-feeding concentrations of ghrelin (p=1.0000), leptin (p=0.8930), GLP-1 (p=0.0887), GIP (p=0.7422), and PYY (p=0.7140) at visit one and visit two.

Additionally, pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP and PYY at visit one and visit two were plotted for each subject (**Figure B.3 A-E**) to examine within subject variability in peptide concentrations. The coefficient of variation was calculated for each subject's pre-feeding peptide concentration at visit one and visit two. The median (IQR) coefficient of variation for the satiation and adiposity peptides were: ghrelin 30.4% (IQR 3.6%-92.5%), leptin 25.9% (IQR 7.2%-41.4%), GIP 21.3% (IQR 9.9%-68.8%), GLP-1 25.8% (IQR 6.9%-37.3%), and PYY 11.3% (IQR 6.9%-17.1%).

5.7.2 Baseline (Pre-Feeding) Concentrations of Satiation and Adiposity Peptides by Formula Type

In addition to exploring satiation and adiposity peptides by visit number, pre-feeding concentrations of satiation and adiposity peptides were also explored by formula type. Pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP, and PYY by

formula type can be seen in **Table A.3b**. A paired samples t-test (ghrelin and leptin) or Wilcoxon Signed Rank test (GLP-1, GIP, and PYY), depending on the distribution, were conducted to determine if there were group-level differences in pre-feeding ghrelin, leptin, GLP-1, GIP and PYY concentrations by formula type (EHF vs. CMF). There were no significant differences in pre-feeding concentrations of ghrelin (p=0.1941), leptin (p=0.7666), GLP-1 (p=0.9375), GIP (p=0.3828), and PYY (p=0.4609) by formula type (EHF vs. CMF). Additionally, pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP and PYY by formula type were plotted for each subject (**Figure B.4 A-E**) to examine within subject variability in peptide concentrations. The mean within subject coefficient of variation for pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP and PYY by formula type were 43%, 26%, 26%, 34%, and 13%, respectively, indicating ghrelin had the highest variability and PYY had the lowest.

5.7.3 Change in Concentration of Satiation and Adiposity Peptides Pre- to Post-Feeding

Change in concentration of satiation and adiposity gut peptides were first explored as the difference and then as the relative difference. To determine difference (change), the pre-feeding concentration of each gut peptide was subtracted from the post-feeding concentration of the peptide. The change in concentration of each gut peptide by formula type is shown in **Table A.3c**. Paired samples t-tests, or Wilcoxon Signed Rank test, depending on normality, were used to determine if there were significant differences in the change of each gut peptide by formula type. There were no significant differences in the change in concentration of ghrelin (Wilcoxon Signed Rank test, p=0.0781), leptin (paired samples t-test, p=0.2029), GLP-1 (paired samples t-test, p=0.2255), GIP (paired samples t-test, p=0.1722), or PYY (paired samples t-

test, p=0.1562) by formula type. Next, to determine if the order in which subjects received the test formulas had an effect on the change in concentration of satiation and adiposity peptides, a 2-factor ANOVA was used. Order was not a significant factor in the change in ghrelin (p=0.5934), leptin (p=0.9912), GLP-1 (p=0.3545), GIP (p=0.6830), or PYY (p=0.8328) by formula type.

The change in concentration of satiation and adiposity peptides between CMF feeds and EHF feeds was also explored using the relative difference. Relative difference was calculated by taking the difference in concentration (pre-feeding concentration subtracted from post-feeding concentration) for each formula, divided by the sum of the change for each formula, multiplied by 100 (to express it as a percent). The relative difference for the change in concentration of each gut peptide can be seen in **Table A.3c.** The peptide GIP had a relative difference score of 93.8% indicating a large difference in the peptide response by formula type, whereas leptin has a relative difference of 25.8% indicating a smaller difference in the peptide response by formula type.

5.7.4 Effect of Baseline on Change

After examining pre-feeding concentrations and the change in concentration of satiation and adiposity peptides, the associations between pre-feeding concentration and the change in concentration of each peptide were explored by formula type. Depending on normality, Pearson or Spearman correlations were used to determine associations by formula type. For CMF feeds, there were no significant associations between pre-feeding concentration and change in concentration for ghrelin (Spearman correlation, p=0.0844), leptin (Pearson correlation, p=0.7504), GLP-1 (Pearson correlation, p=0.5949), GIP (Spearman correlation, p=0.2763), or PYY (Pearson

correlation, p=0.1588). For EHF feeds, there were no significant associations between pre-feeding concentration and change in concentration for ghrelin (Spearman correlation, p=0.4984), leptin (Pearson correlation, p=0.3088), GLP-1 (Pearson correlation, p=0.6679), GIP (Spearman correlation, p=0.2646), or PYY (Pearson correlation, p=0.9560).

5.7.5 Effect of Weight for Length Z-Scores on Change in Satiation and Adiposity Peptide Response

All infants had a weight for length z-score (WLZ) within +/-2 standard deviations of the mean. Therefore, to explore the associations between change in satiation and adiposity peptides and weight for length, WLZ was stratified by infants with a WLZ ≥1.0 (n=6) and <1.0 (n=5). Next, t-tests were used to determine if there was a difference in change in satiation and adiposity peptide response by WLZ. There were no significant differences in the change in satiation and adiposity peptides by WLZ (≥1.0 or <1.0) for ghrelin (p=0.7313), leptin (p=0.1768), GLP-1 (p=0.6956), GIP (p=0.8815), or PYY (p=0.1347).

5.7.6 Specific Aim 1

First, to assess the pre- and post-prandial concentrations of anorexigenic (GLP-1, GIP, PYY) and orexigenic (ghrelin) gastrointestinal peptides in response to feedings that differ in protein composition (EHF vs. CMF) in 1-4 months old infants, the minimum, maximum, and median (IQR) concentration of each gut peptide pre- and post-feeding by formula type (EHF vs. CMF) is presented in **Table A.3b.** To assess differences in the change in concentration of each peptide by formula type, the change in concentration was calculated for each peptide by formula type. Next, paired sample t-tests or Wilcoxon Signed Rank tests, depending on normality, were used to

determine if the mean change differed by formula type. While there were no statistically significant differences ($p>0.05$ for all peptides), there was a trend for a greater magnitude of change in GLP-1, GIP, and PYY following CMF feeds compared to EHF feeds.

5.7.7 Specific Aim 2

To explore relationships among dynamics of feeding (volume, duration, and rate) with change in anorexigenic peptide concentrations in response to feeding formulas that differ in protein composition (EHF vs. CMF), the associations between feeding dynamics and the change in concentration of anorexigenic satiation peptides were explored by formula type (EHF vs. CMF). Descriptive statistics, including minimum, maximum, and mean (95% CI), for volume of intake, duration of feeding, total duration, infant feeding rate, and time since last feeding by formula type were outlined above and data can be found in **Table A.3a**. Further, individual level differences in volume of intake and rate of feeding can be seen in **Figure B.2 A-B**.

To explore relationships between feeding dynamics and the change in concentration of anorexigenic satiation peptides (GLP-1, GIP, and PYY) by formula type, Pearson or Spearman correlations were used, depending on normality (**Tables A.4a, A.4b**). There were no significant associations between infant volume of intake and the change in concentration for any of the anorexigenic peptides for either EHF or CMF feeds ($p>0.05$). For duration of infant feeding, there was a significant association between duration of infant feeding and change in PYY concentration for CMF feeds ($r= -0.82$, $p=0.0276$), but not EHF feeds ($p=0.0958$) (**Table A.4b**). Additionally, there was a significant association between duration of infant feeding and change in leptin concentration for EHF feeds ($r= -0.66$, $p=0.0298$) but not CMF

feeds (p=0.2751) (**Table A.4a**). For total duration (duration of infant feeding + time elapsed to the post-feeding heel stick), there was a significant inverse association between total duration and change in PYY concentration for both CMF (r= -0.71, p=0.0443) and EHF (r= -0.75, p=0.0192) feeds (**Table A.4 A-B**). For rate of feeding, there was a significant association between rate of feeding and change in PYY concentration for CMF feedings (r=0.85, p=0.0079), but this association was not seen for EHF feedings (p=0.5807) (**Table A.4 A-B**). Finally, there was a significant association between time since last feeding and change in concentration of leptin (r=0.54, p=0.0417), GIP (r=0.72, p=0.0126), and PYY (r=0.46, p=0.0271) during CMF feeds only.

To explore differences between infant feeding dynamic variables and relative difference in satiation and adiposity peptide concentrations by formula type, Pearson and Spearman correlations were used, depending on normality. For CMF feeds there were no significant associations. For EHF feeds, the relative difference in GLP-1 concentration was significantly and inversely associated with total duration (Pearson correlation, r= -0.78, p=0.0203), and the relative difference in GLP-1 concentration was significantly associated with rate of feeding (Pearson correlation, r=0.81, p=0.0139).

5.8 Power Analysis

After the mean differences in infant feeding dynamic variables (volume, duration, and rate) and satiation and adiposity peptide variables were determined, power calculations were completed to determine if the current sample size (n=11) was sufficient to detect differences between groups in these variables. The mean difference for each variable and the standard deviation of the mean difference for each

variable were used to calculate effect size (dz) of the variables (**Table A.3a, Table A.3b, Table A.3c**). Using the calculated effect size, power set to 0.90 and type one error (α) for the null hypothesis set to 0.05, the required samples size was calculated for each variable. To detect differences in the volume of feeding (mL), duration of feeding (min), and rate of feeding (mL/min) for EHF vs. CMF feeds, the sample sizes required are 31, 29, and 27 infants, respectively. To detect differences in the change in concentration of ghrelin, leptin, GLP-1, GIP, and PYY under the same power and error assumptions, the required sample sizes are 25, 38, 50, 39, and 36 infants, respectively.

Chapter 6

DISCUSSION

This study is the first of its kind exploring satiation and adiposity gut peptide response to diets of different composition in healthy, term formula fed infants. Although this is a study was underpowered for all outcomes, our preliminary analysis of the pilot study found the following. Consistent with studies of satiation and adiposity peptides from the literature, [34-38] we found that concentrations of anorexigenic peptides (GLP-1, GIP, and PYY) increase in response to feeding in healthy, formula fed infants. The change in concentration of anorexigenic peptides from pre- to post-feeding was further examined by formula type (EHF vs. CMF). There were no significant differences in the mean change in concentration of the anorexigenic peptides by formula type. However, power analysis reveals the present analysis was underpowered to detect such differences, and as such we caution that the subsequent discussion of satiation and adiposity peptides in response to diets of different composition is preliminary. Since these data are part of an on-going study and power calculations reveal peptide variables require 25-50 infants, these analyses will be repeated upon completion of 30 subjects.

Despite no statistically significant differences in the mean change in concentration of GLP-1, GIP, or PYY by formula type, there was a tendency for the magnitude of the mean change to be greater following CMF feedings. This was contrary to our hypothesis, however, Diepvens et al. also found that in adults, GLP-1 concentrations were highest following ingestion of a milk protein shake compared to

whey protein, pea protein hydrolysate, and a combination of whey protein and pea protein hydrolysate[38], suggesting intact milk protein may have a different effect on satiation peptides compared to other proteins. However, the Diepvens study was conducted in adults and post-feeding blood samples were collected at 30, 60, 90, and 120 minutes after shake consumption, with a significant difference in the GLP-1 concentration found at 60 minutes. As such it is difficult to draw further conclusions about the present findings with CMF feedings and the milk protein shake in the Diepvens et al. study.

Studies of ghrelin, the only orexigenic satiation peptide, have shown ghrelin concentrations decrease in response to feeding.[36-38] In the present study, at the group level, ghrelin concentrations appear to trend towards decreasing from pre- to post-feeding. However, when stratified by formula type (EHF vs. CMF), ghrelin concentrations appear to increase in response to EHF feedings and decrease in response to CMF feedings. The increase in ghrelin concentration in response to EHF feedings is contrary to findings in previous studies of ghrelin concentrations in response to feedings.[38] However, previous studies of ghrelin in response to feeding were conducted in children or adults, not infants. Further, it is possible an analytic error occurred with the ghrelin assay. We therefore plan to repeat the ghrelin analysis in these subjects to confirm or refute the finding.

Feeding dynamics (volume of infant feeding, duration of infant feeding, and rate of infant feeding) were also explored in this study. We chose a model system of investigation that has repeatedly shown in both within and between subject studies that there are differences in intake and feeding dynamics based on the formula in the bottle. Infants feed less to satiation[11-13] and signal satiation[12,47] earlier when feeding

EHF compared to feeding CMF. While not significant, consistent with these findings was a tendency for infants to consume a lower volume of infant feeding (mL) and at a slower feeding rate (mL/min) during EHF feedings compared to CMF feedings. In this analysis, eight of the ten infants who completed both EHF and CMF feeds consumed a lower volume and had a slower feeding rate during the EHF feed. This probability was significantly different than chance (50/50), further supporting the trends for differences in feeding dynamic variables at the group level seen in this analysis. With 11 infants in the present sample, this analysis was underpowered to detect differences in feeding dynamics, and once approximately 30 infants have completed this study, this analysis will be repeated.

Literature examining the association between eating rate and anorexigenic peptide response suggests a slower eating rate results in a more pronounced anorexigenic peptide response.[39-41] In this study, the association between rate of feeding and change in GLP-1, GIP, and PYY was examined by formula type, EHF vs. CMF. The rate of feeding and change in PYY concentration following a CMF feed was significantly correlated. This association was positive, suggesting in this study the faster the rate of CMF feeding, the greater the change in PYY concentration. This finding is unlike studies of eating rate in the literature.[40,41] However, the eating rate studies presented in the review of literature were conducted in adults and adolescents, who, unlike infants, are able to eat a prescribed rate. To better understand the influence of infant feeding rate on intake and peptide response, time spent actively feeding (not including rests between sucking) will also be determined.

Ventura et al. demonstrated infants consumed a significantly lower volume to satiation following an EHF feed than a CMF feed.[12] Given infants consume a lower

volume to satiation when fed EHF, the present study was undertaken with the hypothesis that EHF feeding led to a larger rise in one of the satiation peptides leading to earlier meal termination compared to CMF feeding. Therefore, in this study, blood was drawn immediately after feeding to determine if ghrelin, GLP-1, GIP, PYY, or a combination of peptides were driving early satiation following EHF feedings. However, in this small sample size we found there were no significant differences in the change of any of the satiation gut peptides by formula type. One possible explanation for this is that perhaps none of the satiation or adiposity peptides individually are responsible for the lower volume of intake observed following EHF feedings, and with a larger sample size we will be able to model the effect of all the satiation peptides combined. A second possible explanation is that the timing of the post feeding blood draw in this study was either too soon or too late. Padidela et al. and Lomineck et al. both drew pre-feeding blood samples at time zero, subjects took 20 and 30 minutes, respectively to complete the meal and blood samples were drawn. By contrast, Divipens et al. took a fasting blood sample, subjects had fiver minutes to consume the meal/shake, and the next blood sample was drawn 30 minutes later. In the present study, the time elapsed from the start of the feeding until the post feeding blood draw averaged 27 minutes (18 minutes of feeding and 9 minutes to the blood draw). Future work may consider providing infants a short fixed time to consume the meal and an earlier post-feeding blood collection.

A strength of the present study is the counterbalanced, cross-over design, enabling both within and between subject comparisons. The biggest limitation of this study is its small sample size. The present study is on going and all analyses will be repeated when approximately 30 infants have completed the study. Additionally, with

a greater sample size, more complex models of the change in all satiation and adiposity peptides in response to a feeding can be explored. Finally, future studies could provide either a fixed volume of intake or a fixed duration of feeding time, and a fixed time elapsed until the post-feeding blood draw to better assess satiation and adiposity gut peptide responses. Further, additional gut peptides or hormones such as cholecystokinin (CCK) or pro-uroguanylin could be added to the study as potential markers of satiation. CCK is an anorexigenic peptide secreted in the small intestine in response to meals containing fat and protein.[29,31] CCK may perhaps peak earlier or have a greater effect on satiation. Similarly, pro-uroguanylin, the pro-hormone form of uroguanylin, is a recently discovered anorexigenic peptide that influences satiation; serum concentrations of pro-uroguanylin rise quickly after consuming a meal, and fall after meals.[51,52] These additional peptides may provide improved insight into the satiation response to diets of different protein composition in healthy, term, formula fed infants.

Chapter 7

CONCLUSION

Infancy is a sensitive period in development during which feeding practices and weight gain trajectories can have a lasting effect on child- and adulthood weight status. Infants with rapid weight gain in early life are at a greater risk for overweight and obesity later in life, and diet composition in infancy plays a role in infant weight gain. Breastfed infants, as a group, have been shown to gain less weight in the first year of life and are at lower risk for later life overweight and obesity compared to formula fed infants. However recent studies have demonstrated formula fed infants cannot be considered a homogenous group, since different types of infant formulas result in differential weight gain trajectories in the first year of life. Infants fed cow milk infant formula (CMF), the most commonly consumed infant formula, gain weight at an accelerated rate compared to breastfed infants, whereas infants fed an extensive protein hydrolysate formula (EHF) gain weight at a rate more similar to breastfed infants. Further, it has been demonstrated in cross-over studies, that infants satiate with lower volumes of EHF compared to CMF, suggesting lower overall energy intake in EHF fed infants may lead to the more normalized weight gain.

The mechanism by which EHF leads to earlier satiation and hence lower intake volume during infant feedings is unknown. It is hypothesized that the small peptides and free amino acids found in EHF may result in a differential effect on the peripheral peptides that stimulate satiation in the hypothalamus. In the present study we sought to determine if concentrations of satiation peptides in healthy, term, formula fed

infants change from pre-feeding to post-feeding in response to formula feedings of differing protein composition. Since there is little data on satiation and adiposity peptides in healthy term infants, data from this pilot study of 11 infants was used to calculate the true sample size needed to detect differences by formula type. Power calculations revealed the present analysis was underpowered to detect such differences, and as such the results herein are preliminary. There were no significant differences in the change in concentration of any of the satiation and adiposity peptides by formula type (EHF vs. CMF). There were trends towards a lower volume of intake, a longer duration of feeding, and an overall slower rate of feeding during EHF feeds compared to CMF feeds, but these trends did not reach significance perhaps due to the small sample size. Duration of infant feeding and rate of infant feeding for CMF feeds but not EHF feeds were associated with the change in PYY concentration. Further analysis with a larger sample size is needed to confirm feeding dynamic trends, and future studies should consider providing either a fixed volume of feeding or a fixed duration of feeding to better assess satiation and adiposity peptide responses in healthy, term, formula fed infants.

