I, Anne C Minter, hereby submit this original work as part of the requirements for the degree of Master of Science in Nutrition.

It is entitled:
The relationship between human milk adiponectin, maternal measures of metabolic health and anthropometrics

Student’s name: Anne C Minter

This work and its defense approved by:

Committee chair: Seung-Yeon Lee, Ph.D.

Committee member: Laurie Ann Nommsen-Rivers, Ph.D.

Committee member: Ardythe Luxion Morrow, Ph.D.

Committee member: Abigail Peairs, Ph.D.
The relationship between human milk adiponectin, maternal measures of metabolic health and anthropometrics

A thesis proposal submitted to the Graduate School of the University of Cincinnati

In partial fulfillment of the requirements for the degree of Master of Science in the Department of Nutritional Sciences of the College of Allied Health Sciences at the University of Cincinnati

By
Anne Minter
B.A. Miami University, 2002

Committee Chair: Seung-Yeon Lee, PhD
Committee member: Laurie Nommsen-Rivers, PhD
Committee member: Ardythe Morrow, PhD
Committee member: Abigail Peairs, PhD
Abstract

Adiponectin modulates a range of human physiological processes related to metabolism and inflammation. Human milk is known to contain adiponectin in a wide range of concentrations. Variation in adiponectin concentration remains poorly understood; thus we sought to determine its maternal anthropometric and metabolic correlates. The morning after an overnight fast, lactating mothers (N=20) provided an aliquot of milk from a complete breast expression, and underwent an oral glucose tolerance test (OGTT) and anthropometric measurement. Fasting serum (total and HMW) adiponectin and human milk adiponectin were determined by ELISA. Insulin and glucose concentrations were determined by standard clinical assays. Correlates of human milk adiponectin were evaluated using Spearman rank-order correlation coefficient (r). Adiponectin concentrations (mean [min-max] ng/mL) were: human milk, 30.7 (6.9-92.49) and serum total, 6489 (1356-20257). Human milk adiponectin and serum total adiponectin were positively correlated (r=0.45, p=0.048). Human milk adiponectin was not correlated with other full-body measures of maternal metabolic status (fasting glucose, fasting insulin, insulin sensitivity, or insulin secretion). Likewise, full-body anthropometrics (weight and BMI) were not correlated with milk adiponectin concentration. Reflective of more localized fat depots, body circumference measures became more strongly correlated with human milk adiponectin concentration with increasing proximity to the breast. Spearman correlations of circumferences with human milk adiponectin concentration were hip (r=-0.32, p=0.164); iliac crest (r=-0.46, p=0.039); waist (r=-0.50, p=0.025); under breast (r=-0.56, p=0.011). Our novel finding that under-breast circumference was the strongest correlate of human milk adiponectin suggests that the localized fat depots of the breast may be an important source of adiponectin in human milk. Further studies of how the specific fat depots of the breast and upper body independently influence the breast macro-environment and the bioactives in human milk are warranted.
Acknowledgements

This thesis grew out of a series of dialogues with Dr. Seung-Yeon Lee and Dr. Laurie Nommsen-Rivers. Many thanks to Seung-Yeon Lee, whose discipline, decisiveness, and energetic support enabled me to pull this thesis down from the ether and connect me to the socratic mind of Laurie Nommsen-Rivers. I am deeply indebted to Laurie-Nommsen Rivers, who works with divine commitment to ensure healthy breasts for all women and optimal nutrition for all infants, for the countless hours she contributed to the creation and completion of this work. She is an impassioned soul with worldly skills, whose editing suggestions and precise sense of language contributed to clarity throughout. Her patience and presence undoubtedly made me a much better researcher than I would otherwise have been. I would also like to thank Dr. Abby Peairs whose delightful demeanor, inquisitive mind, and clear communication provided me with multifaceted support throughout the research process. I am also beholden to Dr. Ardythe Morrow for including me in her tutelage of young female researchers. She mentors and nourishes the emerging feminine and scientific prowess in us all, and her brilliance and astute insights enriched my research and self-realization extensively. I would also like to thank Nathan Weikert for his unwavering commitment to divine partnership and for his steadfast support of my chosen work- no matter how serpentine and nonlinear the path may appear to be to the critical eye. Finally, deepest gratitude to John Minter and Cynthia Minter, for sharing their own intellectual passions with the author when she was a child, and for providing a boundless model of attainment.

This research was further made possible by the financial support of the University of Cincinnati Graduate Student Summer Fellowship (the author), the University of Cincinnati Center for Clinical and Translational Science and Training (LNR), and the Cincinnati Diabetes and Obesity Center (LNR).
# Table of Contents

Introduction ........................................................................................................................................... 1

Purpose ............................................................................................................................................... 3

Hypotheses .......................................................................................................................................... 3

Literature Review ............................................................................................................................ 4

Methods ............................................................................................................................................ 14

Results .............................................................................................................................................. 25

Discussion ......................................................................................................................................... 29

Bibliography ...................................................................................................................................... 32

Appendix ........................................................................................................................................... 38
List of Tables & Figures

Table 1- Blood Sample Collection..................................................................................................................15
Table 2- Milk Sample Collection......................................................................................................................16
Table 3- Anthropometric, Biochemical (serum & human milk), and Calculated Measures...............................17
Table 4- Characteristics of Study Participants................................................................................................25
Table 5- Maternal Anthropometrics................................................................................................................25
Table 6- Maternal Measures of Metabolic Health.............................................................................................26
Table 7- Milk Adiponectin Concentration.........................................................................................................26
Table 8- Spearman Correlation Coefficients (r) for Maternal Measures of Metabolic Health & Milk Adiponectin......27
Table 9- Spearman Correlation Coefficients (r) for Maternal Anthropometrics & Milk Adiponectin.......................27
Table 10- Multivariate Linear Regression........................................................................................................28
Figure 1- Location of Female Circumference Measures.......................................................................................38
Introduction

In-utero and the first year directly succeeding birth are sensitive periods of development. Nutritional and hormonal exposures (or lack thereof) during these time periods have significant effects on the physiological development of the infant and child, and are mediated through the mechanisms of developmental programming.\textsuperscript{1-3} When a human infant is fed in accordance with the American Academy of Pediatrics (AAP) guidelines, their earliest nutritional and hormonal exposures come in the form of human milk.\textsuperscript{4} The AAP recommends exclusive breastfeeding for 6 months, and this recommendation is supported by a large amount of data on the health outcomes of exclusively breastfed infants, compared to infants who never, or only partially, breastfed.\textsuperscript{5,6} One important finding from this previous research is the significant association of being breastfed with a reduced risk of metabolic disease later in life.\textsuperscript{7-9} The reduced risk of metabolic disease in the breastfed infant is thought to be due to the effect of specific hormones in human milk, including the adipocyte-derived hormone adiponectin.\textsuperscript{10} The adiponectin concentration in human milk is positively associated with infant adiposity and adiposity at two years of age.\textsuperscript{11,12} And, adiposity at two years of age is strongly correlated with adiposity as an adult.\textsuperscript{13}

The concentration of adiponectin in human milk varies greatly, but the full effect of this variation on the metabolic health of the infant consuming the milk is still unknown.\textsuperscript{14-16} In addition, the specific fat-depot source(s) of human milk adiponectin are still unknown. Given the known, and perceived, relationship of adiponectin to the immediate and future health of the breast-fed infant, it is crucial to develop our understanding of the factors that influence the concentration of adiponectin in human milk. Previous studies have examined the association between full-body anthropometrics (i.e. maternal BMI and weight) and milk adiponectin concentration, and found inconclusive results.\textsuperscript{17-19} To the best of our knowledge, no studies have evaluated the association between specific fat-depot anthropometrics (i.e. maternal hip, iliac crest, waist, and breast measurements) and milk adiponectin concentration. One previous study examined the association between maternal metabolic measures (i.e. fasting glucose, fasting insulin, insulin sensitivity, and insulin secretion) and milk adiponectin concentration.\textsuperscript{20} This study found no association between maternal metabolic measures and milk adiponectin concentration. However, this finding may have been the result of obtaining each woman’s milk sample 1-3 months after the collection of her serum sample.\textsuperscript{20} To date, no study has examined the association of maternal metabolic measures and milk adiponectin concentration using milk
samples and serum samples taken at the same time point. This study duplexes existing research by examining the relationship of maternal full-body anthropometrics and metabolic measures to human milk adiponectin concentration, but extends existing research by examining the association of maternal metabolic measures to human milk adiponectin concentration using milk and serum samples taken at the same time point, the ideal. This study also extends the existing research by examining the association of specific fat-depot measures (including circumferences of the hip, iliac crest, waist, and under-breast) to human milk adiponectin concentration.

