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Infant feeding, appetite and satiety regulation, and adiposity during infancy: Study design and protocol of the "MAS-Lactancia" birth cohort

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ABSTRACT

Introduction. Childhood obesity prevalence has risen dramatically in recent years. A proportion of this burden has been attributed to factors that occur during the first 1,000 days of life such as maternal nutrition, genetic predisposition, breastfeeding and complimentary feeding. The mechanisms by which these factors affect weight and adiposity are less well understood. However, appetite and satiety regulation may be key to understand them. This cohort study aims to investigate pre and postnatal factors that influence appetite and satiety regulation, which in turn may be associated with unhealthy eating habits, adiposity, and cardiometabolic risk factors later in life.

Methods and analysis. The MAS-Lactancia birth cohort is an open ongoing prospective cohort of mother-child pairs living in the city of Cuernavaca, Mexico and affiliated to the Mexican Social Security Institute. Women are enrolled into the cohort between 16 and 22 weeks' gestation. Enrollment began in 2016. Cohort participants are followed during the second half of their pregnancies, at birth, and throughout the infant's first 48 months of life (at 1, 3, 6, 9, 12, 18, 24, 36, 48 months) through a series of clinic and home visits with interviewer-administered questionnaires, anthropometric measurements and biospecimen collection. The main exposure variable is infant feeding (breastfeeding and complementary feeding), and outcome variables are satiety and appetite indicators (leptin, adiponectin, and insulin concentrations, appetite and satiety perception by mothers and dietary intake), growth, adiposity and metabolic risk factors. We will conduct longitudinal models and perform path analysis to identify the potential mediating role of satiety and appetite indicators.

Ethics and dissemination. The study protocol, data collection instruments and consent forms and procedures were approved by the Institutional Review Boards of the National

Institute of Public Health, and Mexican Social Security Institute in Mexico. Findings will be disseminated through conferences, peer-reviewed publications and meetings with stakeholders.

Keywords: Infant feeding, appetite, satiety, growth, adiposity, childhood obesity

ARTICLE SUMMARY

Strengths and limitations of this study

- The strengths of this cohort study are its intensive follow-up during pregnancy and the child's first 48 months and its thorough longitudinal collection of infant feeding information, appetite and satiety regulation indicators and anthropometry measures.
- This study will contribute to a better understanding of the effects of maternal obesity and unhealthy diets during pregnancy as well as infant feeding practices on relative weight gain and metabolic alterations in children from birth to 48 months.
- The results of this study are likely to provide evidence regarding early determinants of obesity and windows for action to prevent obesity throughout the life course.
- Limitations include low response rate in recruitment up-to-date and attrition rates which are comparable to those in other birth cohort studies.

INTRODUCTION

Excess body fat (overweight or obesity) affects 41 million children under 5 years globally [1] and is associated with increased metabolic risk factors later in life. Obesity results from a positive energy balance which is modulated by environmental and genetic factors.[2] A proportion of the obesity burden has been attributed to factors that occur during the first 1,000 days of life (i.e. from conception to age 2 years) such as maternal nutrition, excessive weight gain during pregnancy, genetic predisposition, birth weight, breastfeeding and complimentary feeding.[3-5] However, the mechanisms by which these factors affect weight and adiposity are less well understood; appetite and satiety regulation (ASR) being one of the most promising. Alterations to these mechanisms during gestation and the first two years of life potentially increase susceptibility to develop obesity throughout the life course (Figure 1).[6]

Maternal nutrition and appetite and satiety regulation

Maternal nutrition has been associated with offspring's appetite in animal studies.[7 8] Prenatal undernutrition may result in a cascade of genetic, biochemical and cellular alterations that affect appetite regulation in the developing offspring, for example alterations in hypothalamic neuropeptide gene expression and reduced sensitivity to leptin's anorexigenic effects.[7-9] Nutrient availability during pregnancy appears to be especially important in programming of offspring's appetite.[7 8] Observational studies in humans that aim to understand the association between maternal nutrition and ASR are scarce.

Early feeding practices and appetite and satiety regulation

Early feeding (< 6 months of age) is thought to be key for ASR[10] because it occurs during a period of biological plasticity and of behavioral modeling, which can determine long-term eating habits, growth outcomes and future metabolic responses. Further, breastmilk contains

adipokines such as leptin and adiponectin which in turn have been associated with insulin sensitivity, body composition and ASR.[11-13] Breastfed babies self-regulate intake in a more efficient way than bottle fed babies, which has also been proposed as a driver of ASR later in life. During the complementary feeding period ─the transition process between breastfeeding (or formula feeding) to the family diet─ the age at which new foods are introduced and the parents' infant feeding styles, along with genetic predisposition, determine food preference patterns and consumption that may influence the ASR in childhood and throughout the life span.[14-16]

Genetic polymorphisms and appetite and satiety regulation

Several genes have been linked to obesity development including polymorphisms in leptin, adiponectin[17] and FTO (Fat mass and obesity gene).[18] Evidence shows that expression of these genes is particularly relevant in the hypothalamus, which is consistent with the hypothesis that these genes are involved in the regulation of appetite and food intake. In observational studies, genetic susceptibility to childhood obesity seems to be partially explained by appetitive traits in infancy.[19 20]

Appetite and satiety regulation, growth, adiposity and metabolic risk factors

Appetitive traits such as emotional overeating and food responsiveness in early childhood have been associated with overeating, weight and metabolic risk factors in later life.[21 22] The cellular phenotype in obesity modifies the function of adipocytes and influences their micro-environment, increasing the secretion of pro-inflammatory cytokines, reactive oxygen species (ROS) and a parallel response in adipokines production. Moreover, ROS can also decrease insulin sensitivity and damage B cells in the pancreas which can in turn lead to glucose intolerance and even type II diabetes mellitus.[23] Once oxidative stress is established, a cascade of events is generated that can predict the rapid progression of the disease and the

development of complications. This suggests that oxidative stress could be used as an early indicator of the risk of cardiometabolic alteration associated with obesity.