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Appendix A

TABLES

Table A.1 – Nutritional composition of the test formulas, Enfamil (CMF) and Nutramigen (EHF)[27,43,53]

Nutrient	CMF Enfamil	EHF Nutramigen
Calories (kcal/100mL)	67.6	67.6
Protein (g/100mL)	1.3	1.8
Free Amino Acids (µmol/100mL)	86.4	8037.5
Free Glutamate (µmol/100mL)	12.5	723.8
Carbohydrate (g/100mL)	7.6	6.9
Fat (g/100mL)	3.5	3.5
DHA (mg/100mL)	11.4	11.4
ARA (mg/100mL)	22.7	22.7
Sources of Carbohydrate	Lactose, polydextrose, galactooligosaccharides	Corn syrup solids, modified corn starch
Sources of Protein	Nonfat milk, whey protein concentrate	Casein hydrolysate
Sources of Fat	Palm olein oil, coconut oil, soy oil, and high oleic sunflower oil	Palm olein oil, coconut oil, soy oil, and high oleic sunflower oil

Table A.2a – Infant demographic and anthropometric characteristics

Infant Characteristics	**(N) or Mean**	**% or (95% CI)**
Gender		
Male	(5)	45.4%
Female	(6)	54.5%
Race		
White/Caucasian	(6)	54.5%
Black/African American	(5)	45.4%
Visit 1		
Age (days)	86.0	(70.7-101.4)
Weight (kg)	5.8	(5.3-6.4)
Weight for age z-score	-0.1	(-0.8-0.6)
Length (cm)	58.5	(56.4-60.5)
Length for age z-score	-0.7	(-1.6-0.1)
Weight for length z-score	0.7	(0.0-1.5)
Visit 2		
Age (days)	91.1	(75.0-107.2)
Weight (kg)	6.0	(5.5-6.6)

Table A.2b – Maternal demographic and anthropometric characteristics

Maternal Characteristics	**(N) or Mean**	**% or (95% CI)**
Age (years)	27.6	(23.2-32.0)
BMI (kg/m^2)	32.3	(29.8-35.1)
Normal	(0)	0.0%
Overweight	(3)	27.2%
Obese	(8)	72.7%
Race/Ethnicity		
White/Caucasian	(6)	54.5%
Black/African American	(5)	45.4%
Marital Status		
Single	(2)	18.1%
Married	(4)	36.3%
Co-habitating	(5)	45.4%
Education Level		
High school or below	(5)	45.4%
Some college	(2)	18.1%
Trade school	(1)	9.0%
College	(2)	18.1%
Graduate education	(1)	9.0%
Family Income		
Under $10,000	(4)	36.3%
$10,000-$14,999	(0)	0.0%
$15,000-$24,999	(0)	0.0%
$25,000-$34,999	(1)	9.0%
$35,000-$49,999	(2)	18.1%
$50,000-$74,999	(2)	18.1%
$75,000-$99,999	(1)	9.0%
$100,000 or more	(1)	9.0%

Table A.3a – Feeding dynamics by formula type (EHF vs. CMF) and difference and relative differences

Feeding Dynamics	CMF Feeding	EHF Feeding	Difference (CMF-EHF)	Relative Difference (CMF-EHF/ CMF+EHF)	Effect Size
Volume of Feeding (mL)					
Mean (95% CI)	147.2 (104.9-189.4)	128.7 (83.0-174.4)	18.5	6.7%	0.54
Minimum	88.3	19.4	68.9		
Maximum	281.5	233.9	47.6		
Volume of Feeding per Kilogram (mL/kg)					
Mean (95% CI)	24.2 (18.5-30.0)	21.4 (14.6-28.2)	2.8	6.1%	0.52
Minimum	16.5	3.2	13.3		
Maximum	40.9	34.2	6.7		
Duration of Feeding (min)					
Mean (95% CI)	16.5 (10.2-22.7)	21.1 (13.1-29.0)	-4.6	-12.2%	0.49
Minimum	5.0	10.0	-5.0		
Maximum	34.0	44.0	-10.0		
Rate of Feeding (mL/min)					
Mean (95% CI)	11.3 (6.8-15.8)	7.2 (3.8-10.7)	4.1	22.1%	0.58
Minimum	4.1	1.2	2.9		
Maximum	25.8	16.8	9.0		
Total Duration (min)					
Mean (95% CI)	29.7 (23.1-36.4)	29.4 (21.3-37.4)	0.3	0.5%	0.50
Minimum	16.0	16.0	0.0		
Maximum	45.0	49.0	-4.0		
Time Since Last Feeding (min)					
Mean (95% CI)	191.5 (167.2-215.8)	163.5 (119.0-208.0)	28.0	7.8%	0.54
Minimum	148.0	60.0	88.0		
Maximum	270.0	250.0	20.0		
Time from Blood Sample Collection to Start of Feed (min)					
Mean (95% CI)	7.1 (4.7-9.4)	*5.0 (5.0-7.0)	2.1	17.3%	0.03
Minimum	2.0	1.0	1.0		
Maximum	12.0	20.0	-8.0		

Time from End of Feed to Blood Sample Collection (min)					
Mean (95% CI)	9.5 (6.7-12.3)	*10.0 (5.0-11.0)	-0.5	-2.5%	0.47
Minimum	4.0	5.0	-1.0		
Maximum	17.0	13.0	4.0		

There were no significant differences between EHF and CMF feedings for any of the feeding dynamic variables listed in the table (paired samples t-test or Wilcoxon Signed Rank test $p>0.05$).
* Denotes median (IQR) instead of mean (95% CI)

Table A.3b – Pre-feeding and post-feeding concentrations of satiation and adiposity peptides by formula type (EHF vs. CMF) and difference and relative differences

	CMF Feeds	EHF Feeds	Difference (CMF-EHF)	Relative Difference (CMF-EHF/ CMF+EHF)	Effect Size
Ghrelin (pg/mL)					
Pre-Feeding					
Median (IQR)	45.2 (11.9 - 78.9)	20.2 (13.2 - 49.0)	25	38.2%	0.50
Minimum	3.2	5.6	-2.4		
Maximum	1176.8	73.7	1103.1		
Post-Feeding					
Median (IQR)	25.8 (13.6 - 65.7)	31.2 (13.7 - 49.5)	-5.4	-9.5%	0.16
Minimum	5.6	8.7	-3.1		
Maximum	93.7	92.3	1.4		
Leptin (pg/mL)					
Pre-Feeding					
Median (IQR)	3915.3 (2449.5 - 4631.4)	3295.4 (2109.5 - 5295.5)	619.9	8.6%	0.11
Minimum	1114.1	851.8	262.3		
Maximum	6689.6	7080.4	-390.8		
Post-Feeding					
Median (IQR)	3385 (1762.9 - 4050.2)	2851 (1955.3 - 4118.3)	534	8.6%	0.16
Minimum	1309.3	993.3	316		
Maximum	7719.7	6962.3	757.4		
GLP-1 (pg/mL)					
Pre-Feeding					
Median (IQR)	56.1 (19.4 - 103.5)	58.8 (28.8 - 124.9)	-2.7	-2.3%	0.07
Minimum	15.5	23.8	-8.3		
Maximum	241.2	158.3	82.9		
Post-Feeding					
Median (IQR)	203.5 (54.8 - 246.8)	118.8 (62.6 - 173.9)	84.7	26.3%	0.76
Minimum	39.7	39.6	0.1		
Maximum	444.6	243.1	201.5		

GIP (pg/mL)					
Pre-Feeding					
Median (IQR)	414.9 (233.4 - 742.3)	334.1 (244.1 - 922.0)	80.8	10.8%	0.25
Minimum	201.3	144.8	56.5		
Maximum	1176.8	1743.5	-566.7		
Post-Feeding					
Median (IQR)	799 (520.2 - 1000.0)	551.1 (399.0 - 1105.7)	247.9	18.4%	0.47
Minimum	473	273.2	199.8		
Maximum	1509.4	1497	12.4		
PYY (pg/mL)					
Pre-Feeding					
Median (IQR)	190.2 (93.6 - 489.2)	166.9 (114.6 - 483.8)	23.3	6.5%	0.34
Minimum	86	68.6	17.4		
Maximum	690.4	543.3	147.1		
Post-Feeding					
Median (IQR)	404.6 (130.7 - 764.3)	240.3 (165.0 - 558.5)	164.3	25.5%	0.79
Minimum	86.86	119.4	-32.54		
Maximum	852.5	609.6	242.9		

Table A.3c – Change in concentration of satiation and adiposity peptides by formula type (EHF vs. CMF) and difference and relative differences

Gut Peptide	Difference		Relative Difference	Effect Size
	EHF Feed (Post-pre)	**CMF Feed (Post-pre)**	**(CMF-EHF/ CMF+EHF)**	
	Mean (95%CI)	Mean (95%CI)	Mean (95%CI)	
Ghrelin (pg/mL)	5.3 (-7.7 - 18.3)	-9.4 (-21.8 - 2.9)	-53.2% (-164.0-57.6)	0.67
Leptin (pg/mL)	-265.1 (-772.3 - 242.1)	-128.1 (-581.8 - 325.5)	25.8% (-67.2-118.9)	0.54
GLP-1 (pg/mL)	51.5 (6.8 - 96.3)	72.7 (30.3 - 115.2)	32.2% (-11.8-76.4)	0.46
PYY (pg/mL)	77.1 (-1.2 - 155.6)	135.8 (61.4 - 210.2)	3.2% (-55.0-61.5)	0.56
GIP (pg/mL)	139.2 (-39.5 - 317.9)	332.6 (163.1 - 502.1)	93.8% (-108.2-295.9)	0.53

Table A.4a – Correlation between change (Δ) in satiation and adiposity peptides and infant feeding dynamics for EHF feedings

Peptide	Volume (mL)	Duration (min)	Total Duration (min)	Rate (mL/min)	Time Since Last Feeding (min)	Time End Feed to Blood Draw (min)
Δ Ghrelin (pg/mL)	-0.14	-0.47	-0.50	0.44	0.27	-0.54
Δ Leptin (pg/mL)	-0.42	***-0.66****	-0.70	0.16	0.21	0.29
Δ GLP-1 (pg/mL)	-0.06	-0.24	-0.19	0.24	0.44	-0.34
Δ GIP (pg/mL)	-0.50	-0.11	-0.06	-0.39	0.00	-0.21
Δ PYY (pg/mL)	-0.46	-0.54	***-0.75****	0.15	0.32	-0.65

Pearson correlation coefficient for Leptin, GLP-1, GIP, and PYY.
Spearman correlation coefficient for Ghrelin.
*Denotes significant association where $p<0.05$.

Table A.4b – Correlation between change (Δ) in satiation and adiposity peptides and infant feeding dynamics for CMF feedings

Peptide	Volume (mL)	Duration (min)	Total Duration (min)	Rate (mL/min)	Time Since Last Feeding (min)	Time End Feed to Blood Draw (min)
Δ Ghrelin (pg/mL)	-0.24	0.08	0.58	-0.40	0.07	0.25
Δ Leptin (pg/mL)	0.39	-0.42	-0.45	***0.81****	***0.54****	0.16
Δ GLP-1 (pg/mL)	-0.06	-0.52	-0.28	0.20	0.25	0.65
Δ GIP (pg/mL)	-0.15	0.11	-0.30	-0.06	***0.72****	-0.32
Δ PYY (pg/mL)	-0.06	***-0.82****	***-0.71****	***0.85****	***0.46****	0.66

Pearson correlation coefficient for Leptin, GLP-1, GIP, and PYY.
Spearman correlation coefficient for Ghrelin.
*Denotes significant association where $p<0.05$.

Appendix B

FIGURES

Study Procedures	Visit 1	Visit 2
Informed Consent	X	
Inclusion Criteria	X	
Exclusion Criteria	X	
Demographics	X	
General Interview Questionnaire	X	
Infant Feeding Questionnaire	X	
Anthropometrics - Infant	X	X
Pre-feeding blood sample	X	X
Post-feeding blood sample	X	X
Test weighing (infant and bottle)	X	X
Videotape feeding for satiation signals	X	X
Subject payment verification signed	X	X

Figure B.1 – Schedule of events by study visit

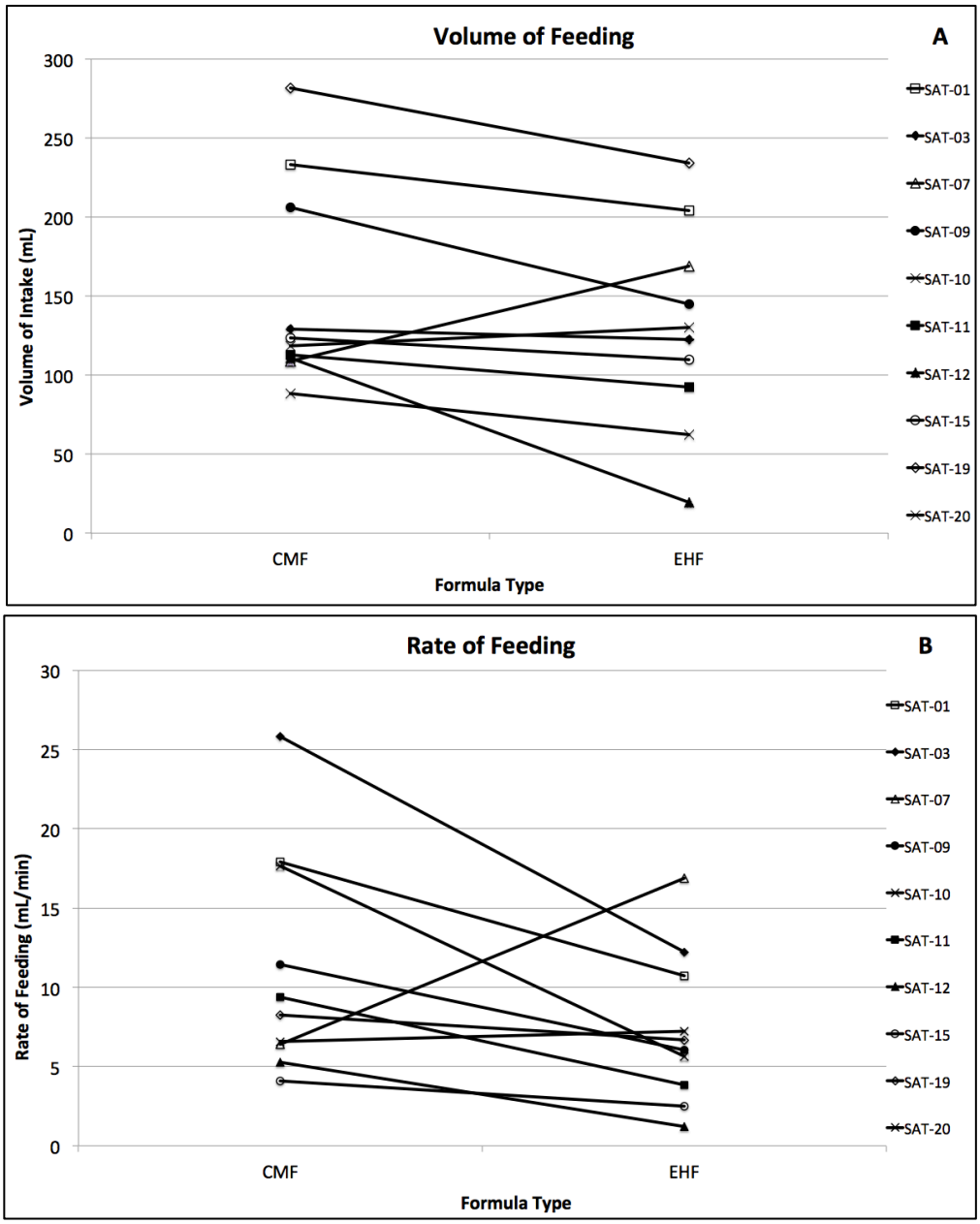


Figure B.2 (A-B) – Volume (mL) of intake and rate of feeding (mL/min) by formula type (EHF vs. CMF) at the subject level. At the individual level, of the 10 infants who completed both feeding types, 8 of 10 infants consumed a lower volume (mL) of EHF compared to CMF and 8 of 10 had a slower feeding rate (mL/min) when feeding EHF compared to CMF.

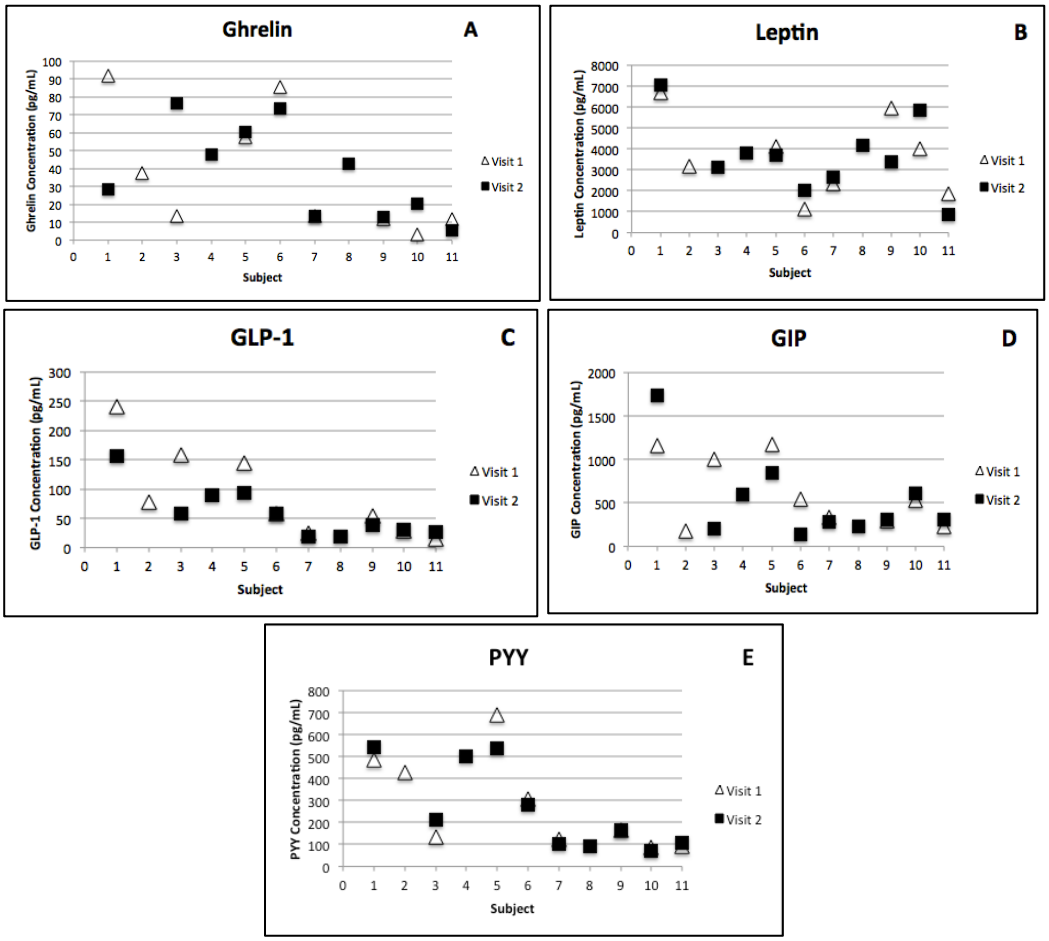


Figure B.3 (A-E) – Pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP, and PYY at visit 1 (Δ) and visit 2 (n) by subject. Mean pre-feeding concentration at the group level did not differ between visits 1 and 2 for ghrelin (Wilcoxon Signed Rank test, p=1.000), leptin (paired samples t-test, p=0.8930), GLP-1 (paired samples t-test, p=0.0887), GIP (Wilcoxon Signed Rank test, p=0.7422), or PYY (paired samples t-test, p=0.7140).