Through the examination of specific fat-depot anthropometrics, we hope to find initial evidence for a new understanding of the maternal correlates and source of adiponectin in human milk. It has been widely assumed that human milk adiponectin originates in serum before it is transported across the mammary epithelial cells (MEC) into the milk supply.\textsuperscript{15,20} This assumption has led to the almost exclusive investigation of maternal correlates that are reflective of the serum adiponectin pool (i.e. full-body anthropometrics and metabolic measures) in prior research on this topic. Meanwhile, physiological research continues to show differences in the regulation and secretion of adiponectin based upon the specifics of the fat-depot location in the human body.\textsuperscript{21-32} This study examines specific fat-depot anthropometrics to preliminarily investigate the assumption that milk adiponectin may be coming from the more localized fat depots closest to the breast, and not exclusively from the full-body fat stores of the mother. We hypothesize that specific fat-depot anthropometrics will be more strongly associated with milk adiponectin concentration than full-body anthropometrics; and that within the examination of specific fat-depots, those closest to the breast will be more strongly associated with milk adiponectin concentration.

This thesis contains four main chapters: Literature Review, Methods, Results, and Discussion. The Literature Review begins with background information on the basic molecular biology of adiponectin, proceeds with an introduction to the lactation physiology of human milk adiponectin, and concludes with a brief review on the relationship of milk adiponectin concentration to known maternal factors. The Methods, Results, and Discussion sections describe the details of the original research study that form the basis of this thesis.
Purpose

The purpose of this study is to determine the association between maternal metabolic and anthropometric correlates and milk adiponectin concentration.

Hypotheses

1. Milk adiponectin will be positively correlated with serum adiponectin

2. Milk adiponectin will be negatively correlated with fasting glucose, fasting insulin, and positively correlated with ISOGTT, and ISSI-2.

3. Milk adiponectin will be negatively correlated with weight and body mass index (i.e. full-body anthropometrics).

4. Milk adiponectin will be negatively correlated with hip, iliac crest, waist, under breast, and full breast circumferences (i.e. specific fat depot anthropometrics); and will be progressively more negatively correlated with increasing proximity to the breast.
Adiponectin, an adipocytokine

Adiponectin is one of over 260 bioactive proteins secreted by the adipocyte. Both the overall size, and the specific location of the adipocyte within the various fat depots of the human body, influence the degree to which the adiponectin gene is expressed and the mechanisms of adiponectin secretion from the adipocyte. Other human cell types express and secrete adiponectin, but to a much lesser extent than adipocytes. Adiponectin modulates a range of human physiological processes related to metabolism and inflammation. A strong and consistent inverse association between serum adiponectin concentration and insulin resistance and inflammatory status has been established. High concentrations of circulating adiponectin in human serum have positive health effects through the reduction of pro-inflammatory cytokines, enhancement of insulin sensitivity, and increased fatty acid metabolism.

Adiponectin was discovered simultaneously by four independent research groups in the 1990s, when it received multiple names including AdipoQ (adipose specific gene adipoQ), ApM1 (adipose most abundant gene transcript 1), GBP28 (gelatin-binding protein of 28kDa) and ACRP30 (adipocyte complement-related protein of 30kDa). Today, adiponectin and ADIPOQ are the accepted names for the human variant and ACRP30 is the accepted name for the murine variant. Although the majority of adiponectin found in circulation is produced by adipocytes, the protein is also secreted by skeletal and cardiac myocytes, osteoblasts, lymphocytes and hepatocytes along with various cells of the adrenal gland, pituitary gland, salivary gland, placenta, ovary, testis, and epithelium.

Adiponectin gene

The human adiponectin gene is on chromosome 3, locus q27. Several single nucleotide polymorphisms at this locus are associated with multiple disease-state susceptibilities including type-2 diabetes (T2D), increased body mass index (BMI), increased waist circumference, dyslipidemia, hypertension, and coronary artery disease. The adiponectin gene consists of three exons and two introns. The promoter region has several regulatory elements including two SRE (sterol regulatory elements), one PPRE (PPAR-responsive element), several CCAAT/enhancer-
binding protein regions (or CCAAT boxes), one LRH-RE (liver receptor homologue 1-response element), and a few E boxes. For optimal expression, the transcription factors SREBP (sterol regulatory element binding protein), PPARγ (peroxisome proliferator activated receptor γ), C/EBP (CCAAAT/enhancer-binding protein), and LXR (liver X receptor) need to be recruited to the promoter region of the adiponectin gene. Also, PPARγ and LXR need to bind their appropriate ligands, and heterodimerize with the RXR (9-cis retinoid X) receptor, before they are able to generate the conformational change necessary to bind to the PPRE and LRH-RE response elements, respectively.

The corresponding natural ligands for PPARγ, LXR, and RXR are fatty acids & their metabolites, oxygenated derivatives of cholesterol & oxysterols, and retinoic acid. For optimal expression of the adiponectin gene, all three natural ligands must be consumed in the diet. Additional dietary and lifestyle factors necessary for the optimal transcription and translation of the adiponectin gene have been thoroughly and fluently reviewed elsewhere.

**Adiponectin monomer and isoforms:**

The gene transcript for adiponectin, or monomeric adiponectin (mAd), is 244 amino acids long and has four distinct structural domains including the: 1) N-terminal signal sequence, 2) variable region, 3) collagenous domain, and 4) C-terminal globular domain. The N-terminal signal sequence consists of 19 amino acids and functions to direct the gene transcript to the endoplasmic reticulum for post-translational modification. The variable region is 23 amino acids long and exhibits structural divergence between species. The collagenous domain is 66 amino acids long and is organized into 22 “GLY-X-Y” repeats, where X and Y represent any amino acid. In contrast to the variable region, the collagenous domain pattern is also highly conserved across species. The C-terminal globular domain is the receptor-binding domain, and the point of initial interaction with the adiponectin receptor. The globular domain exhibits a C1q-like structural homology, which allows for the molecular classification of adiponectin into the soluble defense collagen superfamily of proteins.

Monomeric adiponectin (mAd) is not secreted from the human adipocyte under normal conditions, and has no physiological function in vivo. Only full-length adiponectin isoforms (fAd), generated through post-translational modification of mAd, have physiological function in the human body. Posttranslational modification of mAd by
hydroxylation and glycosylation in the endoplasmic reticulum produces (fAd) trimers, which then assemble into higher-order (fAd) hexamers and (fAd) oligomers. Adiponectin is secreted from the adipocyte in five forms: 1-low molecular weight (LMW) trimer, 2-middle molecular weight (MMW) hexamer, various 3-high molecular weight (HMW) oligomers, 4-LMW bound to serum albumin (Alb-LMW), and 5-globular adiponectin (gAd). gAd is the isolated globular domain of the adiponectin molecule, cleaved from mAd (intracellularly) and fAd (in serum) by the enzyme leukocyte elastase. Leukocyte elastase is secreted from activated monocytes and neutrophils. Thus, inflammation is associated with increased concentrations of gAd in serum. Globular adiponectin can trimerize after cleavage, but cannot oligomerize further.

**Adiponectin in serum**

In non-obese healthy adults, the total adiponectin concentration (all five forms) in human serum is ~5-30 μg/ml. The HMW oligomer accounts for approximately 50% of total adiponectin in human serum, whereas the LMW and MMW multimers constitute another approximate 25% each. Globular adiponectin and Alb-LMW constitute less than 1% of total adiponectin. HMW adiponectin is considered the most metabolically active isoform, and a decrease in the concentration of HMW adiponectin in serum is related to insulin resistance, diabetes, and obesity.