[insert Figure 1 about here]

There are still many gaps in the literature regarding the association of pre and post natal factors with ASR and of ASR with growth, later life adiposity and metabolic risk factors in humans. To fill these gaps, a life-trajectory approach is key in order to consider prenatal factors as well as the feeding history and growth of the child, which are highly interrelated. Studies with reliable longitudinal infant feeding data and robust follow-up protocols are scarce in middle-income countries, which are currently experiencing a nutrition transition and are facing a growing prevalence of overweight and obesity. Understanding the nature of the associations of pre and postnatal factors with growth, adiposity and metabolic risk factors is crucial to design better and more efficient policies and programs for this stage of life within and outside the health system.

Our birth cohort study addresses some of these gaps. The cohort is referred to as "MAS-Lactancia" (the first word is an acronym in Spanish for "Appetite and Satiety Mechanisms" which also means "more" and the second word is "breastfeeding"). Our cohort is based in Mexico, an upper middle-income country that has experienced a fast nutrition transition, and is facing a large burden from obesity and associated chronic diseases.[24 25] Further, the prevalence of breastfeeding is among the lowest in Latin America and complementary feeding does not comply with international recommendations.[26 27] The overarching aim is to establish whether ASR is a mediator between maternal nutrition (dietary patterns and nutritional status during pregnancy), infant feeding (breastfeeding/formula, and CF) and genetic polymorphisms (FTO, leptin and adiponectin) with children's growth, adiposity, and

metabolic risk factors. In this manuscript we describe the design and data collection protocol for the ongoing MAS-Lactancia birth cohort study.

METHODS AND ANALYSIS

Primary Hypothesis

Inadequate infant feeding practices (such as inadequate breastfeeding duration, early food introduction, and high-energy food consumption) will be associated with: (1) appetite and satiety dysregulation in children at 12, 18 and 48 months of age, (2) rapid weight gain and greater adiposity in children at 12, 18 and 48 months of age. Also, (3) the association between feeding patterns and rapid weight gain, adiposity and metabolic risk factors will be partly mediated by appetite and satiety dysregulation and the presence of associated polymorphisms.

Design and study population

The MAS-Lactancia birth cohort is an ongoing, open prospective cohort of mother-child pairs living in the city of Cuernavaca, Morelos, Mexico (Figure 2) who are affiliated to the Mexican Social Security Institute (IMSS for its acronym in Spanish). IMSS provides health care and social protection to private sector formal employees and their families. It serves approximately 60% of the Mexican population. Women are enrolled in the cohort between 16 and 22 weeks' gestation. Cohort participants are followed during the second half of their pregnancies, at birth, and throughout the infant's first 48 months of life through a series of clinic and home visits with interviewer-administered questionnaires, anthropometric measurements and biospecimen collection.

The study aims to follow at least 400 mother-child pairs through the child's first 48 months. This target sample size (N=400) was calculated to be large enough to detect differences between breastfeeding modalities (exclusive and predominant breastfeeding, partial breastfeeding and no breastfeeding) for the primary outcome variables (weight gain, adiposity and appetite and satiety indicators). Based on 80% of power (1-β), with a sample of 400 participants (from which 15% or N= 60 are in the smallest breastfeeding modality group), it is possible to detect a 0.60 kg/m^2 difference in body mass index, 0.12 mm in sum of skinfold thicknesses, 0.30 mm in height and 0.40 mg/dL in insulin concentration between breastfeeding modalities at 95% confidence (α= 0.05, two – side). For the polymorphisms, we estimated that the sample of 400 children had sufficient power (80%) to identify the presence of 11 polymorphisms: FTO (rs9930506, rs9922708, rs17817449, rs7206790, rs9939609, rs7185735), Leptin (rs4731427, rs17151919) and adiponectin (rs266729, rs2241766, rs1501299) genes. Likewise, to identify the differences between explanatory (e.g. presence of polymorphisms) and outcome variables, this sample size can identify linear correlations of up to r=0.14 with a power of 80% and 95% confidence level. For the mediation variables, we considered that a minim sample of 200 will be adequate to identify direct and indirect relationships between main study variables according to Kline's methodology for Structural Equation Models.[28]

Eligibility, enrollment and follow-up

Enrollment into the cohort began March 2016 from two social security clinics (No. 1 and No. 20) providing antenatal care for IMSS-affiliated women in Cuernavaca, Morelos. All pregnant women attending the clinics from March 2016 to date are invited to participate in the study. Women that accept answer a screening questionnaire. If they meet the eligibility

criteria, they are invited to read and sign the informed consent. Women are eligible if they are between 18 and 39 years of age, between 16 and 22 weeks pregnant, living in Cuernavaca's metropolitan area and with plans to remain there over the next three years, planning on giving birth at the local social security hospital (Hospital General no. 1, IMSS), without previous diagnosis of hypertension, preeclampsia, renal, hepatic or cardiovascular diseases, accept to participate and sign the informed consent. Exclusion criteria are applied at the time of birth of the child as follows: preterm birth (<37 weeks' gestation), multiple pregnancy, evidence of maternal substance abuse, intrauterine growth restriction (low birth weight for gestational age), congenital diseases which may affect appetite, feeding and growth (i.e. cleft lip and palate, food allergies) and physical malformations which may affect anthropometric measurements.

All pregnant women enrolled in the study are offered breastfeeding counseling from week 34 of gestation onwards through a series of face-to-face sessions and printed materials. There are two reasons for offering counseling: 1) breastfeeding rates are very low in Mexico therefore, counseling is necessary to achieve sufficient sample with adequate infant feeding practices. There is evidence that breastfeeding advice and counseling can increase exclusive breastfeeding rates up to 3.5 times during the neonatal period and 5.2 times at six months [29]; 2) breastfeeding counseling is an incentive for participation and retention.

Table 1 presents an overview of the data and measurement collection for the cohort. Trained and standardized interviewers administer questionnaires, collect in-person measurements, and biospecimens (blood, breastmilk and saliva).

Table 1. Data collection in the MAS Lactancia Cohort.