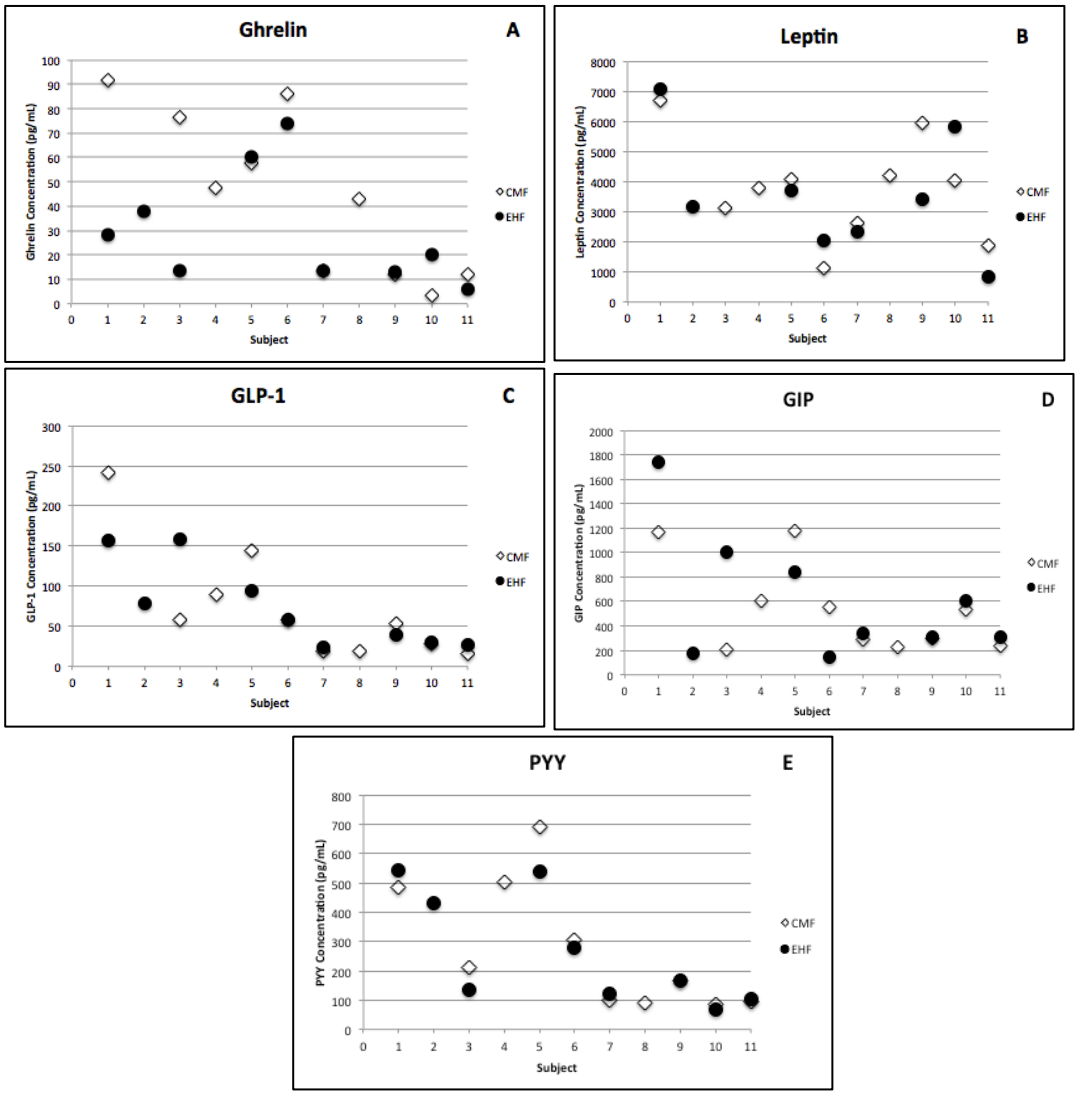


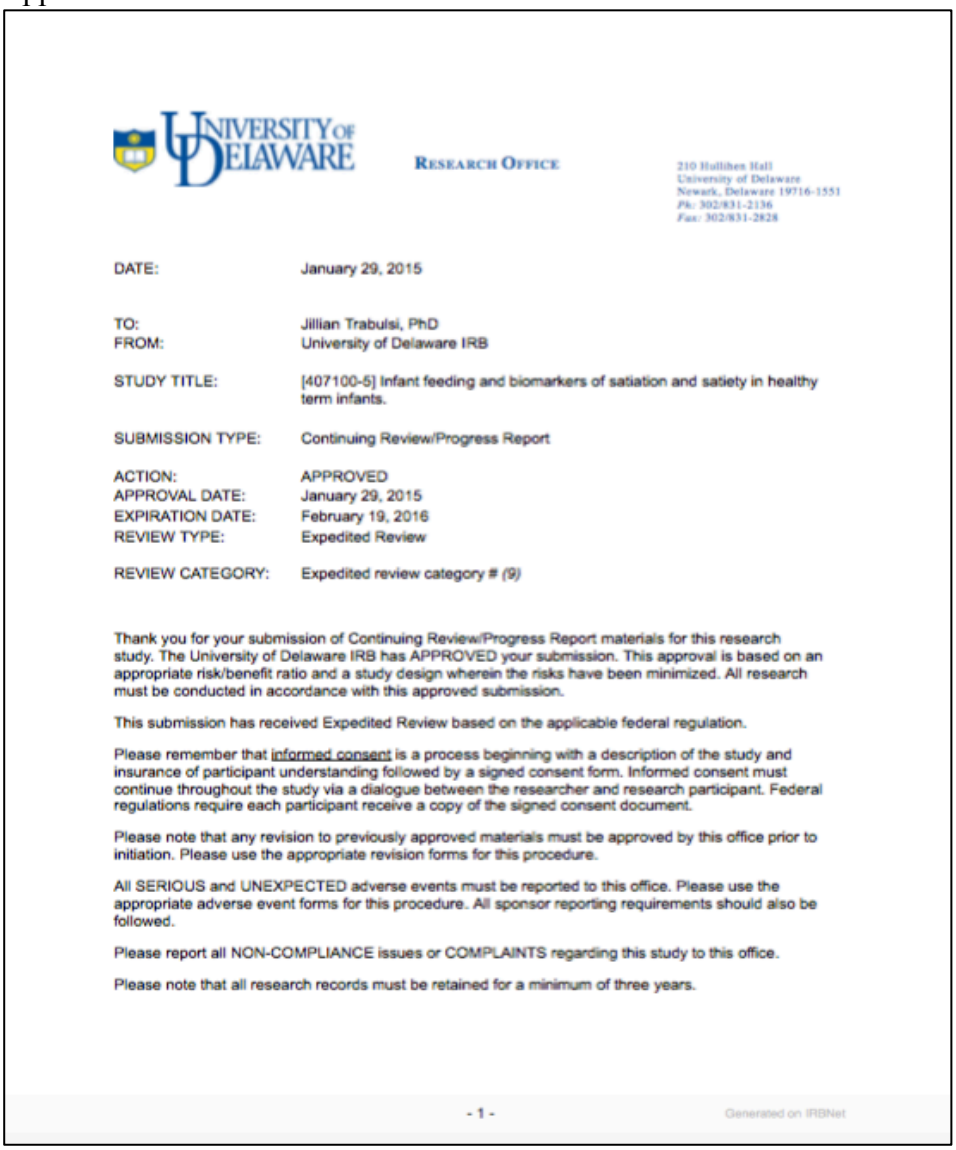
Figure B.4 (A-E) – Pre-feeding concentrations of ghrelin, leptin, GLP-1, GIP, and PYY by formula type (EHF vs. CMF). Mean pre-feeding concentrations are the group level did not differ by formula type (EHF vs. CMF) for ghrelin (paired samples t-test, p=0.1941), leptin (paired samples t-test, p=0.7666), GLP-1 (Wilcoxon Signed Rank test, p=0.9375), GIP (Wilcoxon Signed Rank test, p=0.3828), or PYY (Wilcoxon Signed Rank test, p=0.4609).

Appendix C

STUDY VISIT DOCUMENTS

C.1 Institutional Review Board Approval Letters

Approval Letter 2015



UNIVERSITY OF DELAWARE

RESEARCH OFFICE

210 Hullihen Hall
University of Delaware
Newark, Delaware 19716-1551
Ph: 302/831-2136
Fax: 302/831-2828

DATE: January 29, 2015

TO: Jillian Trabulsi, PhD
FROM: University of Delaware IRB

STUDY TITLE: [407100-5] Infant feeding and biomarkers of satiation and satiety in healthy term infants.

SUBMISSION TYPE: Continuing Review/Progress Report

ACTION: APPROVED
APPROVAL DATE: January 29, 2015
EXPIRATION DATE: February 19, 2016
REVIEW TYPE: Expedited Review

REVIEW CATEGORY: Expedited review category # (9)

Thank you for your submission of Continuing Review/Progress Report materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

Please note that any revision to previously approved materials must be approved by this office prior to initiation. Please use the appropriate revision forms for this procedure.

All SERIOUS and UNEXPECTED adverse events must be reported to this office. Please use the appropriate adverse event forms for this procedure. All sponsor reporting requirements should also be followed.

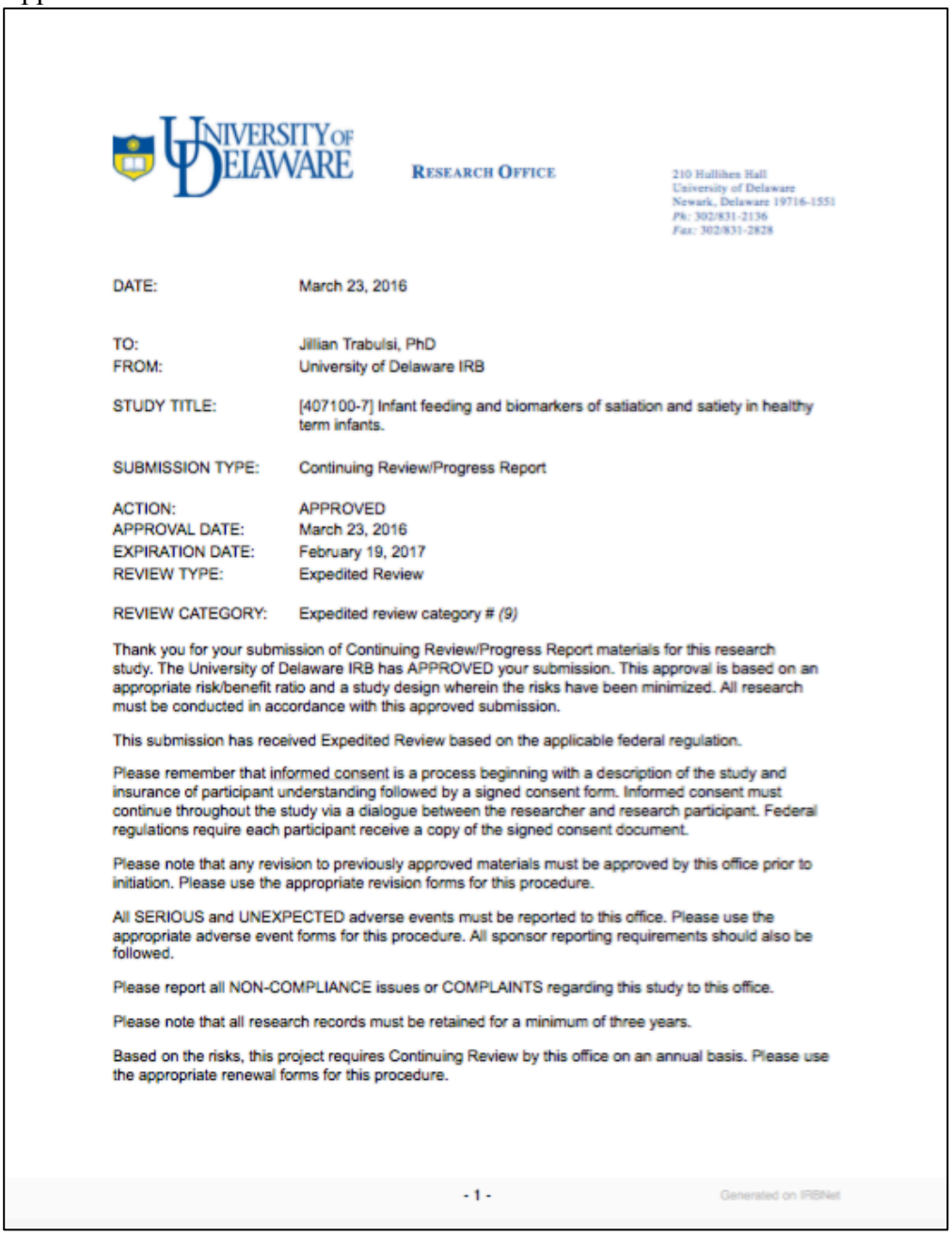
Please report all NON-COMPLIANCE issues or COMPLAINTS regarding this study to this office.

Please note that all research records must be retained for a minimum of three years.

Based on the risks, this project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Nicole Farnese-McFarlane at (302) 831-1119 or nicolefm@udel.edu. Please include your study title and reference number in all correspondence with this office.

Approval Letter 2016



UNIVERSITY OF DELAWARE

RESEARCH OFFICE

210 Hullihen Hall
University of Delaware
Newark, Delaware 19716-1551
Ph: 302/831-2136
Fax: 302/831-2828

DATE: March 23, 2016

TO: Jillian Trabulsi, PhD
FROM: University of Delaware IRB

STUDY TITLE: [407100-7] Infant feeding and biomarkers of satiation and satiety in healthy term infants.

SUBMISSION TYPE: Continuing Review/Progress Report

ACTION: APPROVED
APPROVAL DATE: March 23, 2016
EXPIRATION DATE: February 19, 2017
REVIEW TYPE: Expedited Review

REVIEW CATEGORY: Expedited review category # *(9)*

Thank you for your submission of Continuing Review/Progress Report materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

Please note that any revision to previously approved materials must be approved by this office prior to initiation. Please use the appropriate revision forms for this procedure.

All SERIOUS and UNEXPECTED adverse events must be reported to this office. Please use the appropriate adverse event forms for this procedure. All sponsor reporting requirements should also be followed.

Please report all NON-COMPLIANCE issues or COMPLAINTS regarding this study to this office.

Please note that all research records must be retained for a minimum of three years.

Based on the risks, this project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Nicole Farnese-McFarlane at (302) 831-1119 or nicolefm@udel.edu. Please include your study title and reference number in all correspondence with this office.

C.2 Informed Consent for Formula Feeding Mother-Infant Dyads

UD IRB Approval from 01/29/2015 to 02/19/2016

Principal Investigator: Jillian Trabulsi PhD, RD
Assistant Professor, Department of Behavioral Health and Nutrition
University of Delaware
25 North College Avenue
304 McDowell Hall
Newark, Delaware 19716
Telephone: 302-831-4991
Email: Trabulsi@udel.edu

Co-Investigator: Julie Mennella PhD/ Psychobiologist
Monell Chemical Senses Center
3500 Market Street
Philadelphia, PA 19104-3360
Telephone: 267-519-4880
E-mail: mennella@monell.org

Other study personnel: Caitlin McEwen / Graduate Student, Nutrition
University of Delaware
338 McDowell Hall
Newark, DE 19716
Phone: 302-831-2241
Mobile phone: 773-678-3555
E-mail: cgmcewen@udel.edu

Mother's Name:_____

Infant's Name: _____

1. PURPOSE / DESCRIPTION OF THE RESEARCH

The purpose of this study is to learn more about how much babies eat. We are also interested in learning more about what makes babies start and stop eating during a feeding. A total of 45 mother infant pairs will participate in this study.

2. WHAT YOU WILL DO

You will be asked to read this informed consent completely. You may ask as many questions as needed to make your decision. If you decide to allow your infant to participate, you will initial the bottom of each page, and sign the last page.

If you decide to allow your infant to participate in this study, you and your infant will have **two** study visits at the feeding lab at the University of Delaware. Each visit will take approximately 3 hours and each visit will be within 7 days of each other. At these visits, your baby's weight, length, and head circumference will be measured, we will collect a sample of their saliva and a sample of your baby's blood by a heel stick. You will feed your baby as you usually do using the commercially available formula provided by the study team. After the feeding, another saliva sample and blood sample will be collected. Infant feedings will be videotaped in the feeding laboratory, the video will focus on infant feeding (infant's face) and the types of fullness cues that

Mother's Initials: _____

infants exhibit. This video will be used for research purposes only. You will also be asked to complete a questionnaire about feeding your baby

3. CONDITIONS OF SUBJECT PARTICIPATION

Subjects must meet all of the following criteria to be enrolled in the study.
At birth:

1. Infants must be healthy, term (≥37 and ≤42 week gestation at birth), singleton, appropriate for gestational age infant.

At time of enrollment:

2. ≥30 days and ≤120 days old (Date of birth = day 0)
3. If formula fed, be primarily receiving a standard (intact protein) cow's milk infant formula and have no allergies to cow's milk formula.
4. If formula fed, must not have ever received an extensive protein hydrolysate formula (Nutramigen, Alimentum, Pregestimil or PurAmine).
5. If human milk fed, must be primarily receiving human milk (≤1 formula feeding/day)

Mother must be:

6. ≥18 years of age.

Presence of any of the following criteria will exclude the subject from enrollment in the study.
Infant must not:

1. Have major congenital malformations (i.e. cleft palate, extremity malformation) or genetic disorders.
2. Have suspected or documented systemic or congenital infections (e.g., human immunodeficiency virus, cytomegalovirus).
3. Have evidence of significant cardiac, respiratory, endocrinologic, hematologic, gastrointestinal, or other systemic diseases.
4. Be receiving any prescription medication.

All records and videotapes related to your infant's participation in this study will be stored in password protected electronic files and or a locked file cabinet that only research personnel have access to. Study data will be kept for seven years. Your infant's identity on these study records will be indicated by a number rather than by name. The information linking your infant's name and study number will be kept separate from these records. After seven years, this information will be destroyed. However de-identified data will be kept in electronic form.

The information gathered in this study will be kept confidential. The information will be aggregated for research purposes and no individuals will be identified.

Your infant's participation in this study is completely voluntary. Without loss or penalty of any kind you can: choose not to have your infant take part in this study, choose not to answer a question on the questionnaire, choose not to have a procedure performed on your infant, or withdraw from the study at any time.

4. RISKS AND BENEFITS

Mother's Initials: _____

There is some risk associated with this study. We are weighing and measuring your baby and taking saliva samples. When taking a sample of your baby's blood by a heel stick, a special cream will be placed on your infant's foot to numb it; however your infant may feel some discomfort. You can give your baby a pacifier (if you use one) before, during, or afterward.

There is no direct benefit you or your baby for participation in this study. You will be compensated $75 cash at each visit for your time and you will be reimbursed for parking expenses; if you complete both study visits the total compensation is $150 cash.

5. CONTACTS

Participants may contact Dr. Jillian Trabulsi, PhD, RD at 302-831-4991 or trabulsi@udel.edu with any questions or concerns.

Any concerns or complaints about the manner or conduct of the project should be directed to the Chairperson, Human Subjects Review Board, 109 Hullihen Hall, University of Delaware, Newark, DE, 19716 or 302-831-2137.

6. SUBJECT ASSURANCES

I have read the above informed consent document. The nature, demands, risks and benefits of the project have been explained to me. I knowingly assume the risks involved, and understand that I may withdraw my consent and stop my participation in this study at any time. By signing this form, I agree for my child and myself to take part in this research study and to allow the use of the described information for the purposes of research until the end of the study.

7. CONSENT SIGNATURES
7a. CONSENT TO PARTICIPATE IN THE STUDY

_____ Today's Date:_____
Infant's Name

_____
Mother's Name (Printed)

_____
Mother's Signature

7b. CONSENT TO VIDEOTAPING
Do we have permission to videotape your infant's feeding session in the laboratory? This decision will not impact your ability to participate in the study.

Mother's Initials: _____

Check box and sign below
☐ Yes ☐ No

_____ Today's Date:_____
Infant's Name

_____
Mother's Name (Printed)

_____
Mother's Signature

7c. CONSENT FOR VIDEO RELEASE
Do we have permission to use your infant's videotape in research presentations? This decision will not impact your ability to participate in this study.

Check box and sign below
☐ Yes ☐ No

_____ Today's Date:_____
Infant's Name

_____
Mother's Name (Printed)

_____
Mother's Signature

7d. CONSENT TO PHOTOGRAPH
Do we have permission to take a photograph of your infant for display in the Infant Feeding Room? This decision will not impact your ability to participate in this study.