**Adipocyte size and location: regulation of adiponectin**

Total adiponectin concentration is regulated by circadian rhythms, exhibiting a significant reduction at night. The half-life of circulating adiponectin is variable (~1 hour to 17.5 hours) and is largely determined by the presence of one final posttranslational modification in the endoplasmic reticulum. This modification is sialylation, the addition of sialic acid to the threonine (Thr) residues (O-linked glycans) of the variable region. Enzymatic removal of sialic acid from adiponectin accelerates its clearance from circulation, increasing with decreasing metabolic health.

**Adiponectin receptors**

Adiponectin exerts its physiological effects predominantly through the adiponectin receptors, AdipoR1 and AdipoR2. Like the classic G-protein coupled receptors (GCPR), AdipoR1 and AdipoR2 have seven transmembrane domains.
Unlike GPCRs, AdipoR1 and AdipoR2 have an internal N-terminus and an external C-terminus. AdipoR1 and AdipoR2 are 80% homologous, but AdipoR1 has a downstream signaling pathway distinct from that of AdipoR2. AdipoR1 acts via the adenosine monophosphate-activated kinase (AMPK) signaling, whereas AdipoR2 acts via peroxisome proliferator-activated receptor alpha (PPAR-\(\alpha\)) signaling. AdipoR1 has a strong affinity for both globular adiponectin and full-length adiponectin. AdipoR2 exhibits an intermediate affinity for globular and full-length adiponectin. Although the relative ratios of AdipoR1 to AdipoR2 vary from tissue to tissue, both are ubiquitously expressed. AdipoR1 and AdipoR2 are predominantly expressed in the muscle and liver tissue and have also been found in macrophages, osteoblasts, testis, endothelial cells of the vascular wall, various tissues of the central nervous system including the pituitary gland and the paraventricular and arcuate nuclei of the hypothalamus, adrenal gland, \(\beta\)-pancreatic cells, adipocytes, placenta, colon, fetal small intestine, and the mammary epithelial cells (MEC) of the human breast. Even though AdipoR1 and AdipoR2 are both found in the transcriptome database for human MEC, adiponectin, itself, is not. The molecule, T-cadherin, has been identified as a third adiponectin receptor. T-cadherin lacks the transmembrane and cytoplasmic domains of AdipoR1 and AdipoR2 and is, instead, bound to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor. T-cadherin is also ubiquitously expressed, but the highest expression is found in the cardiovascular system and the muscular system. T-cadherin specifically binds HMW multimers of adiponectin, but is not considered a signaling receptor, per se, because it lacks an intracellular signaling domain. T-cadherin has known function as a tumor suppressor protein when bound by the adiponectin molecule.

**Adipose tissue depot and adipocyte size influence on adiponectin synthesis and secretion**

Not all adipocytes are equal in regards to adiponectin expression and secretion. Differences in adiponectin expression and secretion are related to both adipocyte size and adipocyte location or depot. Human adipose tissue is 5% to 60% of total body weight and consists of many discrete anatomical depots, with each depot belonging to one of two main distributions: subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT). SAT constitutes >80% of the total adipose organ. The most commonly defined and studied SAT depots are the abdominal, gluteal, and femoral depots, and the SAT breast depot will also be considered here. VAT constitutes 10-20% of the
total adipose organ in men, and 5-10% in women. VAT depots are associated with the organs, are found intra- or retro-peritoneal, and commonly include the omental, mesenteric, epilogic, and perinephric depots. Derived from progenitor cells with different gene expression patterns, visceral and subcutaneous adipocytes are known to be structurally and functionally distinct. Adiponectin gene expression is higher in SAT than in VAT, leading to the difference seen in protein expression between the two depots. In non-diabetic, non-obese humans, the expression of adiponectin is higher in SAT adipocytes, but the secretion of adiponectin is three-fold higher in visceral adipocytes. However, not all findings have been consistent. Significantly, adipocyte metabolic activity within the distinct SAT depots was assumed to be homogenous until regional differences in gene expression among four SAT depots (upper abdomen, lower abdomen, flank, and hip) was shown in normal weight women. An understanding of the depot-differences in adiponectin synthesis and secretion is still ambiguous and requires further investigation.

Adipocytes exhibit a unique cellular morphology whereby lipid occupies 95% of the cell volume. Accumulation of lipid determines the overall cell size, which can range from 20 to 200 µM. As adipocytes grow in size, cell vasculature expands in proportion to expansion of the adipocyte volume. However, cellular volume sometimes exceeds vascular expansion, and the adipocyte becomes hypoxic (oxygen-deprived). Hypoxia activates the transcription factor, hypoxia inducible factor (HIF-1). HIF-1 induces changes in over 1,000 genes including the up-regulation of the inflammatory adipokines TNF-α, IL-6, and IL-12. These inflammatory adipokines down-regulate adiponectin and many enzymes required for the cellular secretion of adiponectin. Continuous increase in adipocyte size also parallels a continuous increase in the synthesis and secretion of monocyte chemoattractant protein-1 (MCP-1) by the adipocyte. MCP-1 attracts monocytes and macrophages, allowing them to infiltrate the adipose tissue. Once activated, the macrophages also secrete pro-inflammatory cytokines (TNF-α, IL-6, IL-8) which further suppress adipocyte production of adiponectin. Through these mechanisms, increasing adipocyte size results in decreasing serum adiponectin concentrations.
Adiponectin in human milk

Adiponectin was discovered in human milk in 2006. Woo et al (2009) distinguished the isomeric forms of adiponectin in human milk, and found the HMW isomer to be the only form present. Adiponectin exists in human milk in a concentration range of ~1-100 ng/mL, and the average concentration is 10-20 ng/mL. The function of adiponectin in human milk is not fully understood, yet the wide range in concentration may influence the long-term health of breast-fed infants in positive or negative ways. Previous studies have shown that adiponectin receptors are present in the human intestine and that the concentration of milk adiponectin consumed by the breast-fed infant is associated with both infant serum adiponectin concentration and infant weight and adiposity throughout the first two years of life. Collectively, these results reveal potential pathways through which maternal milk adiponectin may be influencing the metabolism and endocrine system of the developing infant. Several maternal associations with human milk adiponectin concentration have been examined in prior studies, including serum adiponectin, metabolic hormones, inflammatory markers, weight, and BMI. No prior studies have examined the association of maternal serum correlates taken from the same time-point as milk collection. No prior studies have examined the association between localized anthropometric measures (i.e. circumferences of hip, waist, breast, etc.) and human milk adiponectin concentration. This study fills the existing gap in the literature by investigating the association between maternal metabolic (taken at the same time point) and localized anthropometric correlates with milk adiponectin concentration.

Transport pathways of the mammary gland

Five known transport pathways are utilized by the mammary gland to transport molecules into milk. Four of these pathways are transcellular and one pathway is paracellular.

Transcellular Pathway 1: Exocytosis

Pathway 1 transports most aqueous-phase components into human milk, including proteins: The majority of proteins in human milk are synthesized and secreted by the mammary epithelial cells (MEC). After expression on the ribosome, secretory proteins of the MEC are transferred to the ER lumen where their signal sequences are cleaved.
and the protein molecule folds. Post-translational modifications are completed in the Golgi body. The proteins are then packaged into secretory vesicles. The secretory vesicles empty into the mammary alveoli or are transported through the apical membrane by transporters. MEC gene expression for the adiponectin receptors, but not adiponectin itself, suggests that milk adiponectin is not synthesized in the MEC but instead originates outside of it. Thus, it is assumed that adiponectin does not enter human milk through pathway 1.

Transcellular Pathway 2: Lipids and the Milk Fat Globule
Pathway 2 transports the majority of lipids into human milk. Triglycerides are synthesized in the smooth ER from precursor fatty acids and glycerol. These triglycerides coalesce into large droplets and are drawn into the apex of the cell where they eventually become enveloped by the apical membrane, separating off from the MEC and entering into the milk supply as the "milk fat globule". The occasional inclusion of a crescent of cytoplasm within the membrane enables other substances contained in the MEC membrane, or cytoplasm (e.g. phospholipids and cholesterol), to enter the milk. Since adiponectin is a protein molecule and not a lipid, it is assumed that adiponectin does not enter human milk though pathway 2.