	Pregnancy (weeks)		Postnatal time points (months)										Data source
	16-22	34	Birth	1	3	6	9	12	18	24	36	48	
Maternal variables													
Sociodemographic and demographic	X												Questionnaire
Employment and family composition	X											X	Questionnaire
Medical history	X	X											Questionnaire & MR
Diet	X	X		X				X	X	X	X	X	24-hour dietary recall
Anthropometry	X	X		X				X	X	X	X	X	Measured
Smoking	X	X		X				X	X	X	X	X	Questionnaire
Leptin in breastmilk				X									Lab analysis
Adiponectin in breastmilk				X									Lab analysis
Nutrition composition of breastmilk				X									Lab analysis
Parenting styles[1]				X	X	X	X	X	X	X	X	X	Parenting styles questionnaire
Infant variables													
Birth weight and length			X										Measured/MR
Breastfeeding practices and complementary feeding				X	X	X	X	X	X	X			Questionnaire
Diet				X	X	X	X	X	X	X	X	X	24-hour dietary recall
Sleep duration/sleeping habits [2]				X	X	X	X	X	X	X	X	X	Questionnaire
Appetitive traits[3]				X	X	X	X	X	X	X	X	X	BEBQ and CEBQ
Primary caregivers[4]				X	X	X	X	X	X	X	X	X	Questionnaire

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Infant morbidity				X	X	X	X	X	X	X	X	X	Questionnaire
Physical Activity												X	Questionnaire
Weight				X	X	X	X	X	X	X	X	X	Measured
Length				X	X	X	X	X	X	X	X	X	Measured
Skinfolds				X	X	X	X	X	X	X	X	X	Measured
Abdominal circumference				X	X	X	X	X	X	X	X	X	Measured
Head circumference			X	X	X	X	X	X	X	X	X	X	
Leptin concentrations					X			X				X	Lab analysis
Adiponectin concentrations					X			X				X	Lab analysis
Glucose concentrations					X			X					Lab analysis
Insulin concentrations					X			X				X	Lab analysis
Malondialdehyde												X	Lab analysis
Presence of polymorphisms in obesity related genes (FTO, leptin and adiponectin)								X	X	X	X	X	Lab analysis
Body composition									X	X	X	X	DXA

MR: Medical record [1]Brief Screening Questionnaire for Infant Sleep Problems,[30] [2]Through maternal perception, Child Eating Behavior Questionnaire (CEBQ) and Baby Eating Behavior Questionnaire (BEBQ).[31 32] [3]Questionnaire applied to the mother to identify principal caregivers. [4]Infant Feeding Style Questionnaire (IFSQ)[33]

Primary outcomes

Appetite and satiety regulation

Operationalizing ASR is complex because the physiological regulation of appetite and satiety includes a network of mechanisms interacting with each other (signaling cascades that include hormones and effectors). Therefore, we do not consider ASR as an individual variable that categorically identifies a state of satiety or appetite. Rather we use a series of variables that complement each other and will allow us to understand, at least in part, the regulation of ASR: 1) scales of maternal perception of appetite and satiety of their child; 2) biomarkers in child's blood; 3) child's diet.

We apply the Baby Eating Behavior Questionnaire (BEBQ)[31] and the Child Eating Behavior Questionnaire (CEBQ)[32 34] at months 1, 3, 6, 9, 12, 18, 24, 36 and 48. The CEBQ is a parent-report measure comprised of 35 items, each rated on a five-point Likert scale that ranges from never to always. It is made up of eight scales: food responsiveness, emotional over-eating, enjoyment of food, desire to drink, satiety responsiveness, slowness in eating, emotional under-eating, and food fussiness. The BEBQ was derived from the CEBQ and consists of 18 items that identify four distinct appetitive constructs: enjoyment of food, food responsiveness, slowness in eating and satiety responsiveness.[31] Both instruments are standardized measures of infant appetite designed to characterize appetitive traits that have been associated to excess weight gain.[35] Both questionnaires were validated for the study. The validation involved the translation to Spanish and the retranslation (to English to assess consistency), adaptation, clarification test and psychometric analysis. After the clarification test, the adapted Spanish version of the instruments was submitted to the commissions for approval.

Children's leptin, adiponectin and insulin blood concentrations are measured at months 3 (5mL sample) and 12 (7.5 mL sample). Blood samples are collected by a trained phlebotomist using standard pediatric venipuncture protocols. These hormones are associated with appetite regulation, energy balance and adiposity. Serum, and whole blood are stored at -80°C until analyzed in The National Institute of Medical Science and Nutrition SZ. Hormones are determined by ELISA using commercial kits (Merk-Millipore).

Child's diet information is collected to complement the above indicators using a standardized 24-hour dietary recall (24HR) applied to the mother using an automated software.[36] We follow an iterative multi-step procedure to increase the accuracy of the recall.[37] In the first step, a preliminary list of foods consumed in the last 24 hours is recorded, the interviewer then prompts the mother about commonly omitted foods (e.g. added sugar), this is followed by recalling the time and situation when the food was consumed, finally the interviewer reviews responses and makes final additions or revisions. The 24HR is collected at 1, 3, 6, 9, 12, 18, 24, 36 and 48 months. The 24HR allows estimation of total energy, macronutrient and food group intakes to complement information from the questionnaires and biomarkers.

Infant growth and adiposity

Anthropometric measurements: weight, height, arm and abdominal circumference, and skinfold thicknesses of the child are taken at birth, 1, 3, 6, 9, 12, 18, 24, 36 and 48 months of age using standardized procedures.[38 39] A portable electronic pediatric scale (Tanita BABY MOMMY model 1582) with a precision to the nearest 10g is used to measure weight. A wooden infantometer (Schorr) with a precision of 1mm is used to measure height. Circumferences are measured using a fiberglass tape accurate to 1mm and skinfold thickness using a Langer caliper accurate to 1mm. All measurements are taken twice and averaged, and Skinfold thicknesses are measured in triplicate. We use WHO-Anthro software (v.3.2.2,

2011) to estimate Z-scores of weight-for-height (WHZ) and Z-score of height for age (HAZ) based on World Health Organization (WHO) growth standards.

Birth weight is obtained from two sources: 1) from the medical record and 2) measured by the cohort personnel within 48 hours of birth. Body composition (fat and lean mass) is measured using a (dual-energy X-ray absorptiometry) DXA scan model General Electric Lunar Prodigy at month 18, 24, 36 and 48. The DXA scan provides an accurate measure of body composition,[40] is noninvasive and has been previously used in infants and children.[41 42]

Metabolic risk factors

At 48 months, unstimulated saliva samples will be obtained to determine leptin, adiponectin and insulin which will serve both as biochemical indicators of appetite and satiety regulation and metabolic risk factors, as well as malondialdehyde as an oxidative stress biomarker. We will use the method of TBARS described by Richard *et al.*[43] Remaining saliva samples will be stored and conserved in order to increase determinations in the future of another interesting metabolic risk factors such as inflammatory cytokines.