Check box and sign below
☐ Yes ☐ No

_____ Today's Date:_____
Infant's Name

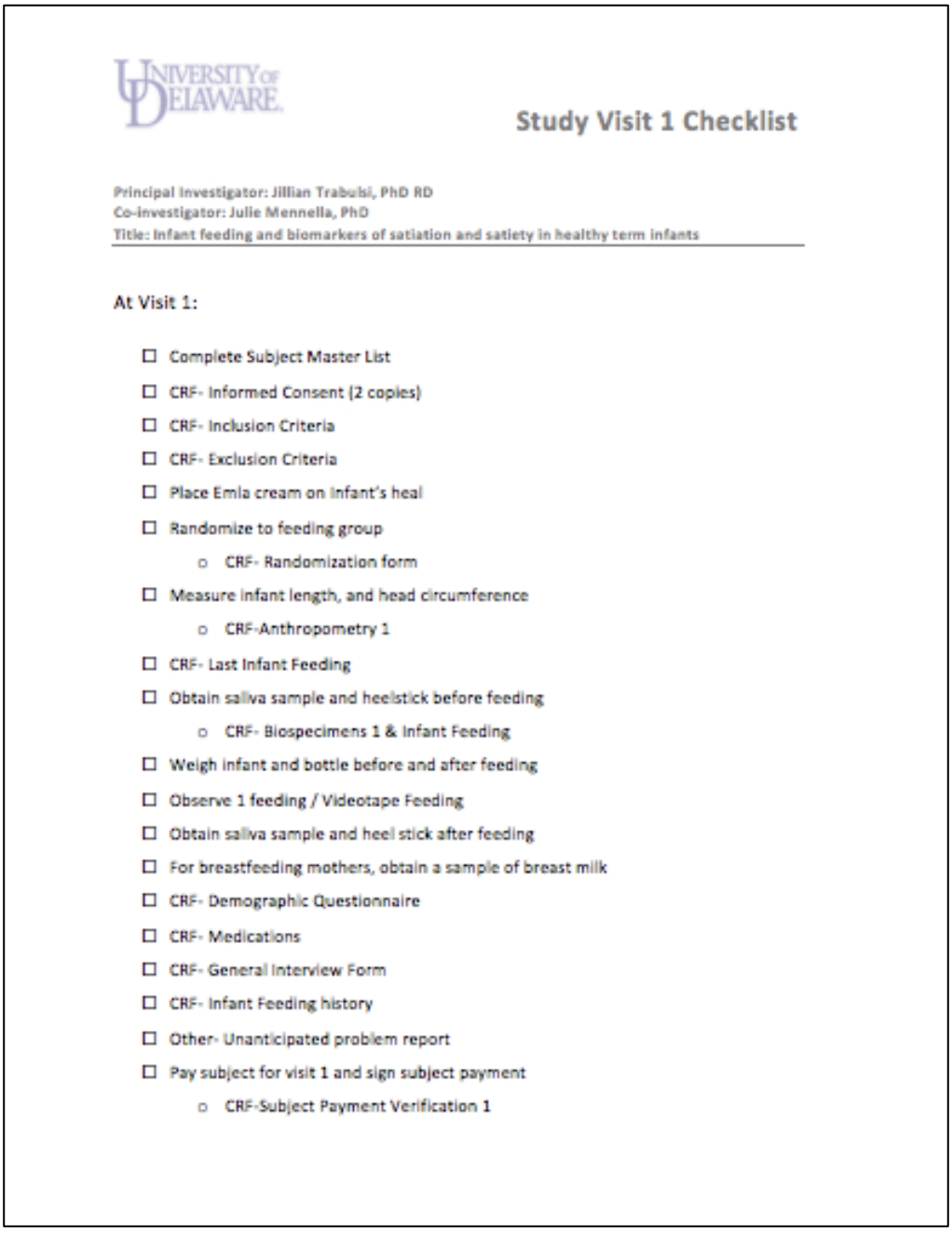
_____
Mother's Name (Printed)

_____
Mother's Signature

Mother's Initials: _____

C.3 Visit 1 Documents

Visit 1 Checklist:



UNIVERSITY OF DELAWARE

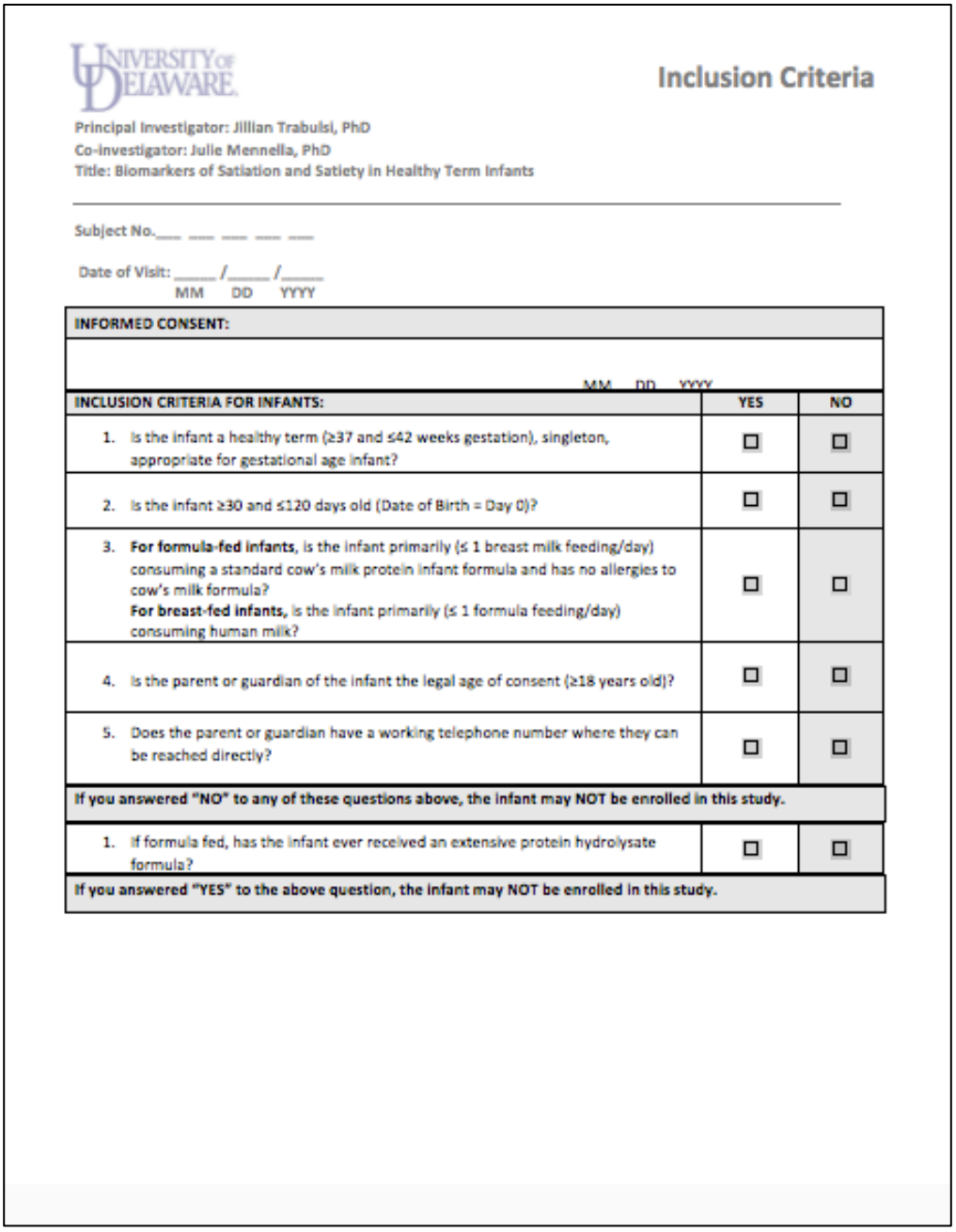
Study Visit 1 Checklist

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

At Visit 1:

- ☐ Complete Subject Master List
- ☐ CRF- Informed Consent (2 copies)
- ☐ CRF- Inclusion Criteria
- ☐ CRF- Exclusion Criteria
- ☐ Place Emla cream on infant's heal
- ☐ Randomize to feeding group
  - o CRF- Randomization form
- ☐ Measure infant length, and head circumference
  - o CRF-Anthropometry 1
- ☐ CRF- Last Infant Feeding
- ☐ Obtain saliva sample and heelstick before feeding
  - o CRF- Biospecimens 1 & Infant Feeding
- ☐ Weigh infant and bottle before and after feeding
- ☐ Observe 1 feeding / Videotape Feeding
- ☐ Obtain saliva sample and heel stick after feeding
- ☐ For breastfeeding mothers, obtain a sample of breast milk
- ☐ CRF- Demographic Questionnaire
- ☐ CRF- Medications
- ☐ CRF- General Interview Form
- ☐ CRF- Infant Feeding history
- ☐ Other- Unanticipated problem report
- ☐ Pay subject for visit 1 and sign subject payment
  - o CRF-Subject Payment Verification 1

Inclusion Criteria:



UNIVERSITY OF DELAWARE

Inclusion Criteria

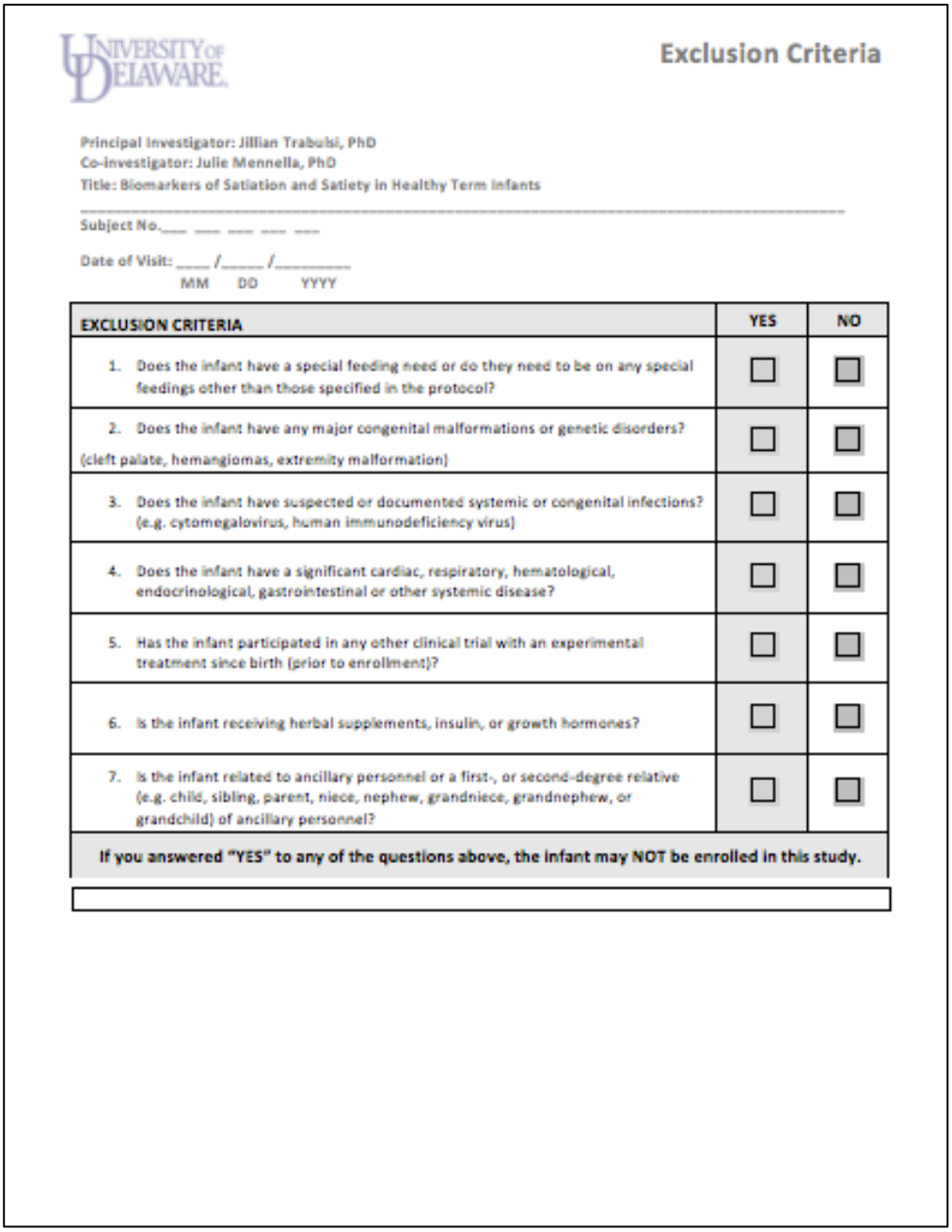
Principal Investigator: Jillian Trabulsi, PhD
Co-investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No.___ ___ ___ ___ ___

Date of Visit: _____ /_____ /_____
MM DD YYYY

INFORMED CONSENT:		
MM DD YYYY		
INCLUSION CRITERIA FOR INFANTS:	**YES**	**NO**
1. Is the infant a healthy term (≥37 and ≤42 weeks gestation), singleton, appropriate for gestational age infant?	☐	☐
2. Is the infant ≥30 and ≤120 days old (Date of Birth = Day 0)?	☐	☐
3. **For formula-fed infants**, is the infant primarily (≤ 1 breast milk feeding/day) consuming a standard cow's milk protein infant formula and has no allergies to cow's milk formula? **For breast-fed infants,** is the infant primarily (≤ 1 formula feeding/day) consuming human milk?	☐	☐
4. Is the parent or guardian of the infant the legal age of consent (≥18 years old)?	☐	☐
5. Does the parent or guardian have a working telephone number where they can be reached directly?	☐	☐
If you answered "NO" to any of these questions above, the infant may NOT be enrolled in this study.		
1. If formula fed, has the infant ever received an extensive protein hydrolysate formula?	☐	☐
If you answered "YES" to the above question, the infant may NOT be enrolled in this study.		

Exclusion Criteria:



Exclusion Criteria

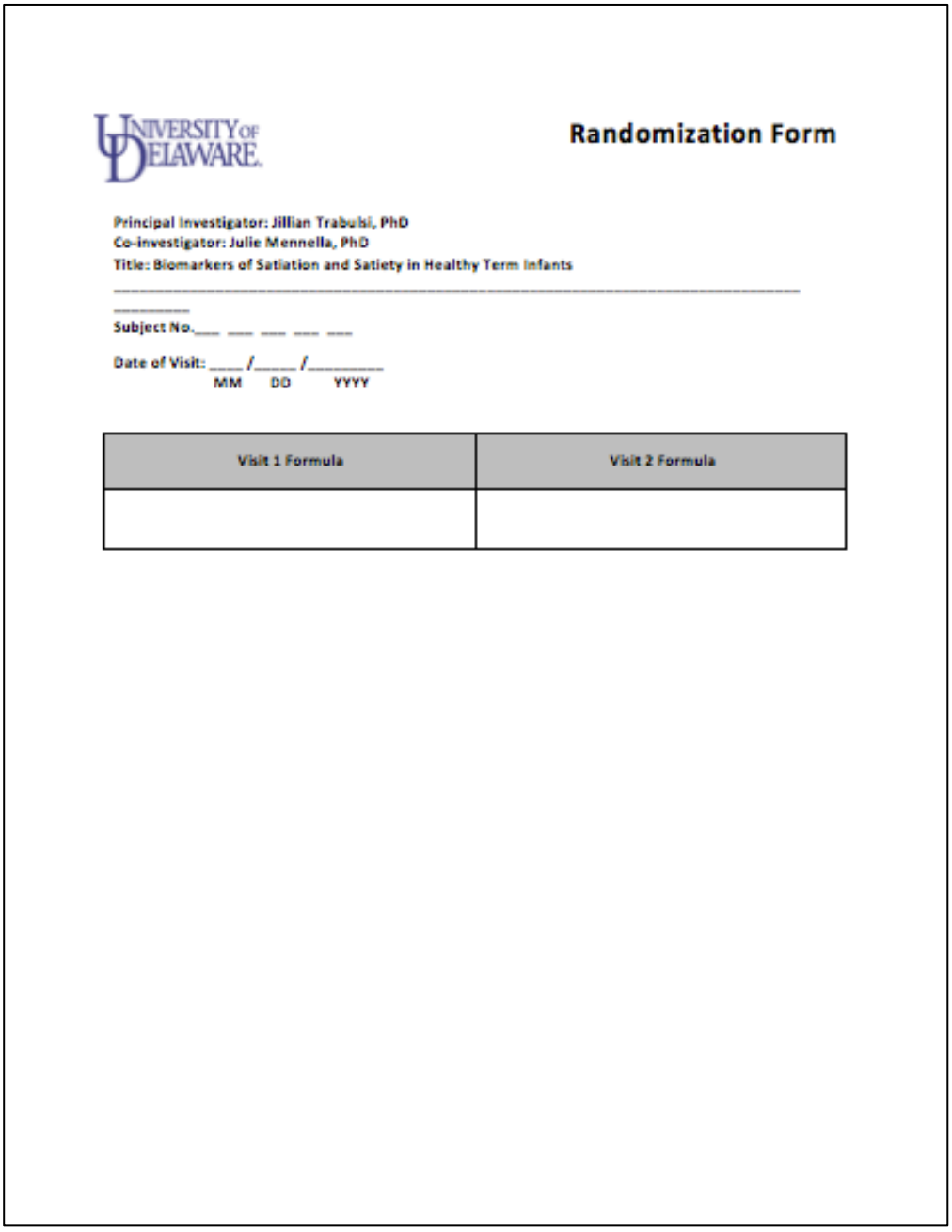
Principal Investigator: Jillian Trabulsi, PhD
Co-investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No.___ ___ ___ ___ ___

Date of Visit: ____ /_____ /_____
MM DD YYYY

EXCLUSION CRITERIA	YES	NO
1. Does the infant have a special feeding need or do they need to be on any special feedings other than those specified in the protocol?	☐	☐
2. Does the infant have any major congenital malformations or genetic disorders? (cleft palate, hemangiomas, extremity malformation)	☐	☐
3. Does the infant have suspected or documented systemic or congenital infections? (e.g. cytomegalovirus, human immunodeficiency virus)	☐	☐
4. Does the infant have a significant cardiac, respiratory, hematological, endocrinological, gastrointestinal or other systemic disease?	☐	☐
5. Has the infant participated in any other clinical trial with an experimental treatment since birth (prior to enrollment)?	☐	☐
6. Is the infant receiving herbal supplements, insulin, or growth hormones?	☐	☐
7. Is the infant related to ancillary personnel or a first-, or second-degree relative (e.g. child, sibling, parent, niece, nephew, grandniece, grandnephew, or grandchild) of ancillary personnel?	☐	☐
If you answered "YES" to any of the questions above, the infant may NOT be enrolled in this study.		

Randomization Form:



UNIVERSITY OF DELAWARE

Randomization Form

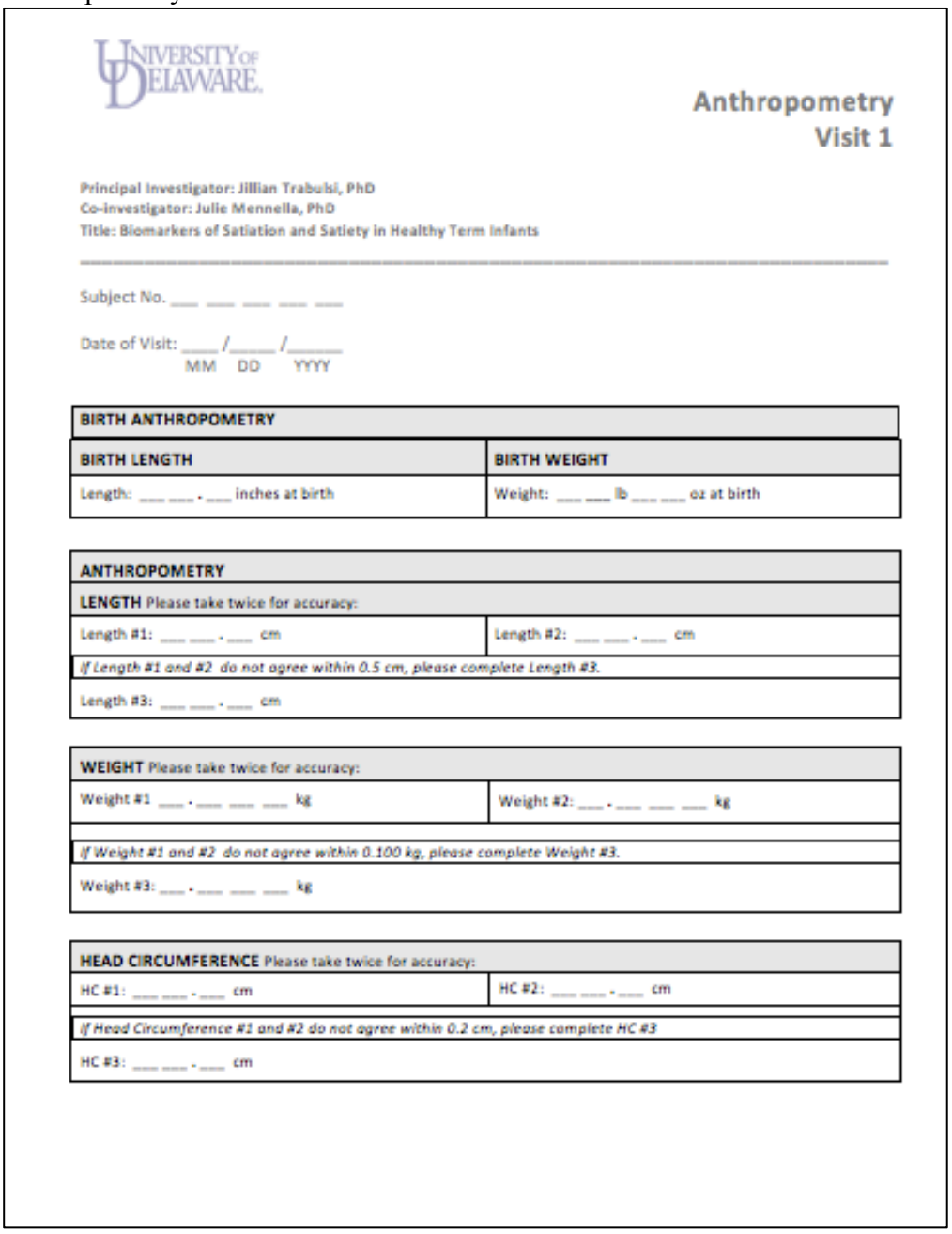
Principal Investigator: Jillian Trabulsi, PhD
Co-investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No.___ ___ ___ ___ ___