Transcellular Pathway 3: Direct transport across the Basolateral and Apical Membranes
Pathway 3 transports small substances across the basolateral and apical membrane of the MEC, but it is still poorly understood. Pathway 3 is utilized by a limited number of small molecules including sodium, potassium, chloride, certain monosaccharides, and by most drugs and drug metabolites that enter the milk supply. Since adiponectin is a large molecule, it is assumed that adiponectin does not enter human milk through pathway 3.

Transcellular Pathway 4: Transcytosis
Pathway 4 transports intact proteins from sources outside the MEC. Intact proteins can cross into, and through, the MEC from the capillaries and interstitial fluid via Pathway 4. Pathway 4 is the predominant pathway utilized by intact
proteins from external sources, during full lactation. Many proteins, hormones, and growth factors utilize Pathway 4 to enter the milk supply from maternal plasma and interstitial fluid. Immunoglobulins are the paragon example of a molecule that utilizes this pathway. It is also assumed that adiponectin utilizes this pathway for entry into the human milk supply. Molecules that utilize this pathway bind to their receptors on the basolateral surface of the MEC cell. The entire ligand-receptor complex is then endocytosed and transferred across the cell. At the apical membrane of the MEC cell the extracellular portion of the receptor is cleaved and secreted along with the molecule (ligand) being transported. It is thought that Pathway 4 is predominantly utilized by adiponectin during full lactation. MEC cells do express AdipoR1 and AdipoR2, and AdipoR1 can be internalized and endocytosed by the cellular membranes of the MEC cells. However, no adiponectin transport role for MEC AdipoR has been observed, as of yet.

Paracellular Pathway 5
This pathway also allows for the direct transfer of molecules, including intact proteins, from the plasma to the milk supply. However, this pathway is closed during full lactation, and only open to molecular transport during pregnancy, infection (i.e. mastitis), involution (weaning), and during non-exclusive breastfeeding. During full lactation, even the passage of small molecular weight molecules between the MEC are impeded by the gasket-like tight junctions (Zonula occludens) that join the MEC cells tightly together. During pregnancy, with mastitis, and after involution- the tight junctions become leaky and allow components of the interstitial space to pass, unimpeded, into the milk. At the same time, milk components can enter the plasma. When the junctions are open, the mammary secretion has high sodium and chloride concentrations, a fact that is useful for determining when the paracellular pathway is open or closed. An increase in milk adiponectin has been observed at month 6 and month 12 of lactation, potentially reflecting an increased use of pathway 5 during this time period for adiponectin transport.

In summary, indirect evidence points to adiponectin most likely utilizing route 4 and/or route 5. In full lactation, route 4 would predominate.

**Association of milk adiponectin with serum adiponectin**
The adiponectin concentration in maternal serum is 1000-fold higher than the adiponectin concentration in human milk. Four separate studies report milk adiponectin concentration to be associated with serum adiponectin concentration. Savino et al. found a positive correlation $r=0.60$ ($p<0.001$) between serum adiponectin concentration and milk adiponectin concentration in milk expressed within the first 6 months postpartum in 60 lactating mothers. Weyermann et al. found a positive correlation $r=0.43$ (Spearman) ($p<0.0001$) between serum adiponectin concentration and milk adiponectin concentration expressed at 6 weeks postpartum. Woo et al. also found a positive correlation ($r=0.37$, $P<0.0001$) between serum adiponectin concentration and median milk adiponectin concentration in milk expressed at week 1 and at 3 months postpartum. Ley et al. found higher maternal serum adiponectin concentration in pregnancy to be associated with higher adiponectin concentration in early milk ($\beta + \text{SEE}: 0.102 \pm 0.032$; $P=0.002$) and in mature milk ($0.045 \pm 0.020$; $P=0.03$). It is sometimes assumed that milk adiponectin is sourced from maternal serum adiponectin. Ley’s finding— that 10% of the variation in week 1 postpartum milk adiponectin concentration can be explained by serum adiponectin (collected during pregnancy), while only 5% of the variation in 3 month postpartum milk can be explained by serum adiponectin— suggests that the predominant source of milk adiponectin may not be from systemic circulation. A large proportion of the variation in human milk adiponectin may be explained by factors other than serum adiponectin.

**Association of milk adiponectin with full-body anthropometrics**

The relationship between maternal full-body anthropometrics and milk adiponectin concentration is not clear. Maternal full-body anthropometrics, such as body weight and BMI, have been positively associated with milk adiponectin concentration in two studies, but no association was found in two others. Bronsky et al. found a positive association between pre-pregnancy body weight and milk adiponectin concentration $r=0.288$ ($p=0.027$). Martin et al. found a positive association between post-pregnancy BMI and milk adiponectin concentration ($\beta= 0.08 \pm 0.02$, $P<0.0001$) such that each unit increase in maternal BMI equated to an 8.33% increase in milk adiponectin concentration. However, Bronsky et al. also found no association between body weight at the time of delivery and milk adiponectin concentration (from milk collected 48h after the start of lactation). Likewise, Ley et al. found no
association between pregravid (30wk.) BMI and milk adiponectin concentration in early (1 wk. postnatal, $\beta=0.003 \pm 0.014, P=0.81$) and late (3 month, $\beta=0.019 \pm 0.010, P=0.06$) milk.\textsuperscript{19}

**Association of milk adiponectin with specific fat depot anthropometrics**

No studies have investigated the association between more localized measures of fat deposition (i.e. hip, waist, or breast) and milk adiponectin concentration.

**Association of milk adiponectin with prenatal metabolic measures**

Two research groups investigated the association between select measures of maternal metabolic health and milk adiponectin concentration. Ley et al. examined the association between prenatal metabolic measures (hyperglycemia, insulin resistance, and insulin sensitivity) and milk adiponectin concentration in milk samples taken during early lactation (1 wk. postnatal) and mature milk (3 m. postnatal), and found no association between prenatal metabolic measures and milk adiponectin concentration.\textsuperscript{19} Ozarda et al. investigated the association between maternal serum hormones and milk adiponectin concentration (serum and milk samples taken at the same time point, on any day between 1-180 postpartum) and found no correlation between milk adiponectin concentration and serum insulin ($r=0.011, p=0.910$), leptin (-0.103, $p=0.231$), or resistin (-0.143, $p=0.122$). Milk adiponectin concentration was found to be negatively correlated with serum estradiol ($r=0.366 p<0.001$), prolactin ($r=-0.444 p<0.001$), thyroxine T4 ($r=-0.355 p<0.001$), triiodothyronine T3 ($r=-0.291 p<0.001$), and cortisol ($r=-0.537 p<0.001$); and positively correlated with serum ghrelin ($r=0.458, p=0.01$).\textsuperscript{83}

**Association of milk adiponectin with temporally matched metabolic measures**

Outside of Ley et al (2012) no studies have investigated the association between milk adiponectin concentration and maternal metabolic measures; and none have done so where serum samples and milk samples were taken at the same point in time.
Methods

This is a cross-sectional study from a larger follow-up of two prospective cohort studies, examining the biochemical indicators of lactogenesis (BIL follow-up study, Nommsen-Rivers, PI). The BIL follow-up study examined the association between various measures of maternal metabolic health at 1-12 months postpartum and lactation outcomes. The BIL-follow up study was approved by the Cincinnati Children’s Hospital Institutional Review Board. All study visits were conducted in the Clinical and Translational Research Center (CTRC) at Cincinnati Children’s Hospital Medical Center.

Participants

The participants in this report are a subset of the BIL follow-up study. The subjects for the original two cohorts were recruited from a local hospital after delivering a healthy baby in the hospital’s mother-baby unit in the spring and summer of 2010 (BIL-I) and 2011 (BIL-II) Selection criteria for the original cohorts were: 1) breastfeeding mother of a full-term, healthy, singleton infant; 2) English-speaking; 3) aged >18 years; 4) no history of major breast surgery. Women who participated in these original prospective cohort studies were secondarily recruited over the phone at 1-12 months postpartum for participation in the BIL follow-up study. A brief screening questionnaire was administered over the phone to determine eligibility. Inclusion criteria were: 1) participation in the original BIL prospective cohort study; 2) not currently pregnant; and 3) no contraindication to glucose tolerance testing.