Primary exposures

Infant feeding including breastfeeding and complimentary feeding

We use a questionnaire previously used in the National Nutrition and Health Surveys of Mexico[44] to elicit information on breastfeeding and complimentary feeding practices. It enquires about feeding practices the day before the interview. Questions related to breastfeeding include: when and if breastfeeding was initiated after birth, type of milk fed to infant, frequency, duration, mode (breast/bottle) and reasons for breast/bottle feeding. The complimentary feeding questionnaire includes questions related to: type of foods consumed during the previous day, and age at which certain foods were introduced i.e. water, milk,

sugar sweetened beverages, cereals, pulses, vegetables, animal protein and dairy. This questionnaire is applied at 1, 3, 6, 9,12, 18, and 24 months of age.

Breastfeeding is classified according to WHO recommendations as: 1) exclusive, when only breast milk is fed to the infant; 2) predominant, when in addition to breastmilk, the infant is given water, unsweetened tea and other beverages excluding formula milk; 3) partial, when in addition to breastmilk the infant is given formula, solid foods or other milks; and 4) not breastfeeding.

Adequate complimentary feeding is defined as follows: the child's diet must include at least one food from each food group (fruits and vegetables; pulses, eggs and meats; dairy; cereals), it must not include more than 12% of total energy from the summation of the following groups: sugar sweetened beverages, milk or formula with added sugar, processed foods with >15% added sugars or >13% saturated fat or >275 kcal/100g. Additionally, consumption of estimated added sugar[45] must not exceed 5% of total energy intake.

Adequate infant feeding is defined as follows: exclusive or predominant breastfeeding up to 6 months of age, some breastfeeding and adequate complimentary feeding between 6 and 11 months of age, and adequate complimentary feeding with or without breastfeeding from 12 to 18 months of age.

Other variables: Maternal characteristics

Demographics. At enrollment, information on marital status, mother and father's education level, place of birth, occupation, household income and family composition is collected. Household characteristics and asset ownership such as TVs or vehicles are also recorded.

Medical history. Women are asked about previous diseases, medicines taken and nutrition supplements. Pre-pregnancy weight and height are self-reported. First day of last menstrual

period, bleeding during pregnancy, diagnosed chronic diseases are obtained from clinical records.

Anthropometric measurements. Weight, height, arm and waist circumference and skinfold thickness are measured at recruitment, 34 weeks' gestation, 1, 12, 18, 24, 36 and 48 months after giving birth. Anthropometric measurements are taken using Lohman's Anthropometry Manual as Reference.[39] Personnel were trained and standardized at the beginning of the study to ensure consistent and accurate measurements.[38] Maternal weight is measured on a Tanita scale, model 1582 accurate to the nearest 10g. Standing height is measured using a Schorr stadiometer accurate to the nearest 1mm. Circumferences are measured using a fiberglass tape accurate to 1mm and skinfold thickness using a caliper accurate to 1mm. All measurements are taken twice and averaged.

Diet. A 24-hour recall is applied at 34 week's gestation, months 1, 18, 24, 36 and 48 postpartum. The same procedure described for children is followed.

Parenting styles. The Infant Feeding Style Questionnaire (IFSQ)[33] is applied to women when their child is 1, 3, 6, 9, 12, 18, 24, 36, and 48 months old. The IFSQ measures feeding beliefs and behaviors among mothers of infants and young children. It identifies five feeding styles: laissez-faire, restrictive, pressuring, responsive and indulgent.[33]

Breast milk composition*:* A sample of breastmilk is collected using an electric pump one month postpartum, during the morning in our research facility. The total amount of milk extracted from one breast (approximately 35-50 mL) is collected in sterilized glasses and it is homogenized and stored at -80°C.[46 47] Leptin, adiponectin and nutrient composition of breastmilk is then measured.

Other variables: Child Characteristics

Sleep. Sleep duration Questionnaire at months 1, 3, 6, 9, 12, 18, 24 and the Brief Screening Questionnaire for Infant Sleep Problems[30] is applied to women to elicit information about their child's sleeping habits at 48 months. This instrument has been previously validated against objective sleep measurements.[30]

Morbidity. Morbidity information includes a list of infectious diseases and accidents collected at months 1, 3, 6, 9, 12, 18, 24, 36 and 48.

Polymorphisms in obesity-related genes. Using the infant blood drawn at month 12 (or 18, 24, 36 or 48 moths) we will genotype polymorphisms: LEP rs4731427 y rs17151919, ADIPOQ rs266729, rs2241766 y rs1501299 y FTO rs9930506, rs9922708, rs17817449, rs7206790, rs9939609 y rs7185735. Blood samples are being stored at -70°C. Once we achieve the study sample size, we will extract DNA using commercial kits (Quiagen).

Other variables: Environment

Environmental and area-level data can be linked to participants by leveraging public use datasets and geographic information systems. Participant's households are georeferenced and area-level information regarding poverty, education, public services, built and food environment, and safety among others will be linked to participants' home addresses.

Patient and public involvement

Participants were not invited to comment on the study design of this cohort study. However, there is a Facebook group that participants can join to share their experience of participating in the study. The results of the study will also be disseminated through this channel to ensure that participants benefit from the research.

Statistical analyses

We will conduct adjusted regression analyses to understand the associations between our exposure and our outcome variables at different time points. We will integrate all time points into a system of regressions with structural equation models to conduct a longitudinal, life-course analysis. This type of analyses will enable us to understand the effect at each follow-up period. Given that this age period is very dynamic in terms of growth and the feeding modes, we will perform longitudinal models that will capture these transitions. In addition, we will perform path analysis to identify the mediation role for some variables.

When the associations investigated require the use of numerous models, we will consider multiple comparison analyses, such a Bonferroni correction.

STRENGTHS AND LIMITATIONS OF THIS STUDY

The main strengths of this cohort lie in its intensive follow-up during pregnancy and the first 48 months postpartum, its thorough longitudinal collection of infant feeding information, ASR and anthropometry measures. The core project will allow us to answer a number of research questions related to the cohort aims which will significantly expand what is known on these topics. Looking ahead, we have managed to secure two additional grants, from CONACYT and Rio Arronte Foundation. These grants will allow for an extension of the follow-up period and to address new research questions related to complimentary feeding and sweetness preferences.