Date of Visit: ____ /_____ /_____
MM DD YYYY

Visit 1 Formula	Visit 2 Formula

Anthropometry Visit 1:



UNIVERSITY OF DELAWARE

Anthropometry Visit 1

Principal Investigator: Jillian Trabulsi, PhD
Co-investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____ /____ /_____
MM DD YYYY

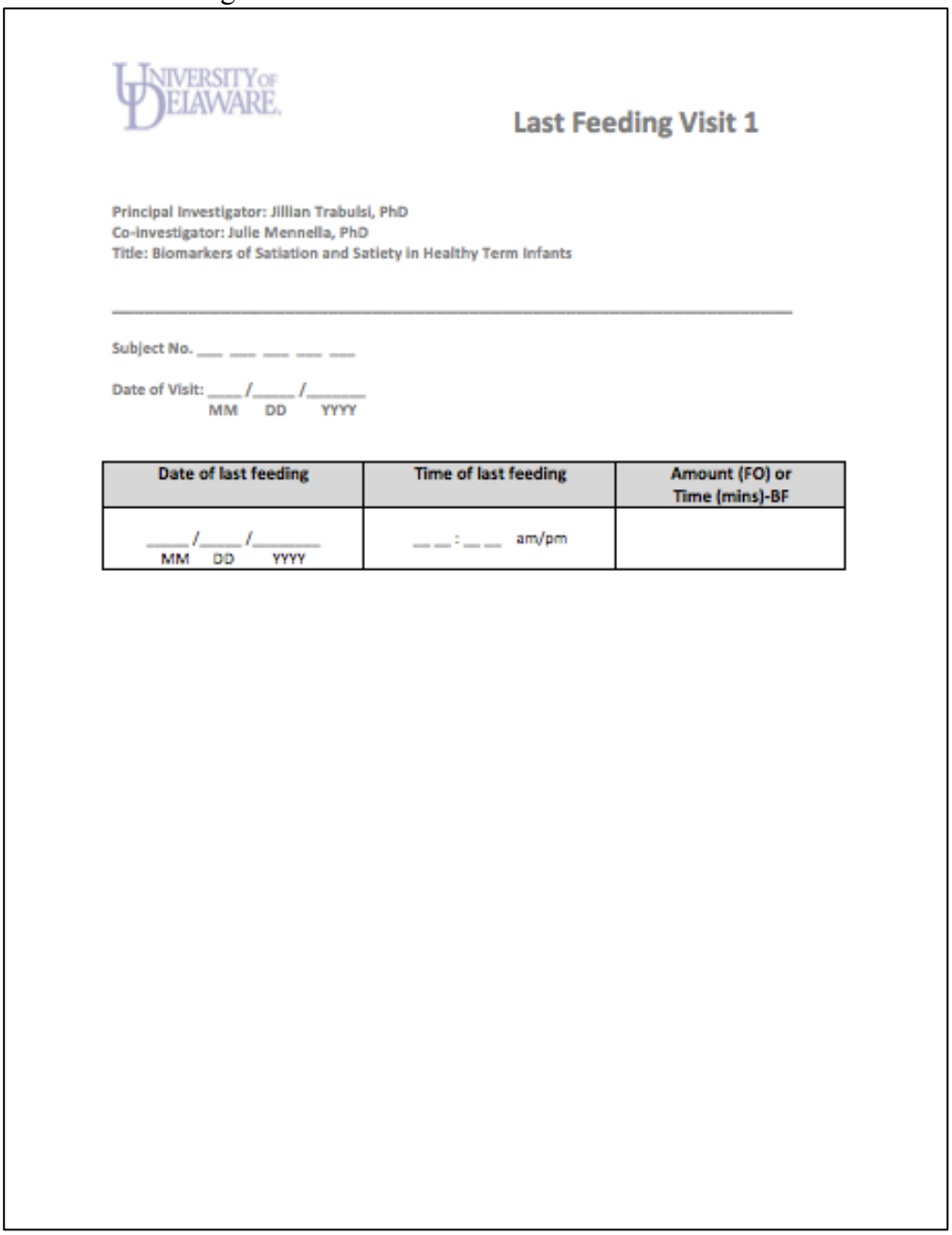
BIRTH ANTHROPOMETRY	
BIRTH LENGTH	**BIRTH WEIGHT**
Length: ___ ___ . ___ inches at birth	Weight: ___ ___ lb ___ ___ oz at birth

ANTHROPOMETRY	
LENGTH Please take twice for accuracy:	
Length #1: ___ ___ . ___ cm	Length #2: ___ ___ . ___ cm
If Length #1 and #2 do not agree within 0.5 cm, please complete Length #3.	
Length #3: ___ ___ . ___ cm	

WEIGHT Please take twice for accuracy:	
Weight #1 ___ . ___ ___ ___ kg	Weight #2: ___ . ___ ___ ___ kg
If Weight #1 and #2 do not agree within 0.100 kg, please complete Weight #3.	
Weight #3: ___ . ___ ___ ___ kg	

HEAD CIRCUMFERENCE Please take twice for accuracy:	
HC #1: ___ ___ . ___ cm	HC #2: ___ ___ . ___ cm
If Head Circumference #1 and #2 do not agree within 0.2 cm, please complete HC #3	
HC #3: ___ ___ . ___ cm	

Last Infant Feeding Visit 1:



UNIVERSITY OF DELAWARE

Last Feeding Visit 1

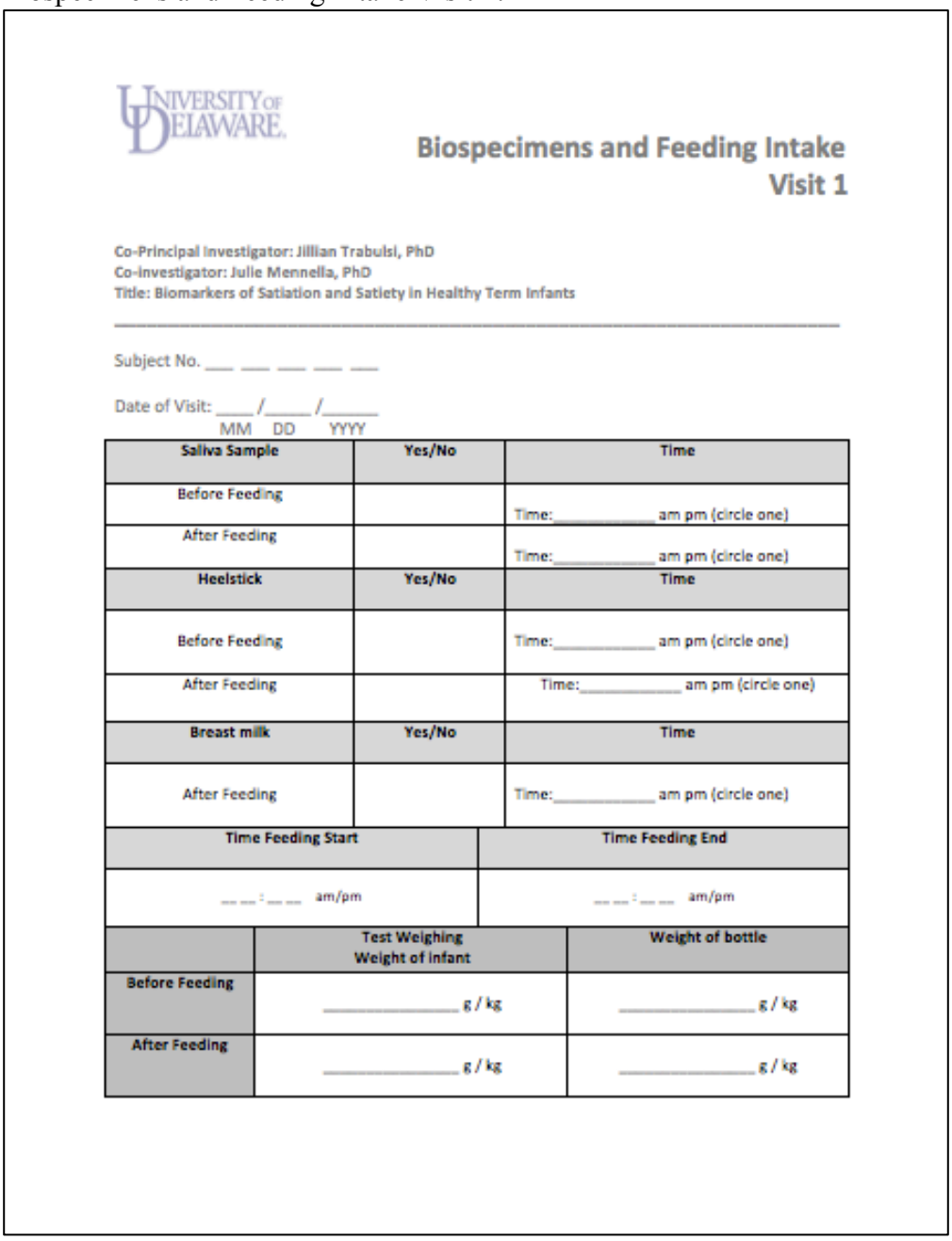
Principal Investigator: Jillian Trabulsi, PhD
Co-investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____/____/_____
MM DD YYYY

Date of last feeding	Time of last feeding	Amount (FO) or Time (mins)-BF
____/____/_____ MM DD YYYY	__ __ : __ __ am/pm	

Biospecimens and Feeding Intake Visit 1:



UNIVERSITY OF DELAWARE

Biospecimens and Feeding Intake Visit 1

Co-Principal Investigator: Jillian Trabulsi, PhD
Co-Investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No. ___ ___ ___ ___ ___

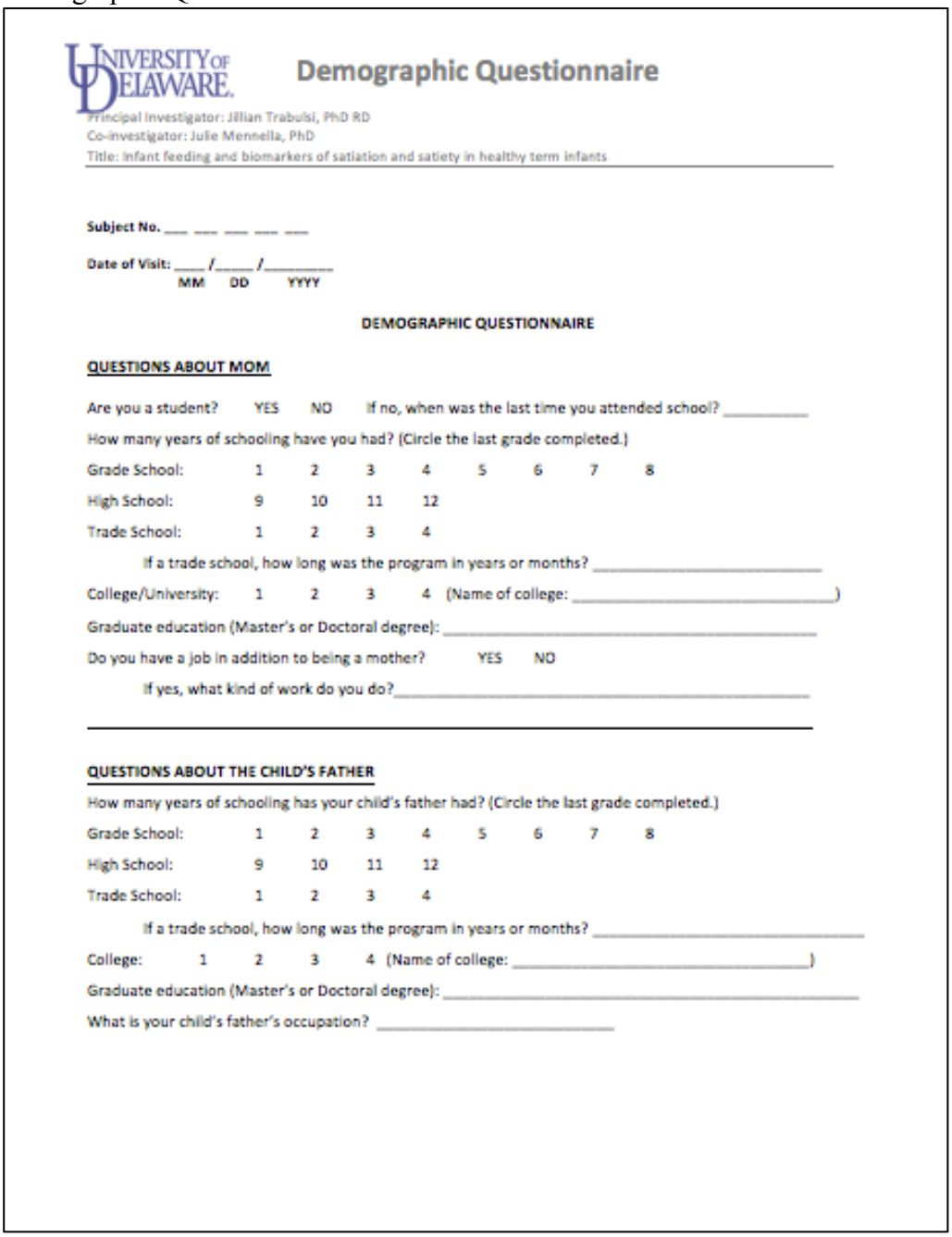
Date of Visit: ____ /_____ /_____
MM DD YYYY

Saliva Sample	Yes/No	Time
Before Feeding		Time:_____ am pm (circle one)
After Feeding		Time:_____ am pm (circle one)
Heelstick	**Yes/No**	**Time**
Before Feeding		Time:_____ am pm (circle one)
After Feeding		Time:_____ am pm (circle one)
Breast milk	**Yes/No**	**Time**
After Feeding		Time:_____ am pm (circle one)

Time Feeding Start	Time Feeding End
__ __ : __ __ am/pm	__ __ : __ __ am/pm

	Test Weighing Weight of Infant	Weight of bottle
Before Feeding	_____ g / kg	_____ g / kg
After Feeding	_____ g / kg	_____ g / kg

Demographic Questionnaire:



UNIVERSITY OF DELAWARE

Demographic Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____ /____ /_____
MM DD YYYY

DEMOGRAPHIC QUESTIONNAIRE

QUESTIONS ABOUT MOM

Are you a student? YES NO If no, when was the last time you attended school? _____

How many years of schooling have you had? (Circle the last grade completed.)

Grade School: 1 2 3 4 5 6 7 8

High School: 9 10 11 12

Trade School: 1 2 3 4

If a trade school, how long was the program in years or months? _____

College/University: 1 2 3 4 (Name of college: _____)

Graduate education (Master's or Doctoral degree): _____

Do you have a job in addition to being a mother? YES NO

If yes, what kind of work do you do?_____

QUESTIONS ABOUT THE CHILD'S FATHER

How many years of schooling has your child's father had? (Circle the last grade completed.)

Grade School: 1 2 3 4 5 6 7 8

High School: 9 10 11 12

Trade School: 1 2 3 4

If a trade school, how long was the program in years or months? _____

College: 1 2 3 4 (Name of college: _____)

Graduate education (Master's or Doctoral degree): _____

What is your child's father's occupation? _____

Demographic Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

What is your family's total yearly income?

_____ Under $10,000
_____$10,000 - $14,999
_____$15,000 - $24,999
_____$25,000 - $34,999
_____$35,000 - $49,999
_____$50,000 - $74,999
_____$75,000 - $99,999
_____$100,000 or more

Do you currently participate in federal nutrition education programs such as WIC? Yes No

If so, but it is not WIC, please specify the name: _____

If not participating presently, have you participated in the past? Yes No

If yes, when did you participate (dates)? _____

1. A. What is **YOUR** ethnic category?
   - ❑ Hispanic or Latino
   - ❑ Not Hispanic or Latino

   B. If you checked "Hispanic or Latin," do you consider yourself to be any of the following? *Check all that apply*
   - ❑ Mexican American or Mexican
   - ❑ Central American
   - ❑ South American
   - ❑ Puerto Rican
   - ❑ Cuban
   - ❑ Dominican
   - ❑ Spaniard or Portuguese
   - ❑ Other (*please specify country*):_____
   - ❑ Don't Know

2. A. What is **YOUR** racial background? (*Check all that apply*)
   - ❑ White or Caucasian
   - ❑ Black or African American
   - ❑ American Indian or Alaskan Native
   - ❑ Asian or Asian American
   - ❑ Native Hawaiian or Pacific Islander
   - ❑ Other (*please specify*) _____

Demographic Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

B. If you checked "Black or African American," do you consider yourself to be any of the following?
(Check all that apply)

- ❑ American
- ❑ African *(please specify country)*_____
- ❑ Haitian
- ❑ Jamaican
- ❑ Cuban
- ❑ Puerto Rican
- ❑ Dominican
- ❑ Other Caribbean Island
- ❑ Central/South American
- ❑ Other *(please specify country)*:_____
- ❑ Don't Know

C. If you checked "Asian or Asian American," do you consider yourself to be any of the following?
Check all that apply

- ❑ Chinese
- ❑ East Indian/South Asian
- ❑ Japanese
- ❑ Filipino
- ❑ Korean
- ❑ Southeast Asian
- ❑ Other *(please specify country)*:_____
- ❑ Don't Know

3. A. What is **YOUR CHILD'S FATHER'S** ethnic category?

- ❑ Hispanic or Latino
- ❑ Not Hispanic or Latino

B. If you checked "Hispanic or Latin," do you consider the father to be any of the following?
Check all that apply

- ❑ Mexican American or Mexican
- ❑ Central American
- ❑ South American
- ❑ Puerto Rican
- ❑ Cuban
- ❑ Dominican
- ❑ Spaniard or Portuguese
- ❑ Other *(please specify country)*:_____
- ❑ Don't Know

Demographic Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

4. A. What is **YOUR CHILD'S FATHER'S** racial background? (*Check all that apply*)
   - ❑ White or Caucasian
   - ❑ Black or African American
   - ❑ American Indian or Alaskan Native
   - ❑ Asian or Asian American
   - ❑ Native Hawaiian or Pacific Islander
   - ❑ Other (*please specify*) _____

   B. If you checked "Black or African American," do you consider the father to be any of the following? (*Check all that apply*)
   - ❑ American
   - ❑ African (*please specify country*)_____
   - ❑ Haitian
   - ❑ Jamaican
   - ❑ Cuban
   - ❑ Puerto Rican
   - ❑ Dominican
   - ❑ Other Caribbean Island
   - ❑ Central/South American
   - ❑ Other (*please specify country*):_____
   - ❑ Don't Know

   C. If you checked "Asian or Asian American," do you consider the father to be any of the following? *Check all that apply*
   - ❑ Chinese
   - ❑ East Indian/South Asian
   - ❑ Japanese
   - ❑ Filipino
   - ❑ Korean
   - ❑ Southeast Asian
   - ❑ Other (*please specify country*):_____
   - ❑ Don't Know

5. A. What is **YOUR CHILD'S** ethnic category?
   - ❑ Hispanic or Latino
   - ❑ Not Hispanic or Latino

Demographic Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

B. If you checked "Hispanic or Latin," do you consider the child to be any of the following?
Check all that apply

- ❑ Mexican American or Mexican
- ❑ Central American
- ❑ South American
- ❑ Puerto Rican
- ❑ Cuban
- ❑ Dominican
- ❑ Spaniard or Portuguese
- ❑ Other (*please specify country*):_____
- ❑ Don't Know

6. A. What is **YOUR CHILD'S** racial background? (*Check all that apply*)

- ❑ White or Caucasian
- ❑ Black or African American
- ❑ American Indian or Alaskan Native
- ❑ Asian or Asian American
- ❑ Native Hawaiian or Pacific Islander
- ❑ Other (*please specify*) _____

B. If you checked "Black or African American," do you consider the child to be any of the following?
(*Check all that apply*)

- ❑ American
- ❑ African (*please specify country*)_____
- ❑ Haitian
- ❑ Jamaican
- ❑ Cuban
- ❑ Puerto Rican
- ❑ Dominican
- ❑ Other Caribbean Island
- ❑ Central/South American
- ❑ Other (*please specify country*):_____
- ❑ Don't Know