Procedures

If a participant was eligible and interested in the study, a research assistant scheduled a one time, 4-hour study visit at the CTRC. After obtaining informed consent the participant was admitted to an exam room for anthropometric measurements and an Oral Glucose Tolerance Test (OGTT). If the participant was still breastfeeding at the time of the study visit, four breast milk samples were collected after additional consent. Procedure details for serum collection and milk collection are described in Table 1 and Table 2, respectively.
Serum collection (for OGTT and other serum assays):

Participants were asked to consume no food or drink during the 10 hours prior to their study visit. A small catheter was inserted into the participant’s vein for the purpose of performing five blood draws. A baseline blood draw was performed. The participant was then asked to consume a 75g glucose beverage (Glucola). The Glucola was consumed within five minutes. Four more blood draws were performed after complete consumption of the Glucola beverage at 30 minutes, 60 minutes, 90 minutes, and 120 minutes post Glucola-load. Serum glucose, insulin, and adiponectin were assayed at the CTRC lab.

Table 1. Blood sample collection

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mL)</th>
<th>Time of sample collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baseline</td>
<td>4 mL Directly before (~5 minutes) glucose consumption</td>
</tr>
<tr>
<td>2</td>
<td>Time 30</td>
<td>3 mL 30 minutes after glucose consumption</td>
</tr>
<tr>
<td>3</td>
<td>Time 60</td>
<td>3 mL 60 minutes after glucose consumption</td>
</tr>
<tr>
<td>4</td>
<td>Time 90</td>
<td>3 mL 90 minutes after glucose consumption</td>
</tr>
<tr>
<td>5</td>
<td>Time 120</td>
<td>3 mL 120 minutes after glucose consumption</td>
</tr>
</tbody>
</table>

Breast milk collection:

Each participant was asked to donate four breast milk samples (Table 2.). The first milk sample was a complete breast expression from either breast (left (L) or right (R)). The milk was expressed in the PM (between 4 PM and 10PM), the evening before the study visit. The participant was asked to record the time of breast expression, and the breast expressed (L or R), on the milk sample container. The milk sample was stored in the refrigerator overnight and brought by the subject to the study visit in a small cooler packed with ice. The remaining three milk samples were expressed in the AM- between the 60 minute and 90 minute OGTT blood draws- on the morning of the study visit. The second milk sample (AM) was also a full breast expression, taken from the same breast that was expressed for the first milk sample (PM). The participant also expressed the third milk sample (foremilk) and the fourth milk sample (hindmilk) at the time same point as the second milk sample- but the milk expressed for the third and fourth samples
came from the breast not used for the first and second milk samples. The third milk sample was foremilk taken at the start of a breastfeeding or milk expression that occurred during the study visit. The fourth sample was hindmilk taken at the very end of the same feeding or expression. The milk sampling scheme was designed to examine variation in milk adiponectin that may occur with variation in sampling conditions.

Table 2. Milk sample collection

<table>
<thead>
<tr>
<th>Sample</th>
<th>Breast</th>
<th>Volume (mL)</th>
<th>Time of sample collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PM</td>
<td>R (or L)</td>
<td>10 mL</td>
<td>Evening before study visit (4PM-10PM)</td>
</tr>
<tr>
<td>2 AM*</td>
<td>R (or L)</td>
<td>10 mL</td>
<td>Morning of study visit (between time 60 and time 90)</td>
</tr>
<tr>
<td>3 Foremilk</td>
<td>L (or R)</td>
<td>2 mL</td>
<td>Morning of study visit (between time 60 and time 90)</td>
</tr>
<tr>
<td>4 Hindmilk</td>
<td>L (or R)</td>
<td>2 mL</td>
<td>Morning of study visit (between time 60 and time 90)</td>
</tr>
</tbody>
</table>

* The AM milk sample was used in all statistical analyses

Measures

Anthropometric measures and biochemical measures in milk and serum were obtained for each study subject. Additional measures were calculated. Measures obtained and calculated for each subject are described in Table 3.
Table 3. Anthropometric, Biochemical (serum & human milk), and Calculated Measures

<table>
<thead>
<tr>
<th>Anthropometric measures</th>
<th>Instrumentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>Scaletronix 5002 digital scale, to nearest 0.1 kg.</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>Holtain Harpenden Stadiometer, to nearest 0.1 cm.</td>
</tr>
<tr>
<td>Minimal Waist Circumference (cm)</td>
<td>Non-elastic measuring tape, to nearest 0.1 cm</td>
</tr>
<tr>
<td>Hip Circumference (cm)</td>
<td>Non-elastic measuring tape, to nearest 0.1 cm</td>
</tr>
<tr>
<td>Iliac Crest Circumference (cm)</td>
<td>Non-elastic measuring tape, to nearest 0.1 cm</td>
</tr>
<tr>
<td>Full Breast Circumference (cm)</td>
<td>Non-elastic measuring tape, to nearest 0.1 cm</td>
</tr>
<tr>
<td>Under Breast Circumference (cm)</td>
<td>Non-elastic measuring tape, to nearest 0.1 cm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biochemical measures</th>
<th>Instrumentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Glucose, g/dL</td>
<td>Glucose Oxidase Enzymatic Assay</td>
</tr>
<tr>
<td>Serum Insulin, iU/dL</td>
<td>Electrochemiluminescence Sandwich Assay</td>
</tr>
<tr>
<td>Serum Adiponecin (Total) μg/mL</td>
<td>Human Adiponecin Sandwich Elisa</td>
</tr>
<tr>
<td>Milk Adiponecin (Total) ng/ml</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculated measures</th>
<th>Instrumentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>BMI= mass (kg) / height (m)²</td>
</tr>
<tr>
<td>ISOGTT</td>
<td>ISOGTT= 10,000/ √ [(FPG X FPI) X (G X I)]*</td>
</tr>
<tr>
<td>ISSI-2</td>
<td>ISSI-2= AUC_ins/glu* x ISOGTT</td>
</tr>
</tbody>
</table>

*FPG=Fasting plasma glucose, FPI=Fasting plasma insulin, G=mean of all glucose samples, I=mean of all insulin samples. AUC_ins/glu=area under insulin curve/area under glucose curve.

Anthropometric measures:

Based on the National Health and Nutrition Examination Survey (NHANES) standard anthropometric protocols, weight and height were measured by trained clinical research CTRC staff prior to the baseline blood draw. Circumference measurements were taken by a trained research assistant between the 30-minute and 60-minute blood draw. The trajectories of circumference measurement can be seen in Figure 1. in the Appendix.
**Weight:**
The subject removed their shoes and outer clothing layers. Body weight was measured using a calibrated, digital adult stand-on scale (Scaletronix 5002). Weight was measured two times and recorded to the nearest 0.1 kg. If the two measurements differed by more than 0.2 kg, a third weight measurement was taken. The two closest weight measurements were averaged together and the average was recorded in the study data.

**Height:**
The subject was not wearing shoes. Standing height was measured using a calibrated, wall-mounted Harpenden Stadiometer (Holtain). Height was measured two times and recorded to the nearest 0.1 cm. If the two measurements differed by more than 0.5 cm, a third height measurement was taken. The two closest height measurements were averaged together and the average was recorded in the study data.

**Circumferences:**
All circumference measurements were taken with a non-elastic, retractable measuring tape. For each circumference value, the circumference measurements were taken two times and recorded to the nearest 0.1 cm. If the two measurements differed by more than 1 cm, a third circumference measurement was taken. The two closest circumference measurements were averaged together and the average circumference was recorded in the study data. Circumference measurements included: hip, iliac crest, minimal waist, under breast, and full breast.

**Hip circumference:**
The subject stood in a relaxed position with arms crossed over the chest and feet together. The measurement was taken over any clothing that covered the hip. The trained research assistant kneeled at the right side of the subject and placed the tape measure at the level of maximum extension of the buttocks. The measurement was taken in a horizontal plane at the level of maximal circumference around the buttocks, without compressing the skin. The hip circumference measurements were recorded to the nearest 0.1 cm.