It is important to highlight that the cohort was not planned to be representative of the Mexican general population since we are recruiting from the social security system in one city. Our intention with this study is not to make inferences about the frequency and distribution of public health issues (we have nationally representative surveys for that purpose), but to investigate associations between biologic and behavioral exposures with health outcomes. In

terms of limitations of this study, we are observing relatively low response rates compared to national surveys in Mexico and attrition rates which are comparable to other international cohorts. A thorough analysis of the implications of these limitations will be performed once the sample is complete. Selection into the sample may be associated with health consciousness, which can decrease the variability in both exposures and outcomes, and/or bias our results.

ETHICS AND DISSEMINATION

Written informed consent is obtained at study entry for each participant and her child. The study protocol, data collection instruments and consent forms and procedures were approved by the Institutional Review Boards of the National Institute of Public Health, and Mexican Social Security Institute in Mexico. Results will be disseminated through peer review studies and communication with local health authorities.

AUTHOR CONTRIBUTIONS

JRD and IRS were responsible for the conception of the study. JRD, IRS, CBR, ACA, AB, EZ, MS, MTO, ADS, RM, UR, LA and ACP were responsible for the design of the study. JRD as well as IRS, ACA and CBR obtained funding for initial and later follow ups, respectively. IRS, SB and ACA were responsible for acquisition and analysis of data. CPF, IRS, ACA and MTO were responsible for drafting the manuscript. All authors critically revised the manuscript for important intellectual content, approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1. Conceptual framework of the determinants of appetite and satiety regulation in young children and their association with growth, adiposity and metabolic risk factors

Maternal Nutrition
- Nutrition status (Undernutrition/obesity)
- Unhealthy diet

Perinatally programmed appetite

Infant Feeding habits
- Breastfeeding
- Complementary feeding

Postnatal programmed appetite

Appetite and satiety dysregulation

Inadequate Eating Habits (Dietary patterns and eating behaviors)

Rapid weight gain

Greater Adiposity

Genetic predisposition
Genes: FTO, Leptin
Adiponetin
(polymorphisms)

Stress Oxidative

Cardiometabolic alterations

Prenatal stage

Early postnatal stage (0 – 12 months)

Later postnatal stage (Toddlers)

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Infant feeding, appetite and satiety regulation, and adiposity during infancy: Study design and protocol of the "MAS-Lactancia" birth cohort

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Infant feeding, appetite and satiety regulation, and adiposity during infancy: Study design and protocol of the "MAS-Lactancia" birth cohort

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ABSTRACT

Introduction. Childhood obesity prevalence has risen dramatically in recent years. A proportion of this burden has been attributed to factors that occur during the first 1,000 days of life such as genetic predisposition, breastfeeding and complimentary feeding. The mechanisms by which these factors affect weight and adiposity are less well understood, however, appetite and satiety regulation may be key to understand them. This cohort study aims to investigate the role of appetite and satiety regulation as a mediator in the association between infant feeding practices and genetic polymorphisms with children's growth, adiposity and metabolic risk factors.

Methods and analysis. The MAS-Lactancia birth cohort is an open, ongoing, prospective cohort of mother-child pairs living in the city of Cuernavaca, Mexico and affiliated to the Mexican Social Security Institute. Women are enrolled into the cohort between 16 and 22 weeks' gestation. Enrollment began in 2016. Cohort participants are followed during the second half of their pregnancies, at birth, and throughout the infant's first 48 months of life (at 1, 3, 6, 9, 12, 18, 24, 36, 48 months) through clinic and home visits with interviewer-administered questionnaires, anthropometric measurements and biospecimen collection. The main exposure variables are infant feeding (breastfeeding and complementary feeding), and genetic polymorphisms (FTO, leptin and adiponectin) and outcome variables are satiety and appetite indicators (leptin, adiponectin, insulin concentrations, appetite and satiety perception by mothers and dietary intake), growth, adiposity and metabolic risk factors. We will conduct longitudinal models and perform path analysis to identify the potential mediating role of satiety and appetite indicators.

Ethics and dissemination. The study protocol, data collection instruments and consent forms and procedures were approved by the Institutional Review Boards of the National

Institute of Public Health, and Mexican Social Security Institute in Mexico. Findings will be disseminated through conferences, peer-reviewed publications and meetings with stakeholders.

Keywords: Infant feeding, appetite, satiety, growth, adiposity, childhood obesity

ARTICLE SUMMARY

Strengths and limitations of this study

- MAS-Lactancia is an ongoing, open prospective cohort of mother-child pairs living in the city of Cuernavaca, Mexico.
- We aim to investigate whether infant feeding practices and genetic polymorphisms are associated with appetite and satiety regulation, which in turn may be associated with adiposity, and cardiometabolic risk factors later in life.
- The strengths of this cohort study include its intensive follow-up during pregnancy and the child's first 48 months, and thorough longitudinal collection of infant feeding information, appetite and satiety regulation indicators and anthropometry measures.
- Limitations include low response rate in recruitment up-to-date and attrition rates which are comparable to those in other birth cohort studies.

INTRODUCTION

Excess body fat (overweight or obesity) affects 41 million children under 5 years globally [1] and is associated with increased metabolic risk factors later in life. Obesity results from a positive energy balance which is modulated by environmental and genetic factors.[2] A proportion of the obesity burden has been attributed to factors that occur during the first 1,000 days of life (i.e. from conception to age 2 years) such as maternal nutrition, genetic predisposition, breastfeeding and complimentary feeding.[3-5] However, the mechanisms by which these factors affect weight and adiposity are less well understood; appetite and satiety regulation (ASR) being one of the most promising. Alterations to these mechanisms during gestation and the first two years of life potentially increase susceptibility to develop obesity throughout the life course (Figure 1).[6]