C. If you checked "Asian or Asian American," do you consider the child to be any of the following?
Check all that apply

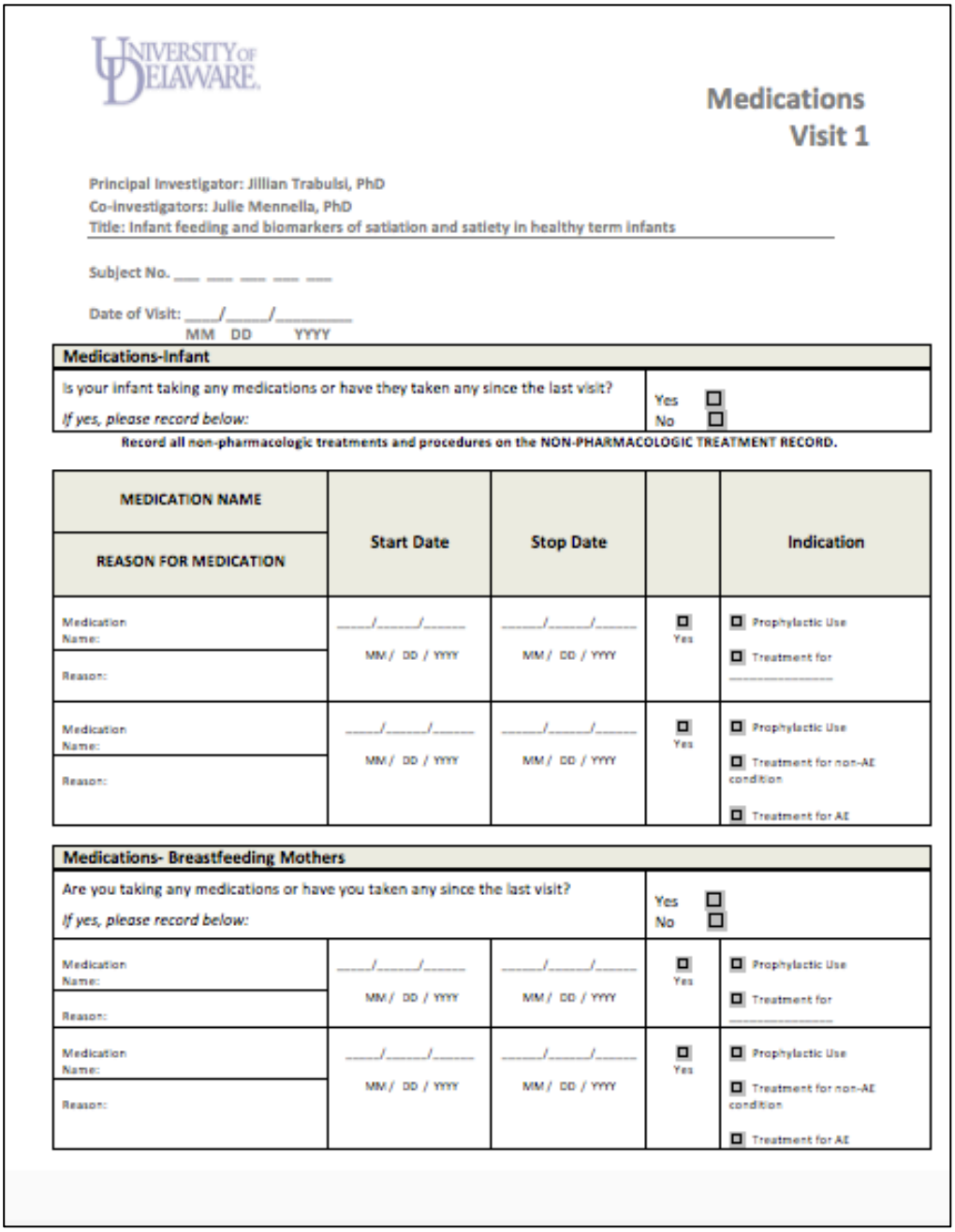
- ❑ Chinese
- ❑ East Indian/South Asian
- ❑ Japanese

Demographic Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

- ❑ Filipino
- ❑ Korean
- ❑ Southeast Asian
- ❑ Other *(please specify country)*:_____
- ❑ Don't Know

Medications Visit 1:



UNIVERSITY OF DELAWARE

Medications Visit 1

Principal Investigator: Jillian Trabulsi, PhD
Co-investigators: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____/____/_____
MM DD YYYY

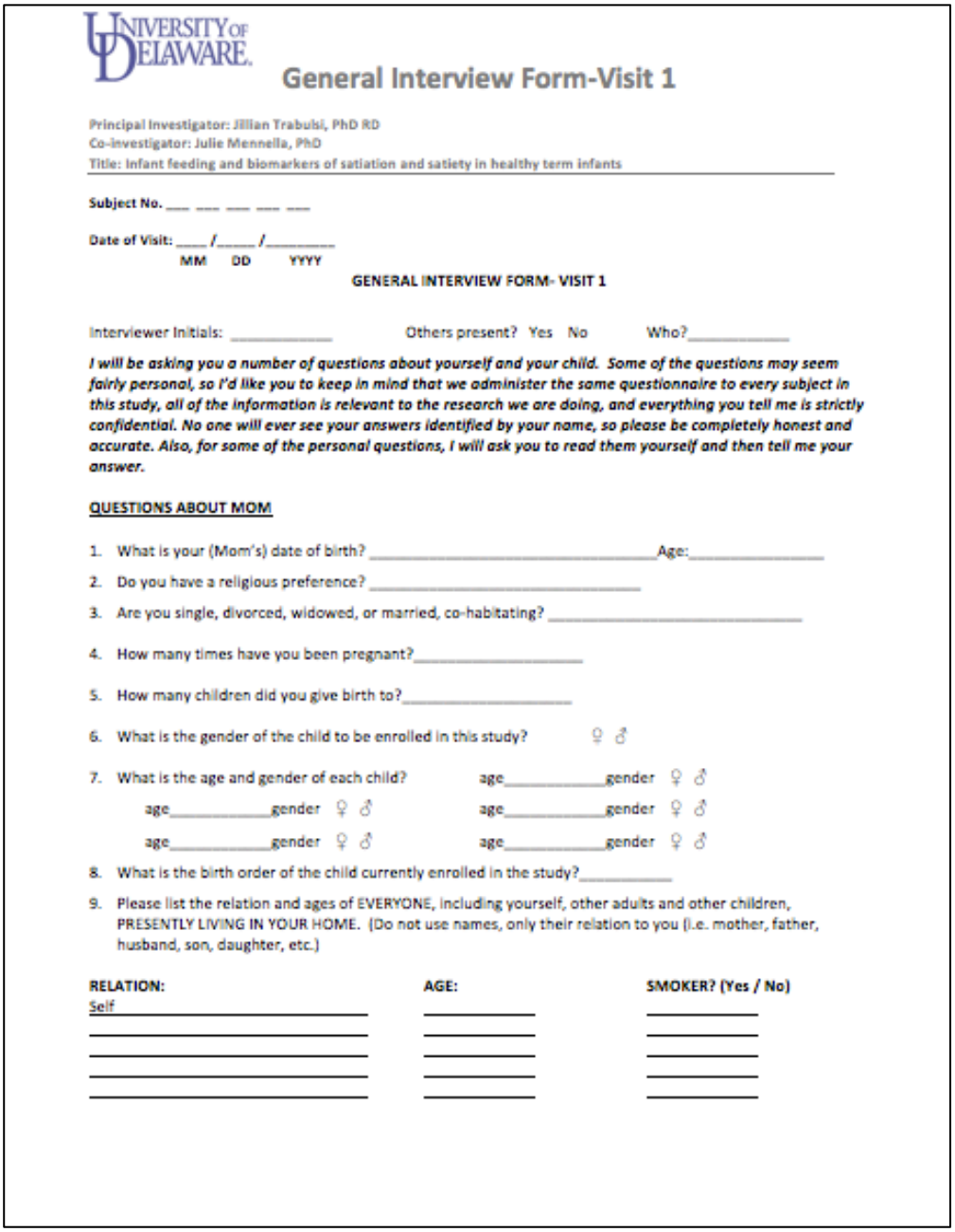
Medications-Infant	
Is your infant taking any medications or have they taken any since the last visit? *If yes, please record below:*	Yes ☐ No ☐

Record all non-pharmacologic treatments and procedures on the NON-PHARMACOLOGIC TREATMENT RECORD.

MEDICATION NAME REASON FOR MEDICATION	Start Date	Stop Date		Indication
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for ____
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for non-AE condition ☐ Treatment for AE

Medications- Breastfeeding Mothers				
Are you taking any medications or have you taken any since the last visit? *If yes, please record below:*			Yes ☐ No ☐	
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for ____
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for non-AE condition ☐ Treatment for AE

General Interview Form Visit 1:



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General Interview Form-Visit 1

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____ /_____ /_____
MM DD YYYY

GENERAL INTERVIEW FORM- VISIT 1

Interviewer Initials: _____ Others present? Yes No Who?_____

I will be asking you a number of questions about yourself and your child. Some of the questions may seem fairly personal, so I'd like you to keep in mind that we administer the same questionnaire to every subject in this study, all of the information is relevant to the research we are doing, and everything you tell me is strictly confidential. No one will ever see your answers identified by your name, so please be completely honest and accurate. Also, for some of the personal questions, I will ask you to read them yourself and then tell me your answer.

QUESTIONS ABOUT MOM

1. What is your (Mom's) date of birth? _____Age:_____
2. Do you have a religious preference? _____
3. Are you single, divorced, widowed, or married, co-habitating? _____
4. How many times have you been pregnant?_____
5. How many children did you give birth to?_____
6. What is the gender of the child to be enrolled in this study? ♀ ♂
7. What is the age and gender of each child? age_____gender ♀ ♂ age_____gender ♀ ♂
   age_____gender ♀ ♂ age_____gender ♀ ♂ age_____gender ♀ ♂
8. What is the birth order of the child currently enrolled in the study?_____
9. Please list the relation and ages of EVERYONE, including yourself, other adults and other children, PRESENTLY LIVING IN YOUR HOME. (Do not use names, only their relation to you (i.e. mother, father, husband, son, daughter, etc.)

RELATION:	AGE:	SMOKER? (Yes / No)
Self		

General Interview Form-Visit 1

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

10. Besides those mentioned in the previous list, is your child currently around anyone who smokes cigarettes, cigars, pipes? YES NO
    a. How much time does the child spend with this person?_____
11. Besides those mentioned, has your child, in the past, been around anyone who has smoked cigarettes, cigars, pipes? YES NO
    a. How often did your child see this person?_____
12. Your length of pregnancy or baby's gestational age (in weeks): _____weeks
13. Your (Mom's) height: _____ ft. _____ in.
14. Pre-pregnancy weight: _____ lbs.
15. How many pounds did you gain during this pregnancy? _____ lbs.
16. Post-pregnancy weight: _____ lbs.
17. Did you have a pre-pregnancy history of diabetes? Yes No
18. Did you develop diabetes during pregnancy? Yes No
19. Did you smoke during pregnancy? Yes No
20. Type of delivery: Natural C-Section

    Were you put on antibiotics during delivery? Yes No If yes, please list:_____

    Was baby put on antibiotics during delivery? Yes No If yes, please list:_____

    Any complications during pregnancy/birth? Yes No If yes, please list:_____
21. Do you have a job, in addition to being a mother? Yes No
    If yes, what kind of work do you do?_____
22. What is the best method to contact you for study updates, reminders, scheduling, etc.?
    Please provide all information, and check which you prefer:
    - ☐ Call:_____ Home Cell Phone
      If you have a cell phone, is it Pre-paid or Contract/Monthly?_____
    - ☐ Email:_____
    - ☐ Text:_____
      If you checked something other than texting, would you still be willing to receive and send texts?
      Yes No

General Interview Form-Visit 1

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

QUESTIONS ABOUT CHILD

23. Your child's date of birth: _____

24. Her/his birth weight: _____ in pounds (_____kg)

25. Her/his birth length: _____ in inches (_____ cm)

QUESTIONS ABOUT THE CHILD'S FATHER

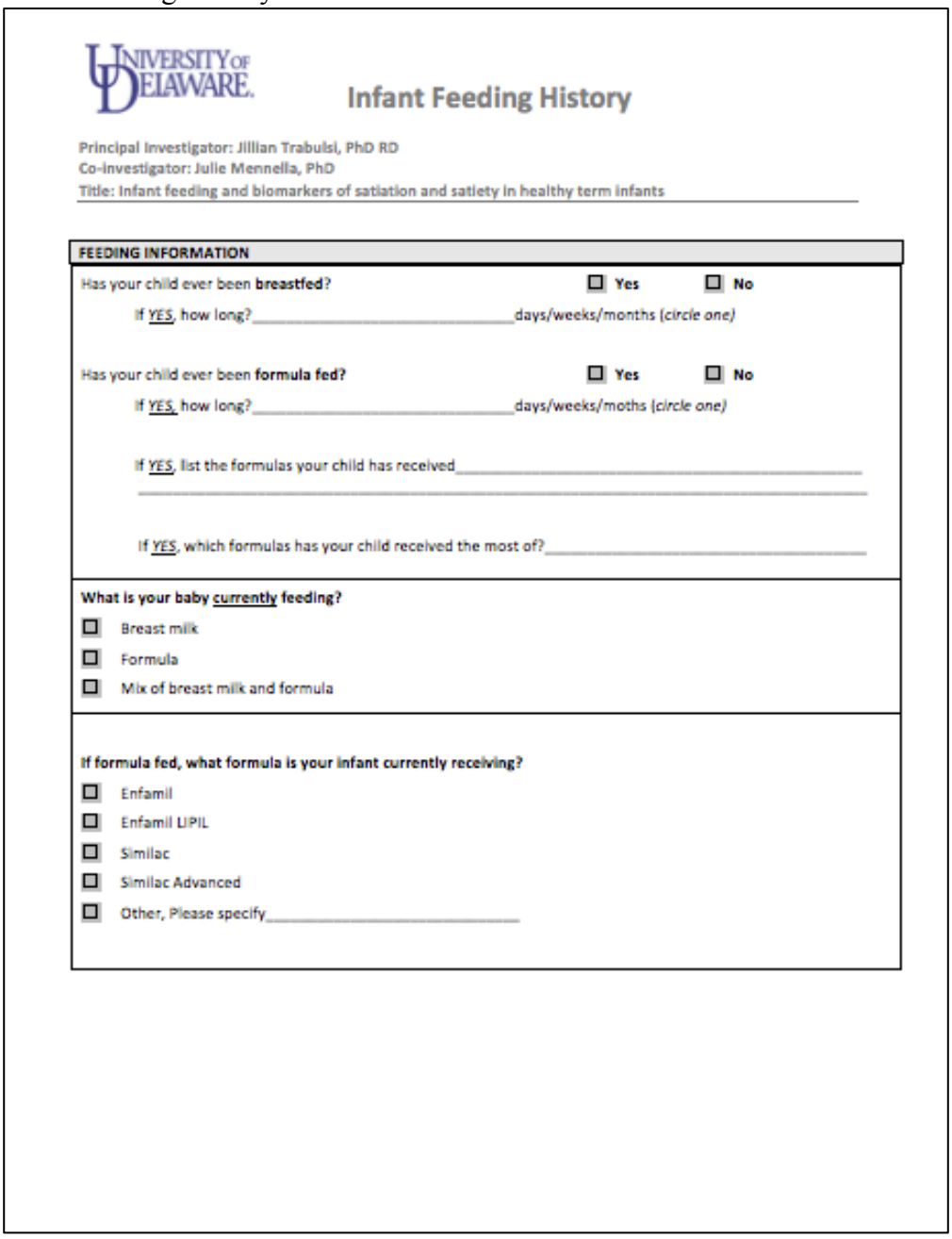
26. How old is your child's father?_____
    How tall is he?_____ How much does he weigh? _____

27. Does the father smoke cigarettes? YES NO
    If yes, how much and how often? _____
    When did he start smoking? _____

28. Has he smoked since your child was born? Yes No
    (If different:) How much was he smoking then per day, week? _____
    When did he cut down (or start smoking more)? _____

29. Do you think you've been able to remember this information about the father fairly accurately?
    YES NO
    If not, which answers are you unsure of?_____
    _____

Infant Feeding History:



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Infant Feeding History

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

FEEDING INFORMATION

Has your child ever been **breastfed**? ☐ **Yes** ☐ **No**

If YES, how long?_____days/weeks/months *(circle one)*

Has your child ever been **formula fed?** ☐ **Yes** ☐ **No**

If YES, how long?_____days/weeks/moths *(circle one)*

If YES, list the formulas your child has received_____

_____

If YES, which formulas has your child received the most of?_____

What is your baby currently feeding?

☐ Breast milk
☐ Formula
☐ Mix of breast milk and formula

If formula fed, what formula is your infant currently receiving?

☐ Enfamil
☐ Enfamil LIPIL
☐ Similac
☐ Similac Advanced
☐ Other, Please specify_____

Infant Feeding History

Principal Investigator: Jillian Trabulsi, PhD RD
Co-Investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

If formula fed, how do you prepare your infant's formula?

Amount of powder _____ scoops

Amount of water _____ oz or cups (please circle one)

Do you add anything to your baby's bottle? ☐ **Yes** ☐ **No**

If *Yes*, what?_____ How much?_____

How often?_____

How many times per day to you feed your infant?_____

How many feedings per day does your child receive **formula**?_____

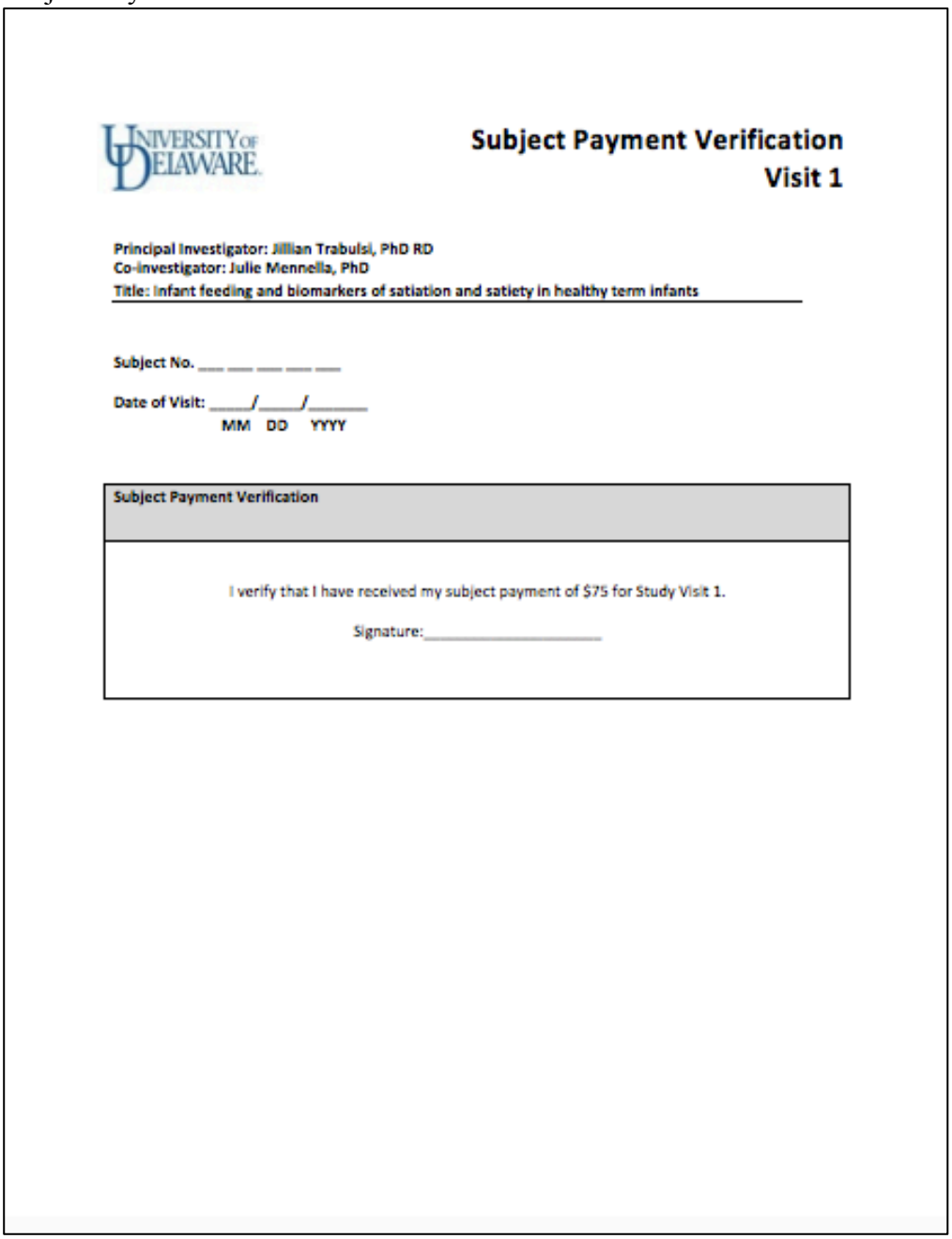
How many feedings per day does your child receive **breast milk**?_____