**Iliac crest circumference:**
The subject stood erect with feet together, abdomen relaxed and arms crossed over their chest with hands resting on their shoulders. The subject removed or lifted any clothing that covered the iliac crest. The trained research assistant palpated the subject’s iliac crest on the right side (directly under the armpit along the mid-axillary line). Using a cosmetic pencil, an “X” was made at this point to mark the location of the right iliac crest. The zero end of the tape measure was held at the “X” on the right iliac crest. The other end of the tape measure was handed to a second research assistant, who was standing at the left side of the subject. A visual check was performed to ensure that the measuring tape was in a horizontal plane, a second “X” was made on the left iliac crest. The tape was pulled around the back of the patient until it met the zero end of the tape on the right iliac crest. The patient exhaled and relaxed the abdomen. The measurement was taken in a horizontal plane at the level of maximal circumference from “X” (right iliac crest) to “X” (left iliac crest), without compressing the skin. The full iliac crest circumference measurements were recorded to the nearest 0.1 cm.

**Minimal waist circumference:**
The subject stood in a relaxed position with arms crossed over the chest and feet together. The subject removed or lifted any clothing that covered the waist. The trained research assistant placed the measuring tape directly on the subject’s skin, without compressing the skin. The measurement was taken at the narrowest part of the torso as seen from the front view (at the natural waist), and at the end of a normal expiration. The minimal waist circumference measurements were recorded to the nearest 0.1 cm.

**Under breast circumference:**
The subject stood in a relaxed position with body erect, arms at sides, palms facing forward, and feet together. The measurement was taken in a horizontal plane at the level of maximal circumference under the breasts, without compressing the skin. The under breast circumference measurements were recorded to the nearest 0.1 cm.

**Full breast circumference:**
The subject stood in a relaxed position with body erect, arms at sides, palms facing forward, and feet together. The measurement was taken under the shirt but over the bra. The measurement was taken in a horizontal plane at the level of maximal circumference across the nipples, without compressing the skin. The full breast circumference measurements were recorded to the nearest 0.1 cm.
Biochemical measures/Serum:

Fasting Serum Glucose:

Serum glucose was assayed using a glucose oxidase enzymatic assay. In the glucose oxidase enzymatic assay, glucose oxidase (GOX) catalyzes the oxidation of glucose to D-glucono-δ-lactone and hydrogen peroxide (H₂O₂). In the presence of peroxidase (POD), H₂O₂ enters into a second reaction with ρ-hydroxybenzoic acid and 4-aminoantipyrine (4-AAP) yielding the quantitative formation of a quinoneimine dye complex that is measured at 500nm. The absorbance at 500 nm is proportional to the concentration of glucose in the sample. The two-step reaction is shown below

\[
\text{(GOX)} \\
1) \quad \beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{--------------------------}} \text{D-glucono-δ-lactone} + H_2O_2 \\
\text{(POD)} \\
2) \quad 2H_2O_2 + \rho\text{-hydroxybenzoic acid} + 4\text{-aminoantipyrine} \xrightarrow{\text{-------------}} \text{quinoneimine dye} + 4H_2O
\]

Fasting Serum Insulin:

Serum insulin was measured using an electrochemiluminescence sandwich immunoassay. Serum was incubated with biotinylated monoclonal insulin-specific antibody for capture, and monoclonal insulin specific antibody labeled with ruthenium for detection. Serum samples were added to the microtiter wells. Steptavidin coated microparticles were also added to the wells. The streptavidin binds with biotin in the solid phase platform. The magnetic microparticles were captured on an electrode and washed to remove any unbound substance. Voltage was applied to the complex/electrode medium inducing a chemiluminescence emission, measured by a photomultiplier. The concentration of insulin in the sample was determined with a calibration curve. The detection range for the assay was 2.6-24.9 µU/mL. Coefficients of variation were between 1.5%-2.5% and 2.1%.

Fasting Serum Adiponectin:
Total adiponectin and high molecular weight (HMW) adiponectin were measured using sandwich ELISAs. The microplate wells were coated with a monoclonal anti-human adiponectin antibody for capture. Serum samples were added to the microtiter wells. A second biotinylated monoclonal anti-human antibody bound to streptavidin horseradish peroxidase was added for detection. Serum adiponectin was quantified by monitoring horseradish peroxidase activities in the presence of the substrate 3,3', 5',5'- tetramethylbenzidine. The enzyme activity was measured spectrophotometrically by the increased absorbance at 450-590nm, with an increase in absorbance shown to be directly proportional to the amount of captured human adiponectin from the serum sample. The concentration of adiponectin in the sample was determined with a calibration curve.

Biochemical measures/Milk:
Milk samples collected during the CTRC study visit were assayed by the thesis author for total adiponectin concentration at CCHMC’s Department of Neonatology Human Milk Laboratory.

Milk Adiponectin:
Milk adiponectin concentration was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) validated for use with human milk (BioVendor-Laboratory Medicine, Czech Republic). After milk collection, whole milk samples were stored at -80°C until analysis. Before analysis the samples were thawed on ice at room temperature. Whole breast milk samples were diluted 3 times with a dilution buffer and assayed according to manufacturer’s directions. The microplate wells were pre-coated with polyclonal anti-human adiponectin antibody for capture. The milk samples were vortexed continuously while pipetting to ensure sample uniformity, and were added to the microplate wells. Polyclonal anti-human antibody conjugated with horseradish peroxidase (HRP) was used for detection. The detection antibody was raised against whole adiponectin and reacts with the whole molecule, including the globular domain. The detection range of the assay was 1-50 ng/mL. Assay sensitivity was 0.5 ng/mL, as reported by the manufacturer. A microplate reader (reference wavelength 630 nm) was used to read absorbance.

Calculated measures:
The following calculations were performed to create new measures that served as additional independent variables:

**Body Mass Index (BMI):**

BMI was calculated as weight in kg divided by the square of height in meters (kg/m$^2$). BMI is also known as the Quetelet index, as it was developed in 1835 by the Belgian mathematician Adolphe Quetelet. The equation used to calculate BMI is indicated below:

$$\text{BMI} = \frac{\text{mass (kg)}}{\text{height (m)}^2}$$

**Insulin Sensitivity (Matsuda index):**

Insulin sensitivity was calculated using the Matsuda index ($IS_{OGTT}$) devised by Matsuda and DeFronzo. The Matsuda index is a well-established measure of whole-body insulin sensitivity that has been validated against the gold standard measure, the euglycemic-hyperinsulinemic clamp. The Matsuda index calculation requires data obtained from an oral glucose tolerance test (OGTT), a 75-g glucose load following a 10-12 hour overnight fast. Blood samples are obtained at baseline (fasting) and at 30, 60, 90, and 120 minutes post-glucose load, for the determination of plasma glucose and insulin concentrations. The results are then entered into the following equation to determine the Matsuda index ($IS_{OGTT}$):

$$IS_{OGTT} \text{ (Matsuda index)} = \frac{10,000}{\sqrt{([\text{FPG} \times \text{FPI}] \times (G \times I))}}$$

Where 10,000 represents a constant that enables $IS_{OGTT}$ results in the range of 0 to 12, FPG= fasting plasma glucose (mg/dl), FPI = fasting insulin ($\mu$U/mL), G= mean of all glucose concentrations obtained from the OGTT, and I= mean of all insulin concentrations obtained from the OGTT.