Early feeding practices and appetite and satiety regulation

Early feeding (< 6 months of age) is thought to be key for ASR[7] because it occurs during a period of biological plasticity and of behavioral modeling, which can determine long-term eating habits, growth outcomes and future metabolic responses. Further, breastmilk contains adipokines such as leptin and adiponectin which in turn have been associated with insulin sensitivity, body composition and ASR.[8-10] Breastfed babies self-regulate intake in a more efficient way than bottle fed babies, which has also been proposed as a driver of ASR later in life. During the complementary feeding period ─the transition process between breastfeeding (or formula feeding) to the family diet─ the age at which new foods are introduced and the parents' infant feeding styles, along with genetic predisposition, determine food preference patterns and consumption that may influence the ASR in childhood and throughout the life span.[11-13]

Genetic polymorphisms and appetite and satiety regulation

Several genes have been linked to obesity development including polymorphisms in leptin, adiponectin[14] and FTO (Fat mass and obesity gene).[15] Evidence shows that expression of these genes is particularly relevant in the hypothalamus, which is consistent with the hypothesis that these genes are involved in the regulation of appetite and food intake. In observational studies, genetic susceptibility to childhood obesity seems to be partially explained by appetitive traits in infancy.[16 17]

Appetite and satiety regulation, growth, adiposity and metabolic risk factors

Appetite traits such as emotional overeating and food responsiveness in early childhood have been associated with overeating, weight and metabolic risk factors in later life.[18 19] Such traits can modify or even impair satiety signals, and along with other factors, can lead to overweight and obesity.[20 21] This latest condition is characterized by a subclinical chronic inflammation and oxidative stress that induce cellular and physiological responses contributing to cardiometabolic comorbidities.[22] The cellular phenotype in obesity modifies the function of adipocytes and influences their micro-environment, increasing the secretion of pro-inflammatory cytokines, reactive oxygen species (ROS) and a parallel response in adipokines production. Moreover, ROS can also decrease insulin sensitivity and damage B cells in the pancreas which can in turn lead to glucose intolerance and even type II diabetes mellitus.[23] Once oxidative stress is established, a cascade of events is generated that can predict the rapid progression of the disease and the development of complications. This suggests that oxidative stress could be used as an early indicator of the risk of cardio-metabolic alterations associated with obesity, with a promising clinical relevance

[insert Figure 1 about here]

There are still many gaps in the literature regarding the association of infant feeding and genetic polymorphisms with ASR and of ASR with growth, later life adiposity and metabolic

risk factors in humans. To fill these gaps, a life-trajectory approach is key in order to consider the feeding history and growth of the child while adjusting for important prenatal factors. Studies with reliable longitudinal infant feeding data and robust follow-up protocols are scarce in middle-income countries, which are currently experiencing a nutrition transition and are facing a growing prevalence of overweight and obesity. Understanding the nature of the association of infant feeding and genetic polymorphisms with growth, adiposity and metabolic risk factors is crucial to design better and more efficient policies and programs for this stage of life within and outside the health system.

Our birth cohort study addresses some of these gaps. The cohort is referred to as "MAS-Lactancia" (the first word is an acronym in Spanish for "Appetite and Satiety Mechanisms" which also means "more" and the second word is "breastfeeding"). Our cohort is based in Mexico, an upper middle-income country that has experienced a fast nutrition transition, and is facing a large burden from obesity and associated chronic diseases.[24 25] Further, the prevalence of breastfeeding is among the lowest in Latin America and complementary feeding does not comply with international recommendations.[26 27] The overarching aim is to establish whether ASR is a mediator between infant feeding (breastfeeding/formula, and CF) and genetic polymorphisms (FTO, leptin and adiponectin) with children's growth, adiposity, and metabolic risk factors while adjusting for important prenatal factors such as maternal nutrition.

We hypothesize that inadequate infant feeding practices (such as inadequate breastfeeding duration, early food introduction, and high-energy food consumption) and genetic polymorphisms will be associated with: (1) appetite and satiety dysregulation in children from 3 to 48 months of age, (2) rapid weight gain, greater adiposity and metabolic risk factors in children from 3 to 48 months of age. Also, (3) the association between feeding patterns

and genetic polymorphisms with rapid weight gain, adiposity and metabolic risk factors will be partly mediated by appetite and satiety dysregulation. In this manuscript we describe the design and data collection protocol for the ongoing MAS-Lactancia birth cohort study.

METHODS AND ANALYSIS

Design and study population

The MAS-Lactancia birth cohort is an ongoing, open prospective cohort of mother-child pairs living in the city of Cuernavaca, Morelos, Mexico who are affiliated to the Mexican Social Security Institute (IMSS for its acronym in Spanish). IMSS provides health care and social protection to private sector formal employees and their families. It serves approximately 60% of the Mexican population. Women are enrolled in the cohort between 16 and 22 weeks' gestation. Cohort participants are followed during the second half of their pregnancies, at birth, and throughout the infant's first 48 months of life through a series of clinic and home visits with interviewer-administered questionnaires, anthropometric measurements and biospecimen collection.

The study aims to follow at least 400 mother-child pairs through the child's first 48 months. This target sample size (N=400) was calculated to be large enough to detect differences between breastfeeding modalities (exclusive and predominant breastfeeding, partial breastfeeding and no breastfeeding) for the primary outcome variables (weight gain, adiposity and appetite and satiety indicators). Based on 80% of power (1-β), with a sample of 400 participants (from which 15% or N= 60 are in the smallest breastfeeding modality group), it is possible to detect a 0.60 kg/m^2 difference in body mass index, 0.12 mm in sum of skinfold thicknesses, 0.30 mm in height and 0.40 mg/dL in insulin concentration between breastfeeding modalities at 95% confidence (α= 0.05, two – side). For the polymorphisms,

we estimated that the sample of 400 children had sufficient power (80%) to identify the presence of 11 polymorphisms: FTO (rs9930506, rs9922708, rs17817449, rs7206790, rs9939609, rs7185735), Leptin (rs4731427, rs17151919) and adiponectin (rs266729, rs2241766, rs1501299) genes. Likewise, to identify the differences between explanatory (e.g. presence of polymorphisms) and outcome variables, this sample size can identify linear correlations of up to r=0.14 with a power of 80% and 95% confidence level. We used the program GPower3 for all sample size calculations. For the mediation variables, we considered that a minim sample of 200 will be adequate to identify direct and indirect relationships between main study variables according to Kline's methodology for Structural Equation Models.[28]