Has your infant ever received any solid foods? ☐ **Yes** ☐ **No**

If *YES*, which foods? *(circle all that apply)* Cereal Fruit Vegetables

If YES, how was it given? *(circle one)* Bottle Spoon

Subject Payment Verification Visit 1:



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Subject Payment Verification
Visit 1

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

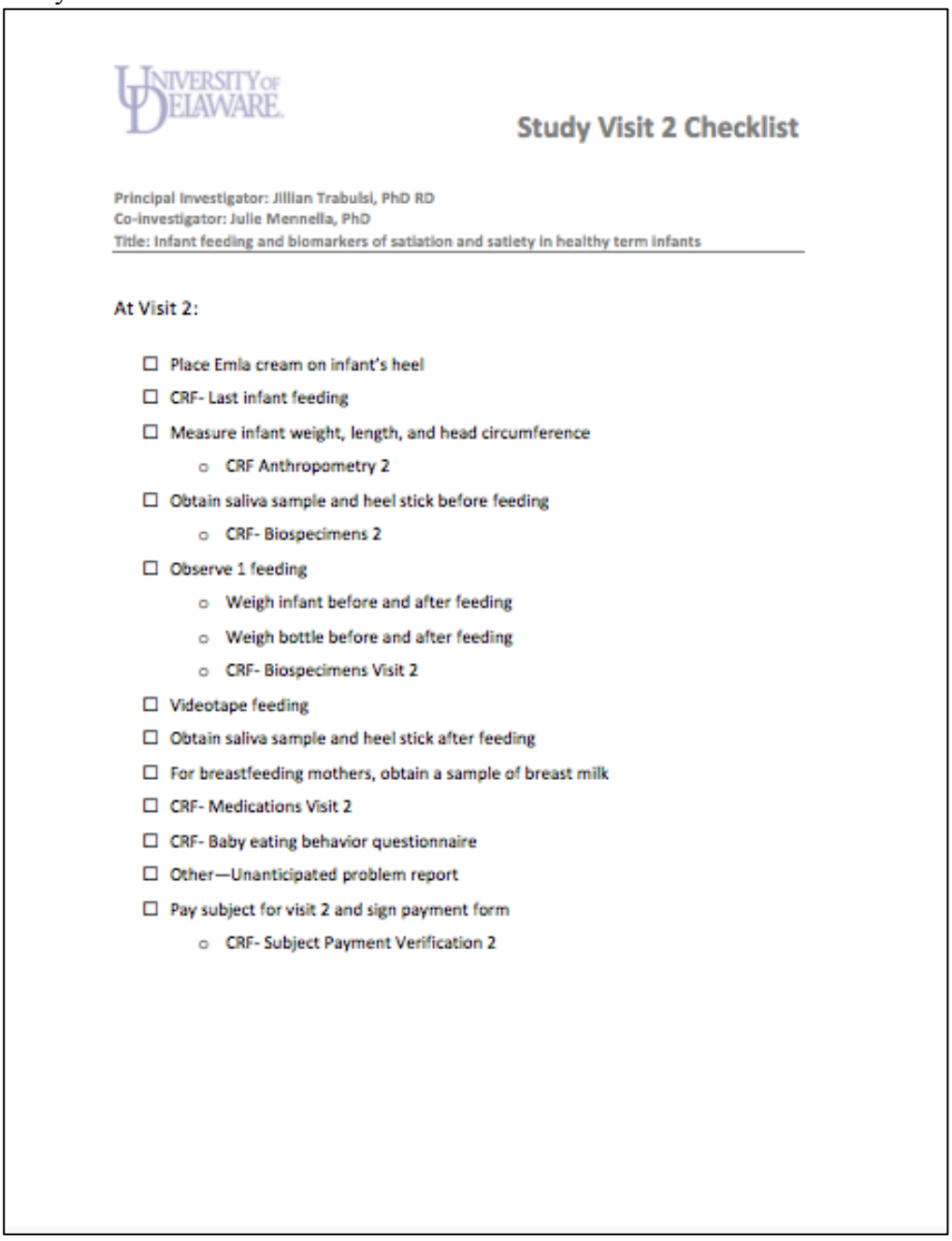
Subject No. ___ ___ ___ ___ ___

Date of Visit: _____/_____/_____
MM DD YYYY

Subject Payment Verification
I verify that I have received my subject payment of $75 for Study Visit 1. Signature:_____

C.4 Visit 2 Documents

Study Visit 2 Checklist:



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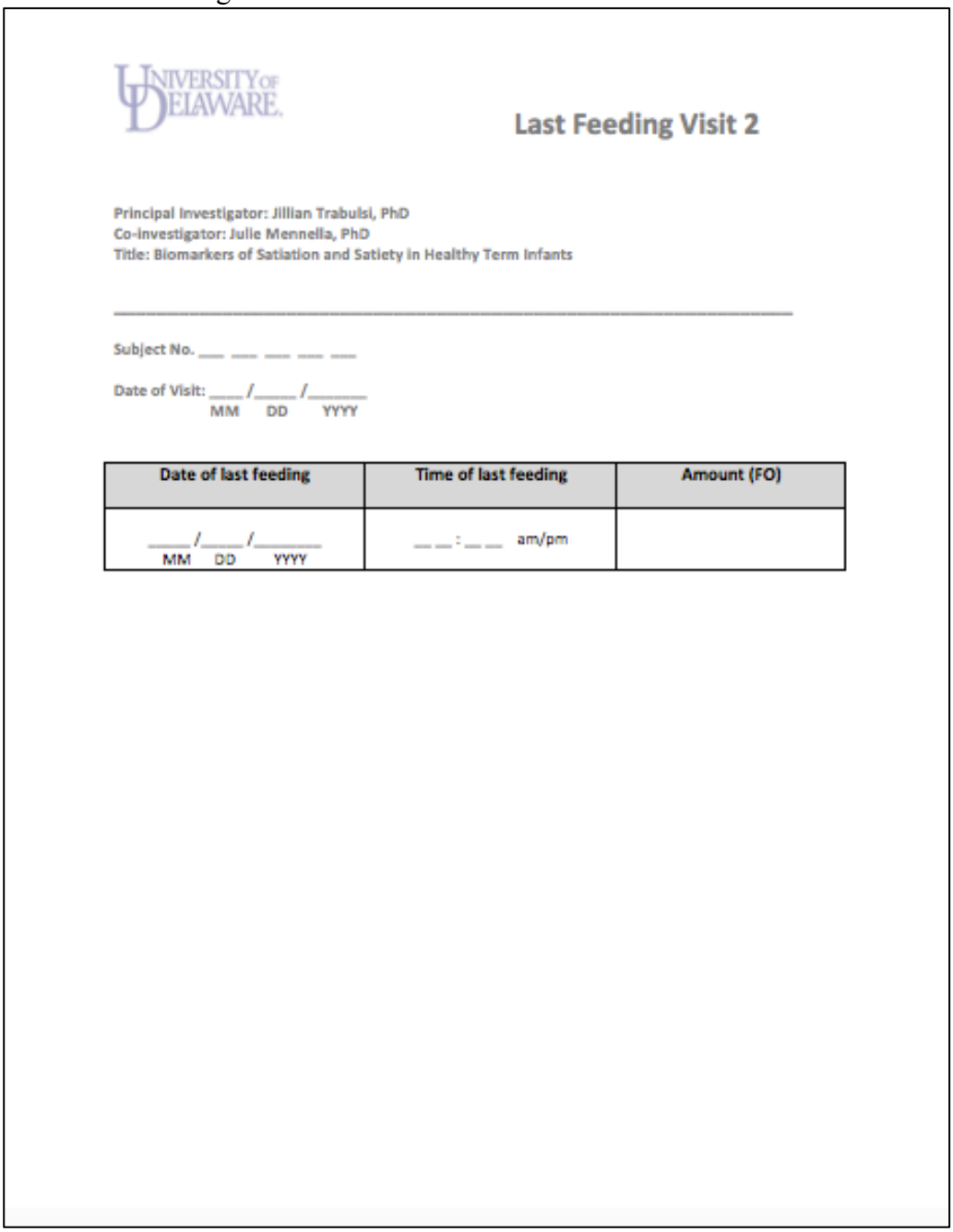
Study Visit 2 Checklist

Principal Investigator: Jillian Trabulsi, PhD RD
Co-Investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

At Visit 2:

- ☐ Place Emla cream on infant's heel
- ☐ CRF- Last infant feeding
- ☐ Measure infant weight, length, and head circumference
  - o CRF Anthropometry 2
- ☐ Obtain saliva sample and heel stick before feeding
  - o CRF- Biospecimens 2
- ☐ Observe 1 feeding
  - o Weigh infant before and after feeding
  - o Weigh bottle before and after feeding
  - o CRF- Biospecimens Visit 2
- ☐ Videotape feeding
- ☐ Obtain saliva sample and heel stick after feeding
- ☐ For breastfeeding mothers, obtain a sample of breast milk
- ☐ CRF- Medications Visit 2
- ☐ CRF- Baby eating behavior questionnaire
- ☐ Other—Unanticipated problem report
- ☐ Pay subject for visit 2 and sign payment form
  - o CRF- Subject Payment Verification 2

Last Infant Feeding Visit 2:



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Last Feeding Visit 2

Principal Investigator: Jillian Trabulsi, PhD
Co-Investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

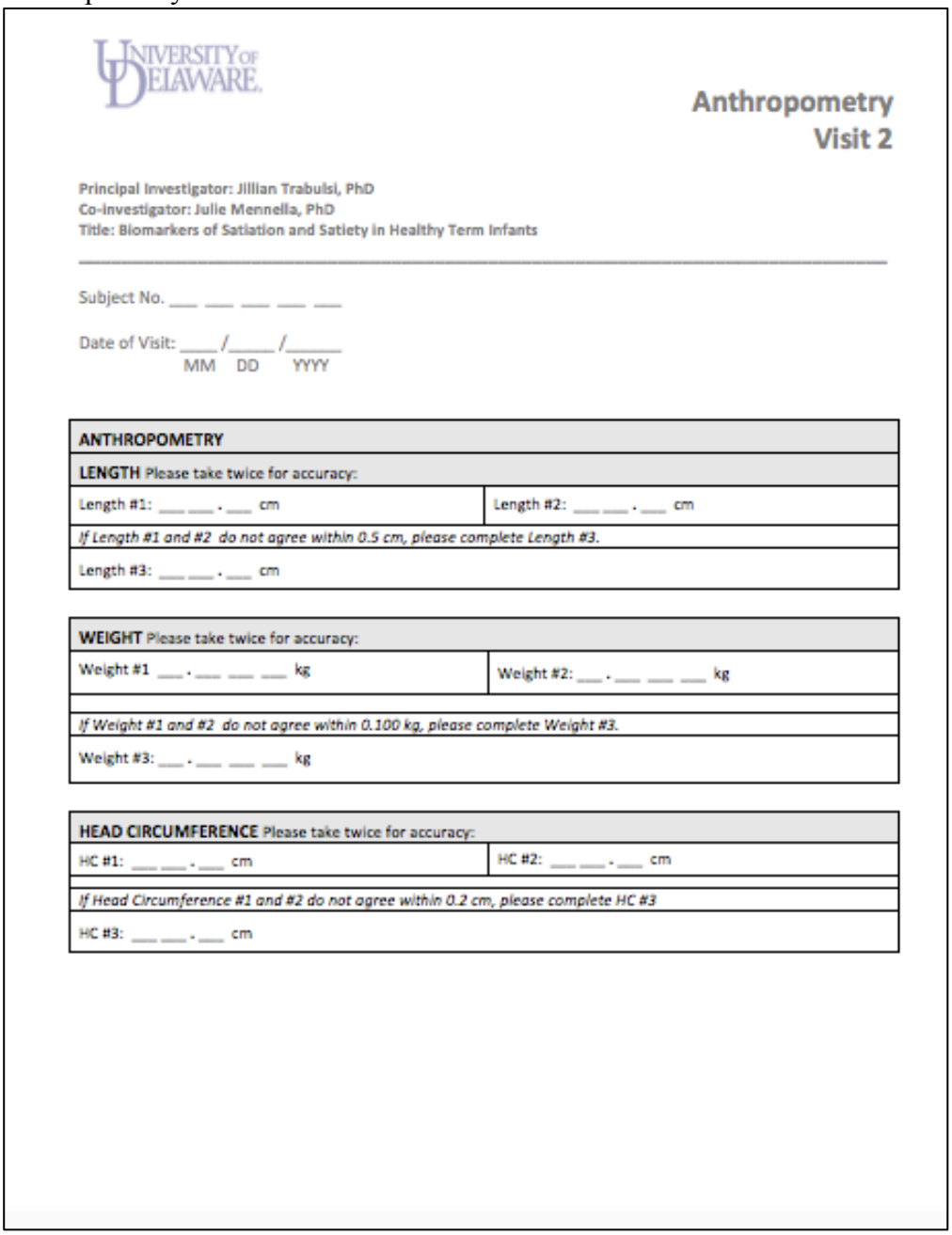
---

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____ /____ /_____
MM DD YYYY

Date of last feeding	Time of last feeding	Amount (FO)
____ /____ /_____ MM DD YYYY	__ __ : __ __ am/pm	

Anthropometry Visit 2:



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Anthropometry Visit 2

Principal Investigator: Jillian Trabulsi, PhD
Co-Investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No. ___ ___ ___ ___ ___

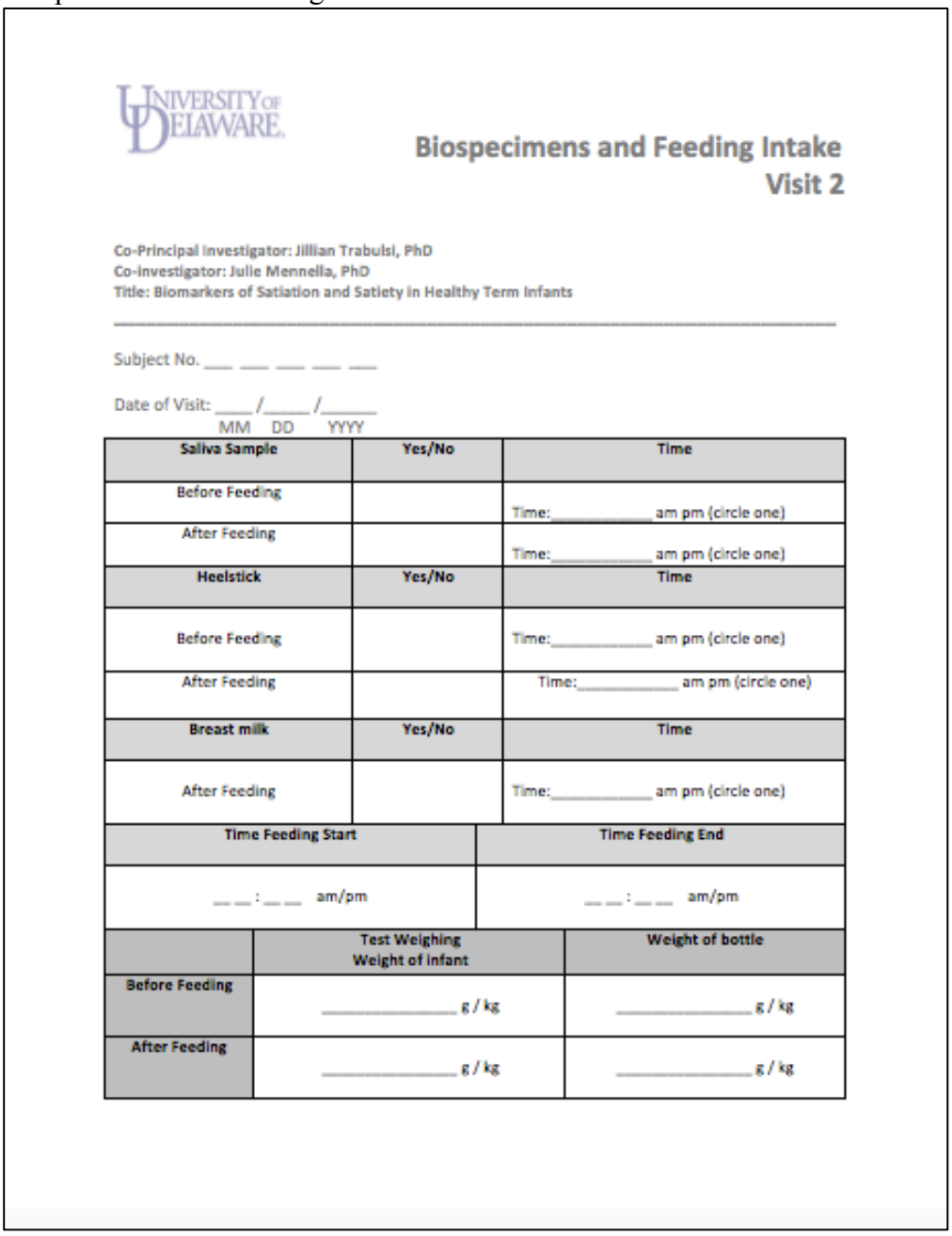
Date of Visit: ____/____/_____
MM DD YYYY

ANTHROPOMETRY	
LENGTH Please take twice for accuracy:	
Length #1: ___ ___ . ___ cm	Length #2: ___ ___ . ___ cm
If Length #1 and #2 do not agree within 0.5 cm, please complete Length #3.	
Length #3: ___ ___ . ___ cm	

WEIGHT Please take twice for accuracy:	
Weight #1 ___ . ___ ___ ___ kg	Weight #2: ___ . ___ ___ ___ kg
If Weight #1 and #2 do not agree within 0.100 kg, please complete Weight #3.	
Weight #3: ___ . ___ ___ ___ kg	

HEAD CIRCUMFERENCE Please take twice for accuracy:	
HC #1: ___ ___ . ___ cm	HC #2: ___ ___ . ___ cm
If Head Circumference #1 and #2 do not agree within 0.2 cm, please complete HC #3	
HC #3: ___ ___ . ___ cm	

Biospecimens and Feeding Intake Visit 2:



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Biospecimens and Feeding Intake Visit 2

Co-Principal Investigator: Jillian Trabulsi, PhD
Co-Investigator: Julie Mennella, PhD
Title: Biomarkers of Satiation and Satiety in Healthy Term Infants