**Insulin Secretion:**

Insulin secretion was calculated using the insulin secretion sensitivity index-2 (ISSI-2) following the method described by Retnarkaran. The ISSI-2 is an OGTT derived measure that is analogous to the gold standard oral disposition index obtained from the frequently sampled intravenous glucose tolerance test (FSIVGTT). The ISSI-2 is a measure of pancreatic beta cell function, defined as the product of 1) insulin secretion measured by the ratio of the area-under-the-insulin-curve to the area-under-the-glucose-curve ($AUC_{ins/glu}$) and 2) insulin sensitivity measured by the Matsuda index ($IS_{OGTT}$).
ISSI-2 = AUC_{ins/glu} \times ISOBT

**Statistical Analysis**

My primary aim is to evaluate the association between the independent variables (fasting serum adiponectin, fasting glucose, fasting insulin, insulin sensitivity (ISOBT), insulin secretion (ISSI-2), maternal weight, BMI, and hip, iliac crest, waist, and breast circumferences) and the dependent variable (milk adiponectin concentration). Descriptive statistics (including mean and standard deviation) were used to summarize the characteristics, primary variables, and covariates of the participants. Scatter plots were created to examine the data for nonlinear relationships between serum adiponectin concentration, fasting glucose concentration, insulin sensitivity, insulin secretion, maternal anthropometric measures, and milk adiponectin concentration. Non-normally distributed variables were log-transformed to improve normality, where possible. Outliers were examined. Spearman (rank-order) and Pearson (linear) correlation analyses were used to examine the correlations between concentrations of serum adiponectin and milk adiponectin. Univariate analyses were conducted to examine the unadjusted relation between serum adiponectin concentration, fasting glucose concentration, fasting insulin concentration, insulin sensitivity, insulin secretion, hip circumference, iliac crest circumference, waist circumference, under-breast circumference, and full-breast circumference and milk adiponectin concentration measures. Among normally distributed variables, multiple linear regression analysis was used to determine the independent effect of serum adiponectin concentration, fasting glucose concentration, fasting insulin concentration, insulin sensitivity, insulin secretion, and maternal anthropometrics on milk adiponectin concentration. Covariates were selected with two steps. First potential covariates and confounding variables were identified based on previous literature. These included stage of lactation, and parity. Then, the association of each covariate with dependent variable was examined using simple linear regression at the significance level $\alpha=0.1$. A confounding variable was defined as one that changed the $\beta$-coefficient for effects of fasting glucose concentration, insulin sensitivity, insulin secretion, and maternal anthropometrics on milk adiponectin concentration by more than 10% when it was added to the model. Only significant covariates and confounding variables were included. Development of the final model proceeded with identification, among the normally distributed variables, of the single strongest predictor of milk adiponectin concentration from among the
maternal metabolic measures and identification of the single strongest predictor of milk adiponectin concentration from among the maternal anthropometric measures. The two strongest predictors were used in the final model to examine their independent effects in a multivariate model. Level of significance was set at $p < 0.05$ for all of the analyses. All statistical analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC).
Results

Participant characteristics

Maternal demographic characteristics are listed in Table 4. The study sample ethnic distribution is reflective of the greater Cincinnati area population. The study sample had a higher education level, higher BMI, and higher maternal age when compared to the general postpartum population in the greater-Cincinnati area.18

Table 4. Characteristics of study participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>30.1± 5.2</td>
</tr>
<tr>
<td>Education, n(%)</td>
<td></td>
</tr>
<tr>
<td>High school graduate or less</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Some college or technical, vocational, or associate degree</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Bachelors degree</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Masters, Doctorate, or Professional degree</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Ethnicity, n(%)</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0 (0)</td>
</tr>
<tr>
<td>White, Non-Hispanic</td>
<td>15 (75)</td>
</tr>
<tr>
<td>Other,Mixed</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Obstetrical measures</td>
<td></td>
</tr>
<tr>
<td>% primiparous, n(%)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Infant birth weight (grams), mean ± SD</td>
<td>3661.20±481.6</td>
</tr>
<tr>
<td>Postpartum day of study visit, median (min-max)</td>
<td></td>
</tr>
<tr>
<td>Early postpartum (day 24-50), n(%)</td>
<td>13 (65)</td>
</tr>
<tr>
<td>Late postpartum (day 347-381), n(%)</td>
<td>7 (35)</td>
</tr>
</tbody>
</table>

Participant anthropometric measures

Participant anthropometry measures are summarized in Table 5.

Table 5. Maternal anthropometrics (n=20)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median [Interquartile range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Anthropometrics:</td>
<td></td>
</tr>
<tr>
<td>Postnatal BMI (kg/m²)</td>
<td>29.2 [25.9-32.3]</td>
</tr>
<tr>
<td>Postnatal Weight (kg)</td>
<td>75.12 [68.4-95.8]</td>
</tr>
<tr>
<td>Under Breast circumference (cm)</td>
<td>86.9 [83.3-98.8]</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>90.3 [85.6-98.0]</td>
</tr>
<tr>
<td>Iliac crest circumference (cm)</td>
<td>100.6 [91.0-109.7]</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>109.7 [103.6-126.3]</td>
</tr>
</tbody>
</table>
**Participant metabolic measures**

Participant metabolic measures are summarized in Table 6.

**Table 6. Maternal measures of metabolic health (n=20)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median [Interquartile range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measures of Metabolic Health:</td>
<td></td>
</tr>
<tr>
<td>Fasting Glucose (mg/dL)</td>
<td>89.0 [85.3-93.7]</td>
</tr>
<tr>
<td>Fasting Insulin (uIU/mL)</td>
<td>8.6 [6.2-10.2]</td>
</tr>
<tr>
<td>Insulin Sensitivity Matsuda Index (ISOGTT)</td>
<td>4.9 [3.7-7.0]</td>
</tr>
<tr>
<td>Insulin Secretion (ISSI-2)</td>
<td>1.9 [1.6-2.5]</td>
</tr>
<tr>
<td>Serum Adiponectin:</td>
<td></td>
</tr>
<tr>
<td>Adiponectin (Total) (ng/mL)</td>
<td>5896 [3901-8032]</td>
</tr>
</tbody>
</table>

**Participant milk**

Milk adiponectin concentrations are summarized in Table 7. The AM milk sample was collected under standardized conditions (fasting, between 8-10 AM) and was more strongly associated with maternal characteristics than the PM milk sample, for which collection was not standardized. There was a positive correlation between adiponectin concentration in the AM milk samples and PM milk samples r=0.84579 (P<0.001). For this reason, AM milk adiponectin concentration, alone, is used in all remaining statistical analyses. One participant was unable to produce a PM milk sample.

**Table 7. Milk adiponectin concentration**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median [Interquartile range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk Adiponectin:</td>
<td></td>
</tr>
<tr>
<td>AM Milk Adiponectin (ng/mL) (n=20)</td>
<td>24.7 [16.0-35.8]</td>
</tr>
<tr>
<td>PM Milk Adiponectin (ng/mL) (n=19)</td>
<td>21.5 [14.1-31.4]</td>
</tr>
</tbody>
</table>

**Correlations**

Correlations between milk adiponectin concentration and measures of maternal metabolic health are shown in Table 8. Milk adiponectin concentration was correlated with serum total adiponectin. Milk adiponectin concentration was not correlated with the other measures of maternal metabolic status including fasting glucose, fasting insulin, insulin sensitivity (ISOGGT), and insulin secretion (ISSI2). Milk adiponectin concentration was correlated with maternal anthropometrics. Correlations between milk adiponectin concentration and maternal anthropometrics are shown in Table 9. Anthropometric circumference measurements become more significantly correlated with human milk...
adiponectin with increasing proximity to the breast. All anthropometric measurements above, the iliac crest are moderately to highly correlated ($R^2 = 0.48\text{-}0.75$) to milk adiponectin concentration. Under-breast circumference was the strongest single correlate of AM human milk concentration. However, under-breast circumference was not normally distributed and therefore was not examined further in multivariate analyses.