Eligibility, enrollment and follow-up

Enrollment into the cohort began March 2016 from two social security clinics (No. 1 and No. 20) providing antenatal care for IMSS-affiliated women in Cuernavaca, Morelos. Recruitment has been interrupted twice for periods of up to one year, due to a strong earthquake in 2017 which damaged the clinics and due to the COVID-19 pandemic. During active recruitment periods, all pregnant women attending the clinics are invited to participate in the study. Women that accept answer a screening questionnaire. If they meet the eligibility criteria, they are invited to read and sign the informed consent. Women are eligible if they are between 18 and 39 years of age, between 16 and 22 weeks pregnant, living in Cuernavaca's metropolitan area and with plans to remain there over the next three years, planning on giving birth at the local social security hospital (Hospital General no. 1, IMSS), without previous diagnosis of hypertension, preeclampsia, renal, hepatic or cardiovascular diseases, accept to participate and sign the informed consent. Exclusion criteria are applied

at the time of birth of the child as follows: preterm birth (<37 weeks' gestation), multiple pregnancy, evidence of maternal substance abuse, intrauterine growth restriction (low birth weight for gestational age), congenital diseases which may affect appetite, feeding and growth (i.e. cleft lip and palate, food allergies) and physical malformations which may affect anthropometric measurements.

All pregnant women enrolled in the study are offered breastfeeding counseling from week 34 of gestation onwards through a series of face-to-face sessions and printed materials. There are two reasons for offering counseling: 1) breastfeeding rates are very low in Mexico therefore, counseling is necessary to achieve sufficient sample with adequate infant feeding practices. There is evidence that breastfeeding advice and counseling can increase exclusive breastfeeding rates up to 3.5 times during the neonatal period and 5.2 times at six months [29]; 2) breastfeeding counseling is an incentive for participation and retention.

Table 1 presents an overview of the data and measurement collection for the cohort. Trained and standardized interviewers administer questionnaires, collect in-person measurements, and biospecimens (blood, breastmilk and saliva).

Table 1. Data collection in the MAS Lactancia Cohort.

	Pregnancy (weeks)		Postnatal time points (months)										Data source
	16-22	34	Birth	1	3	6	9	12	18	24	36	48	
Maternal variables													
Sociodemographic and demographic	X												Questionnaire
Employment and family composition	X											X	Questionnaire
Medical history	X	X											Questionnaire & MR
Diet	X	X		X				X	X	X	X	X	24-hour dietary recall
Anthropometry	X	X		X				X	X	X	X	X	Measured
Smoking	X	X		X				X	X	X	X	X	Questionnaire
Leptin in breastmilk				X									Lab analysis
Adiponectin in breastmilk				X									Lab analysis
Nutrition composition of breastmilk				X									Lab analysis
Parenting styles[1]				X	X	X	X	X	X	X	X	X	Parenting styles questionnaire
Infant variables													
Birth weight and length			X										Measured/MR
Breastfeeding practices and complementary feeding				X	X	X	X	X	X	X			Questionnaire
Diet				X	X	X	X	X	X	X	X	X	24-hour dietary recall
Sleep duration/sleeping habits [2]				X	X	X	X	X	X	X	X	X	Questionnaire
Appetitive traits[3]				X	X	X	X	X	X	X	X	X	BEBQ and CEBQ
Primary caregivers[4]				X	X	X	X	X	X	X	X	X	Questionnaire

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Infant morbidity				X	X	X	X	X	X	X	X	X	Questionnaire
Physical Activity												X	Questionnaire
Weight				X	X	X	X	X	X	X	X	X	Measured
Length				X	X	X	X	X	X	X	X	X	Measured
Skinfolds				X	X	X	X	X	X	X	X	X	Measured
Abdominal circumference				X	X	X	X	X	X	X	X	X	Measured
Head circumference			X	X	X	X	X	X	X	X	X	X	Measured
Leptin concentrations					X			X				X	Lab analysis
Adiponectin concentrations					X			X				X	Lab analysis
Glucose concentrations					X			X					Lab analysis
Insulin concentrations					X			X				X	Lab analysis
Malondialdehyde												X	Lab analysis
Presence of polymorphisms in obesity related genes (FTO, leptin and adiponectin)								X	X	X	X	X	Lab analysis
Body composition									X	X	X	X	Dual Energy X ray Absorptiometry (DXA)

MR: Medical record

[1] Infant Feeding Style Questionnaire (IFSQ)[30]

[2] Brief Screening Questionnaire for Infant Sleep Problems,[31]

[3] Through maternal perception, Child Eating Behavior Questionnaire (CEBQ) and Baby Eating Behavior Questionnaire (BEBQ).[32 33]

[4] Questionnaire applied to the mother to identify principal caregivers.

Primary outcomes

Appetite and satiety regulation

Operationalizing ASR is complex because the physiological regulation of appetite and satiety includes a network of mechanisms interacting with each other (signaling cascades that include hormones and effectors). Therefore, we do not consider ASR as an individual variable that categorically identifies a state of satiety or appetite. Rather we use a series of variables that complement each other and will allow us to understand, at least in part, the regulation of ASR: 1) scales of maternal perception of appetite and satiety of their child; 2) biomarkers in child's blood; 3) child's diet.

We apply the Baby Eating Behavior Questionnaire (BEBQ)[32] at months 1,3, 6 and 9 and the Child Eating Behavior Questionnaire (CEBQ)[33 34] at months 12, 18, 24, 36 and 48. The CEBQ is a parent-report measure comprised of 35 items, each rated on a five-point Likert scale that ranges from never to always. It is made up of eight scales: food responsiveness, emotional over-eating, enjoyment of food, desire to drink, satiety responsiveness, slowness in eating, emotional under-eating, and food fussiness. The BEBQ was derived from the CEBQ and consists of 18 items that identify four distinct appetitive constructs: enjoyment of food, food responsiveness, slowness in eating and satiety responsiveness.[32] Both instruments are standardized measures of infant appetite designed to characterize appetitive traits that have been associated to excess weight gain.[35] Both questionnaires were validated for the study. The validation involved the translation to Spanish and the retranslation (to English to assess consistency), adaptation, clarification test and psychometric analysis. After the clarification test, the adapted Spanish version of the instruments was submitted to the commissions for approval.