Subject No. ___ ___ ___ ___ ___

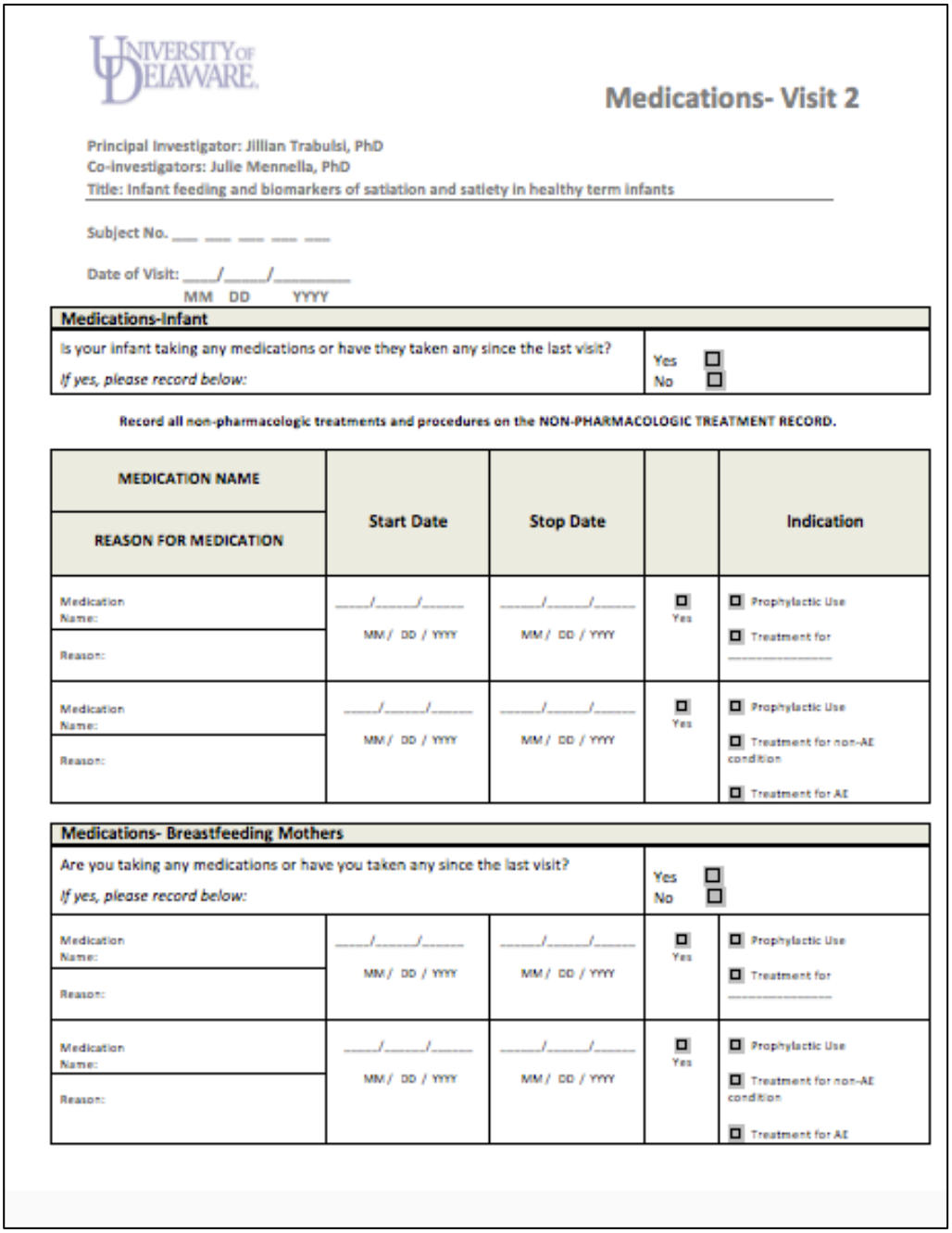
Date of Visit: ____/_____/_____
MM DD YYYY

Saliva Sample	Yes/No	Time
Before Feeding		Time:_____ am pm (circle one)
After Feeding		Time:_____ am pm (circle one)
Heelstick	**Yes/No**	**Time**
Before Feeding		Time:_____ am pm (circle one)
After Feeding		Time:_____ am pm (circle one)
Breast milk	**Yes/No**	**Time**
After Feeding		Time:_____ am pm (circle one)

Time Feeding Start	Time Feeding End
__ __ : __ __ am/pm	__ __ : __ __ am/pm

	Test Weighing Weight of infant	Weight of bottle
Before Feeding	_____ g / kg	_____ g / kg
After Feeding	_____ g / kg	_____ g / kg

Medications Visit 2:



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Medications- Visit 2

Principal Investigator: Jillian Trabulsi, PhD
Co-Investigators: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____/____/_____
MM DD YYYY

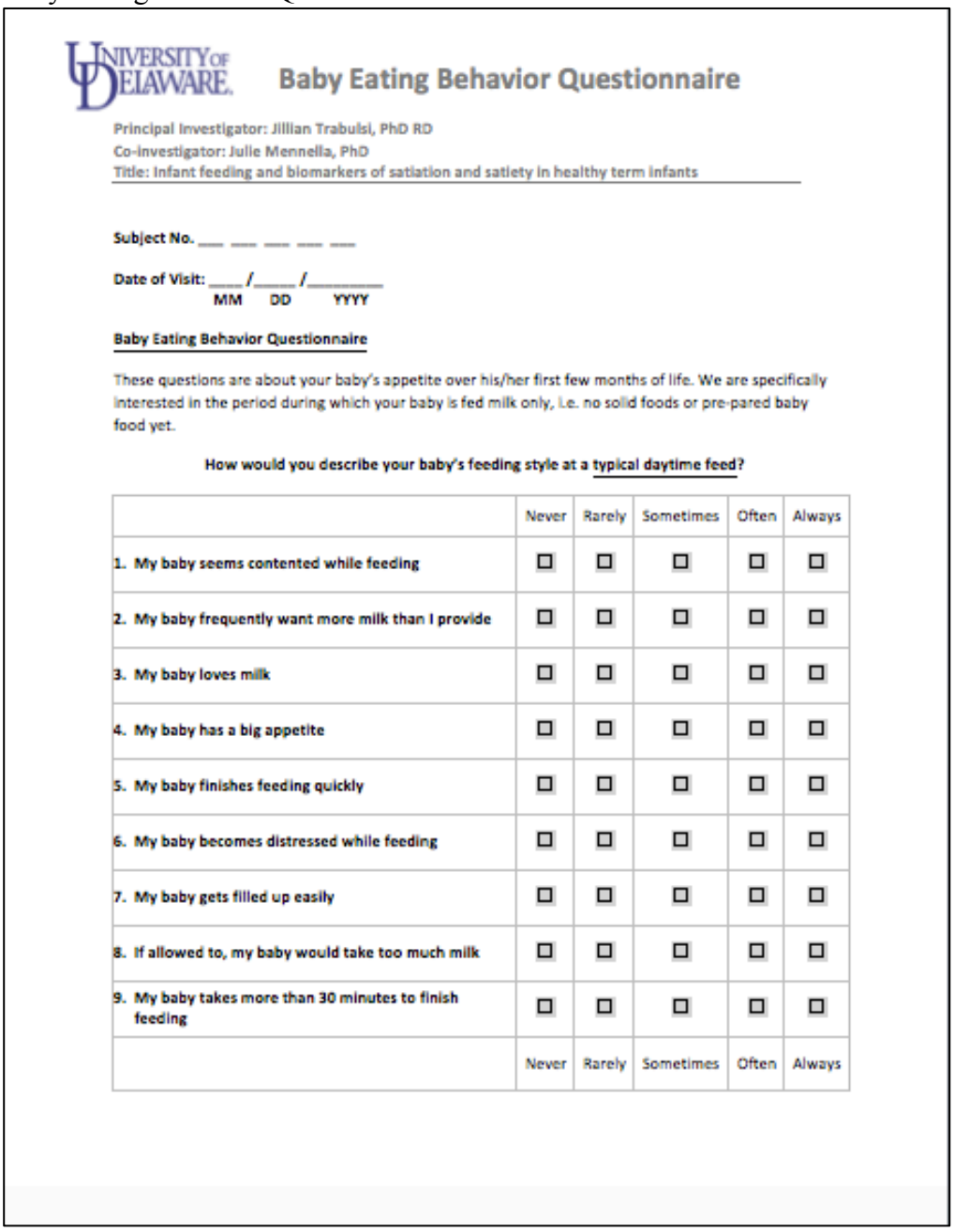
Medications-Infant	
Is your infant taking any medications or have they taken any since the last visit? *If yes, please record below:*	Yes ☐ No ☐

Record all non-pharmacologic treatments and procedures on the NON-PHARMACOLOGIC TREATMENT RECORD.

MEDICATION NAME REASON FOR MEDICATION	Start Date	Stop Date		Indication
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for _____
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for non-AE condition ☐ Treatment for AE

Medications- Breastfeeding Mothers				
Are you taking any medications or have you taken any since the last visit? *If yes, please record below:*			Yes ☐ No ☐	
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for _____
Medication Name: Reason:	____/____/____ MM / DD / YYYY	____/____/____ MM / DD / YYYY	☐ Yes	☐ Prophylactic Use ☐ Treatment for non-AE condition ☐ Treatment for AE

Baby Eating Behavior Questionnaire:



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Baby Eating Behavior Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-Investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: ____ /____ /_____
MM DD YYYY

Baby Eating Behavior Questionnaire

These questions are about your baby's appetite over his/her first few months of life. We are specifically interested in the period during which your baby is fed milk only, i.e. no solid foods or pre-pared baby food yet.

How would you describe your baby's feeding style at a typical daytime feed?

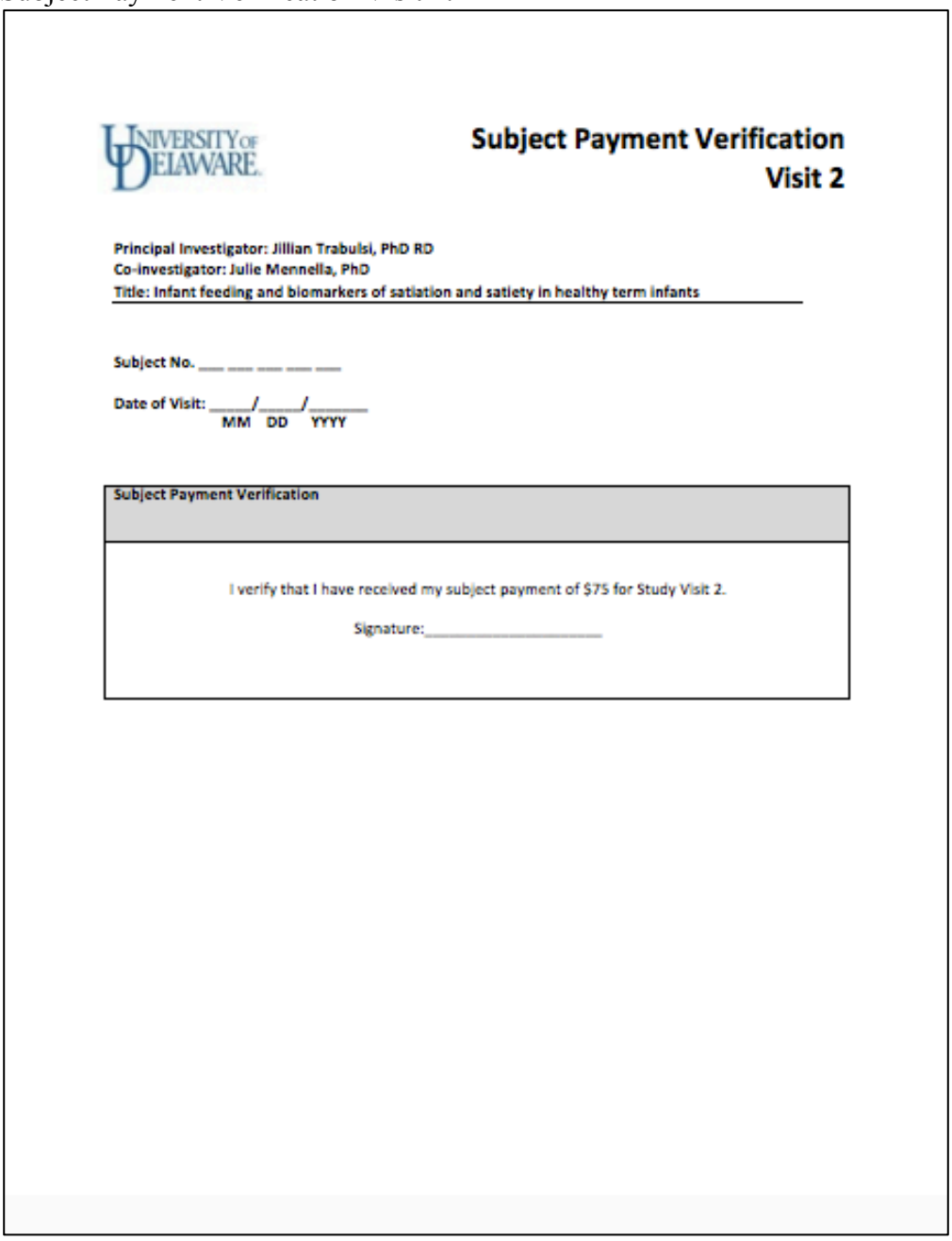
	Never	Rarely	Sometimes	Often	Always
1. My baby seems contented while feeding	☐	☐	☐	☐	☐
2. My baby frequently want more milk than I provide	☐	☐	☐	☐	☐
3. My baby loves milk	☐	☐	☐	☐	☐
4. My baby has a big appetite	☐	☐	☐	☐	☐
5. My baby finishes feeding quickly	☐	☐	☐	☐	☐
6. My baby becomes distressed while feeding	☐	☐	☐	☐	☐
7. My baby gets filled up easily	☐	☐	☐	☐	☐
8. If allowed to, my baby would take too much milk	☐	☐	☐	☐	☐
9. My baby takes more than 30 minutes to finish feeding	☐	☐	☐	☐	☐
	Never	Rarely	Sometimes	Often	Always

Baby Eating Behavior Questionnaire

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

	Never	Rarely	Sometimes	Often	Always
10. My baby gets full before taking all the milk I think he/she should have	☐	☐	☐	☐	☐
11. My baby feeds slowly	☐	☐	☐	☐	☐
12. Even when my baby has just eaten well he/she is happy to feed again if offered	☐	☐	☐	☐	☐
13. My baby finds it difficult to manage a complete feed	☐	☐	☐	☐	☐
14. My baby is always demanding a feed	☐	☐	☐	☐	☐
15. My baby sucks more and more slowly during the course of a feed	☐	☐	☐	☐	☐
16. If given the chance, my baby would always be feeding	☐	☐	☐	☐	☐
17. My baby enjoys feeding time	☐	☐	☐	☐	☐
18. My baby can easily take a feed within 30 minutes of the last one	☐	☐	☐	☐	☐

Subject Payment Verification Visit 2:



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Subject Payment Verification
Visit 2

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

Subject No. ___ ___ ___ ___ ___

Date of Visit: _____/_____/_____
MM DD YYYY

Subject Payment Verification
I verify that I have received my subject payment of $75 for Study Visit 2. Signature:_____

Adverse Event:

UNIVERSITY OF DELAWARE

Adverse Event

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

ADVERSE EVENTS

Did the subject experience any Adverse Events? Yes ☐ No ☐

Adverse Event: ____

Serious Adverse Event (SAE): Yes _____ If Serious, please complete Form 7443

Start Date: _____/_____/_____
DD MMM YYYY

Stop Date: _____/_____/_____
DD MMM YYYY

Severity: Mild ☐ Moderate ☐ Severe ☐ Life Threatening ☐

Is there a reasonable possibility of a causal relationship between the test article and the AE?
☐ Yes
☐ No

Medical History

Condition ____________________	Start Date: _____/_____/_____ DD MMM YYYY ☐ Ongoing Stop Date: _____/_____/_____ DD MMM YYYY	Complete only if **ongoing**. Currently being treated? ☐ Yes ☐ No
Condition ____________________	Start Date: _____/_____/_____ DD MMM YYYY ☐ Ongoing Stop Date: _____/_____/_____ DD MMM YYYY	Complete only if **ongoing**. Currently being treated? ☐ Yes ☐ No
Condition ____________________	Start Date: _____/_____/_____ DD MMM YYYY ☐ Ongoing Stop Date: _____/_____/_____ DD MMM YYYY	Complete only if **ongoing**. Currently being treated? ☐ Yes ☐ No

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Adverse Event

Principal Investigator: Jillian Trabulsi, PhD RD
Co-investigator: Julie Mennella, PhD
Title: Infant feeding and biomarkers of satiation and satiety in healthy term infants

Outcome:

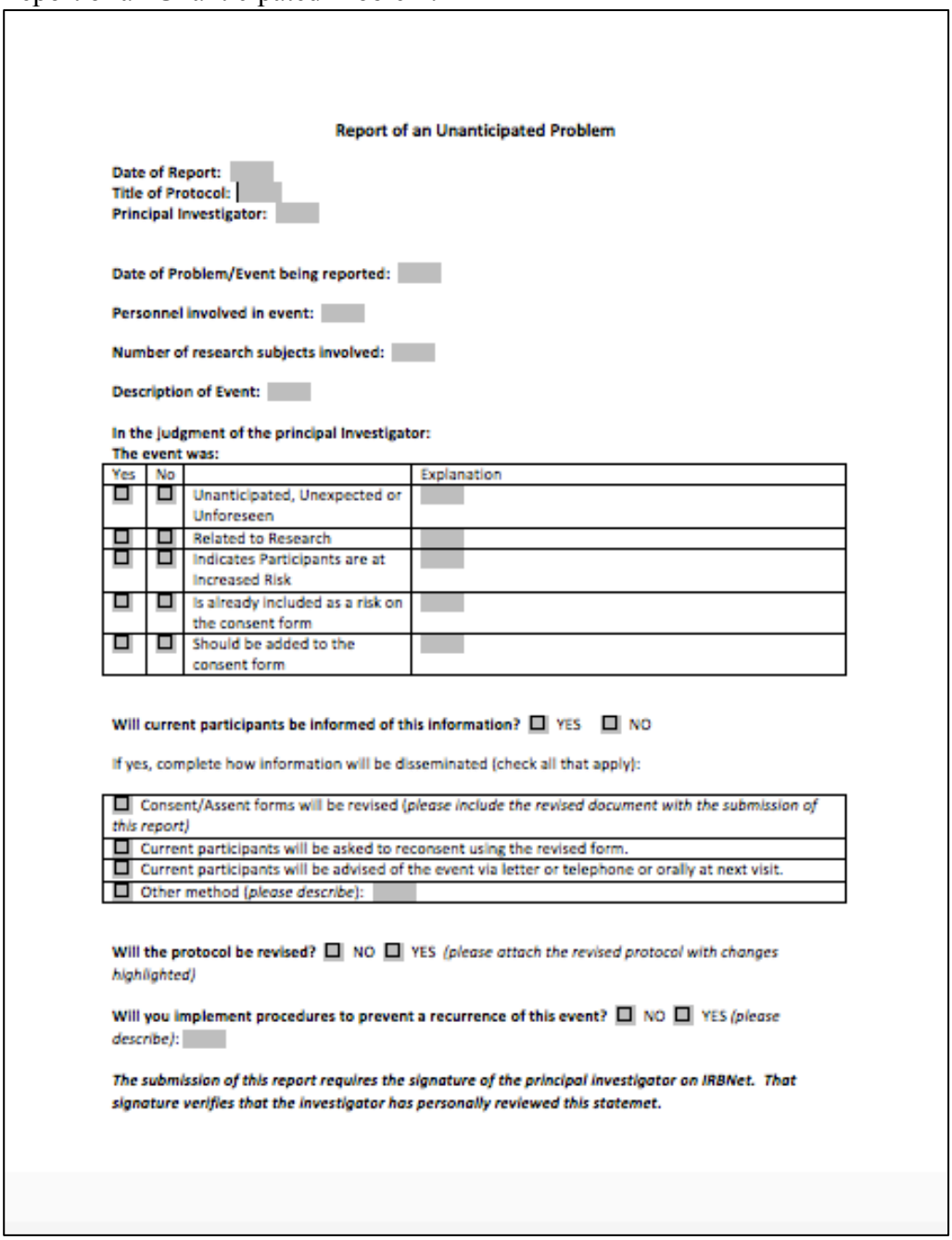
- ☐ Resolved
- ☐ Persisted
- ☐ Death

Action: (Check all that apply)

- ☐ None
- ☐ Withdrawn from study
- ☐ Other, please specify _____

Signature: _____ Date: _____

Report of an Unanticipated Problem:



Report of an Unanticipated Problem

Date of Report:
Title of Protocol:
Principal Investigator:

Date of Problem/Event being reported:

Personnel involved in event:

Number of research subjects involved:

Description of Event:

In the judgment of the principal investigator:
The event was:

Yes	No		Explanation
☐	☐	Unanticipated, Unexpected or Unforeseen	
☐	☐	Related to Research	
☐	☐	Indicates Participants are at Increased Risk	
☐	☐	Is already included as a risk on the consent form	
☐	☐	Should be added to the consent form	

Will current participants be informed of this information? ☐ YES ☐ NO

If yes, complete how information will be disseminated (check all that apply):

☐ Consent/Assent forms will be revised (*please include the revised document with the submission of this report)*
☐ Current participants will be asked to reconsent using the revised form.
☐ Current participants will be advised of the event via letter or telephone or orally at next visit.
☐ Other method (*please describe*):

Will the protocol be revised? ☐ NO ☐ YES *(please attach the revised protocol with changes highlighted)*

Will you implement procedures to prevent a recurrence of this event? ☐ NO ☐ YES *(please describe)*:

The submission of this report requires the signature of the principal investigator on IRBNet. That signature verifies that the investigator has personally reviewed this statemet.