**Table 8. Spearman rank-order correlation coefficient (r) for AM milk adiponectin concentration and maternal metabolic factors**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation to AM Milk Adiponectin</th>
<th>r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Adiponectin (HMW)</td>
<td></td>
<td>0.17</td>
<td>0.482</td>
</tr>
<tr>
<td>Serum Adiponectin (Total)</td>
<td></td>
<td>0.45</td>
<td>0.048</td>
</tr>
<tr>
<td>Fasting Glucose (mg/dL)</td>
<td></td>
<td>0.12</td>
<td>0.618</td>
</tr>
<tr>
<td>Fasting Insulin (uiU/mL)</td>
<td></td>
<td>-0.21</td>
<td>0.380</td>
</tr>
<tr>
<td>Insulin Sensitivity Matsuda Index (ISOGGT)</td>
<td></td>
<td>0.09</td>
<td>0.700</td>
</tr>
<tr>
<td>Insulin Secretion (ISSI-2)</td>
<td></td>
<td>-0.04</td>
<td>0.879</td>
</tr>
</tbody>
</table>

**Table 9. Spearman rank-order correlation coefficient (r) for AM milk adiponectin concentration and maternal anthropometrics**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Correlation to AM Milk Adiponectin</th>
<th>r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnatal Weight</td>
<td></td>
<td>-0.33</td>
<td>0.158</td>
</tr>
<tr>
<td>Postnatal BMI</td>
<td></td>
<td>-0.36</td>
<td>0.121</td>
</tr>
<tr>
<td>Under-breast circumference</td>
<td></td>
<td>-0.56</td>
<td>0.011</td>
</tr>
<tr>
<td>Waist circumference</td>
<td></td>
<td>-0.50</td>
<td>0.025</td>
</tr>
<tr>
<td>Iliac crest circumference</td>
<td></td>
<td>-0.46</td>
<td>0.039</td>
</tr>
<tr>
<td>Hip circumference</td>
<td></td>
<td>-0.32</td>
<td>0.164</td>
</tr>
</tbody>
</table>

**Multivariate Analysis**

The strongest metabolic predictor of milk adiponectin concentration was serum adiponectin concentration. The strongest anthropometric predictor of milk adiponectin concentration was under-breast circumference. However, since under-breast circumference was non-normally distributed, waist circumference was used as its proxy. Neither parity nor stage of lactation were significant covariates. The results of multivariate analyses are shown in Table 10. The final multivariate linear regression model revealed serum adiponectin and waist circumference to be significant independent predictors of milk adiponectin concentration, with the latter increasing with increasing serum adiponectin and decreasing with increasing waist circumference. For every 1 cm increase in maternal waist circumference, milk adiponectin concentration decreased by 0.02 ng/ml. For every 1 ng/ml increase in serum adiponectin, milk
adiponectin increased by 0.61 ng/ml. Together, serum total adiponectin and maternal waist circumference explained 47% of the variation in human milk adiponectin concentration. Notably, the combined predictive value of these two variables in an adjusted model is two-fold greater than expected based on the univariate results.

**Table 10. Multivariate Linear Regression**

<table>
<thead>
<tr>
<th>Model</th>
<th>Variable</th>
<th>β + std error</th>
<th>p-value</th>
<th>Model R² (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>Intercept</td>
<td>0.093 + 0.842</td>
<td>0.9132</td>
<td>0.068 (p=0.139)</td>
</tr>
<tr>
<td></td>
<td>Serum adiponectin (ng/mL)</td>
<td>0.349 + 0.225</td>
<td>0.1389</td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>Intercept</td>
<td>2.634 + 0.554</td>
<td>0.0002</td>
<td>0.176 (p=0.037)</td>
</tr>
<tr>
<td></td>
<td>Waist circumference (cm)</td>
<td>-0.014 + 0.006</td>
<td>0.0372</td>
<td></td>
</tr>
<tr>
<td>Model 3</td>
<td>Intercept</td>
<td>0.950 + 0.672</td>
<td>0.175</td>
<td>0.470 (p=0.002)</td>
</tr>
<tr>
<td></td>
<td>Serum adiponectin (ng/mL)</td>
<td>0.607 + 0.182</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Waist circumference (cm)</td>
<td>-0.019 + 0.005</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

This is the first study to examine the association between maternal metabolic measures and milk adiponectin concentration, taken at the same time-point postnatally. This is also the first study to examine the association between anthropometric measures of localized fat-depots and milk adiponectin concentration. There are two major findings in this study that advance our understanding of sources of variation in human milk adiponectin concentration. First, with the exception of total serum adiponectin, maternal metabolic measures were not associated with milk adiponectin concentration. Second, while full-body anthropometric measures (weight and BMI) were not associated with milk adiponectin concentration, as hypothesized, anthropometric measures of specific fat-depots (circumferences) were associated with milk adiponectin concentration. Circumferences of hip, iliac crest, waist, under-breast, and full-breast were all negatively associated with milk adiponectin concentration. The correlation coefficient for the association between the circumference measure and milk adiponectin concentration increased with increasing proximity to the breast, such that under-breast circumference was the strongest single correlate with human milk adiponectin concentration. Under-breast circumference was not analyzed in multiple regression models because it was non-normally distributed. The next closest normally distributed variable, waist circumference, was analyzed as its proxy. The finding that waist circumference and serum total adiponectin concentration together were able to explain almost half (47%) of the variation in human milk adiponectin concentration suggests that the adiponectin in human milk may be influenced by both systemic and local factors. In fact, when each of these factors was adjusted for the other in a multiple variable model, the predictive value of both strengthened, suggesting that both are important, but potentially counter-regulatory, predictors of milk adiponectin.

The wide range of human milk adiponectin concentrations (1-100 ng/mL) in the current study is reflective of previous reports, which suggest that milk adiponectin concentrations vary greatly among lactating women. As in previous research, this study found a significant, positive association between serum adiponectin concentration and milk adiponectin concentration. Assumptively, the adiponectin that enters human milk from maternal serum does so through Transport Pathway 4, carried by the internalized adiponectin receptor through the basolateral and apical membranes of the MEC cells into the milk supply. It is not known if (or how) this transport mechanism is regulated.
However, the consistent low to moderate correlation of serum adiponectin concentration with milk adiponectin concentration suggests that sources besides human serum may also significantly contribute to the variation in human milk adiponectin concentration. Among all the maternal metabolic correlates examined in previous research, serum adiponectin concentration was the only correlate found to be significantly associated with milk adiponectin concentration. While the other metabolic factors with known relationship to serum adiponectin concentration—including fasting glucose, fasting insulin, insulin secretion, and insulin sensitivity—showed no association with milk adiponectin concentration when they were examined prenatally by Ley et al.\textsuperscript{19} The findings of the current study mirrored those of Ley et al. but with predictor variables and an outcome variable taken from body fluids that were collected at the same time point, both postnatally. In addition, previous reports on the relationship between full-body anthropometrics and milk adiponectin concentration have not been consistent. A positive association between milk adiponectin concentration and pre-pregnancy body weight has been reported, as well as an increase in milk adiponectin concentration with increasing full-body fat deposition. However, multiple findings of no association between milk adiponectin concentration, and pre-pregnancy BMI and maternal body weight at the time of delivery have also been reported.\textsuperscript{11,17,19} Reflective of these findings, the current study found no association between postpartum BMI or weight and milk adiponectin concentration. The apparent lack of association of both metabolic measures and full body anthropometric measures with milk adiponectin concentration further supports the hypothesis of an additional source of adiponectin in human milk, beyond serum. When viewed from an integrated and comprehensive perspective, it can be reasonably postulated that some, but not all, of the adiponectin found in human milk may be coming from serum. Hypertrophic adipocytes produce and secrete less adiponectin than normal-size adipocytes. The finding of an inverse relationship between the circumference measurements of the waist and under-breast further suggest that the source of adiponectin in human milk may be, at least in part, from the localized fat depots closest to the breast.

Because all associations were reported in only 20 women, it will be important to confirm these findings in larger studies. With a larger sample size, it will be possible to look at the percent variation in human milk adiponectin concentration that is explained by serum adiponectin and under-breast circumference together in multivariate analysis. Another limitation of this study reflects the fact that under-breast circumference is a crude measure of the
specific fat depots of the breast. Using dual energy x-ray absorptiometry (DEXA) to assess the ratio of fat tissue to glandular tissue in the breast would be a better quantitative measure of the fat depots of the breast. Also, the current study enrolled breastfeeding mothers throughout their first year postpartum. Limiting the enrollment period to 4-6 months, for example, would better ensure homogeneity in stage of lactation among the study participants.

In summary, our results provide significant evidence that the two major sources of variation in human milk adiponectin, which together explain 47% of its variation, are serum adiponectin and waist circumference. Future studies, in a larger sample with more precise measures of upper body fat depots, are needed to fully elucidate sources of variation in human milk adiponectin concentration and how this variation impacts the developmental trajectory of the breastfeeding infant.


17. Bronsky, J. (2006). Adiponectin, adipocyte fatty acid binding protein, and epidermal fatty acid binding protein:
proteins newly identified in human breast milk. *Clinical chemistry (Baltimore, Md.),* 52(9), 1763-1770.


Appendix

Figure 1. Female Circumference Measurements

- Under-breast circumference
- Waist circumference
- Iliac-crest circumference
- Hip circumference