Children's leptin, adiponectin and insulin blood concentrations are measured at months 3 (5mL sample) and 12 (7.5 mL sample). Blood samples are collected by a trained phlebotomist using standard pediatric venipuncture protocols. These hormones are associated with appetite regulation, energy balance and adiposity. Serum, and whole blood are stored at -80°C until analyzed in The National Institute of Medical Science and Nutrition SZ. Hormones are determined by ELISA using commercial kits (Merk-Millipore).

Child's diet information is collected to complement the above indicators using a standardized 24-hour dietary recall (24HR) applied to the mother using an automated software.[36] We follow an iterative multi-step procedure to increase the accuracy of the recall.[37] In the first step, a preliminary list of foods consumed in the last 24 hours is recorded, the interviewer then prompts the mother about commonly omitted foods (e.g. added sugar), this is followed by recalling the time and situation when the food was consumed, finally the interviewer reviews responses and makes final additions or revisions. The 24HR is collected at 1, 3, 6, 9, 12, 18, 24, 36 and 48 months using the same protocol. The 24HR allows estimation of total energy, macronutrient and food group intakes to complement information from the questionnaires and biomarkers.

Infant growth and adiposity

Anthropometric measurements: weight, height, arm, head and abdominal circumference, and skinfold thicknesses of the child are taken at birth, 1, 3, 6, 9, 12, 18, 24, 36 and 48 months of age using standardized procedures.[38 39] A portable electronic pediatric scale (Tanita BABY MOMMY model 1582) with a precision to the nearest 10g is used to measure weight. A wooden infantometer (Schorr) with a precision of 1mm is used to measure height. Circumferences are measured using a fiberglass tape accurate to 1mm and skinfold thickness using a Langer caliper accurate to 1mm. All measurements are taken twice and averaged, and

Skinfold thicknesses are measured in triplicate. We use WHO-Anthro software (v.3.2.2, 2011) to estimate Z-scores of weight-for-height (WHZ) and Z-score of height for age (HAZ) based on World Health Organization (WHO) growth standards.

Birth weight is obtained from two sources: 1) from the medical record and 2) measured by the cohort personnel within 48 hours of birth. Body composition (fat and lean mass) is measured using dual-energy X-ray absorptiometry (DXA scan) using the General Electric Lunar Prodigy model at month 18, 24, 36 and 48. The DXA scan provides an accurate measure of body composition,[40] is noninvasive and has been previously used in infants and children.[41 42]

Metabolic risk factors

At 48 months, unstimulated saliva samples will be obtained to determine leptin, adiponectin and insulin which will serve both as biochemical indicators of appetite and satiety regulation and metabolic risk factors, as well as malondialdehyde as an oxidative stress biomarker. We will use the method of TBARS described by Richard *et al.*[43] Remaining saliva samples will be stored and conserved in order to increase determinations in the future of other interesting metabolic risk factors such as inflammatory cytokines.

Primary exposures

Infant feeding including breastfeeding and complimentary feeding

We use a questionnaire previously used in the National Nutrition and Health Surveys of Mexico[44] to elicit information on breastfeeding and complimentary feeding practices. It enquires about feeding practices the day before the interview. Questions related to breastfeeding include: if and when breastfeeding was initiated after birth, type of milk fed to infant, frequency, duration, mode (breast/bottle) and reasons for breast/bottle feeding. The

complimentary feeding questionnaire includes questions related to: type of foods consumed during the previous day, and age at which certain foods were introduced i.e. water, milk, sugar sweetened beverages, cereals, pulses, vegetables, animal protein and dairy. This questionnaire is applied at 1, 3, 6, 9,12, 18, and 24 months of age.

Breastfeeding is classified according to WHO recommendations as: 1) exclusive, when only breast milk is fed to the infant; 2) predominant, when in addition to breastmilk, the infant is given water, unsweetened tea and other beverages excluding formula milk; 3) partial, when in addition to breastmilk the infant is given formula, solid foods or other milks; and 4) not breastfeeding.

Adequate complimentary feeding is defined as follows: the child's diet must include at least one food from each food group (fruits and vegetables; pulses, eggs and meats; dairy; cereals), it must not include more than 12% of total energy from the summation of the following groups: sugar sweetened beverages, milk or formula with added sugar, processed foods with >15% added sugars or >13% saturated fat or >275 kcal/100g. Additionally, consumption of estimated added sugar[45] must not exceed 5% of total energy intake.

Adequate infant feeding is defined as follows: exclusive or predominant breastfeeding up to 6 months of age, some breastfeeding and adequate complimentary feeding between 6 and 11 months of age, and adequate complimentary feeding with or without breastfeeding from 12 to 18 months of age.

Polymorphisms in obesity-related genes

Using the infant blood drawn at month 12 (or 18, 24, 36 or 48 moths) we will genotype polymorphisms: LEP rs4731427 y rs17151919, ADIPOQ rs266729, rs2241766 y rs1501299 y FTO rs9930506, rs9922708, rs17817449, rs7206790, rs9939609 y rs7185735. Blood

samples are being stored at -70°C. Once we achieve the study sample size, we will extract DNA using commercial kits (Quiagen).

Other variables: Maternal characteristics

Demographics. At enrollment, information on marital status, mother and father's education level, place of birth, occupation, household income and family composition is collected. Household characteristics and asset ownership such as TVs or vehicles are also recorded.

Medical history. Women are asked about previous diseases, medicines taken and nutrition supplements. Pre-pregnancy weight and height are self-reported. First day of last menstrual period, bleeding during pregnancy, diagnosed chronic diseases are obtained from clinical records.

Anthropometric measurements. Weight, height, arm and waist circumference and skinfold thickness are measured at recruitment, 34 weeks' gestation, 1, 12, 18, 24, 36 and 48 months after giving birth. All anthropometric measurements are taken using Lohman's Anthropometry Manual as Reference.[39] Personnel were trained and standardized at the beginning of the study to ensure consistent and accurate measurements.[38] Maternal weight is measured on a Tanita scale, model 1582 accurate to the nearest 10g. Standing height is measured using a Schorr stadiometer accurate to the nearest 1mm. Circumferences are measured using a fiberglass tape accurate to 1mm and skinfold thickness using a caliper accurate to 1mm. All measurements are taken twice and averaged.

Diet. A 24-hour recall is applied at 34 week's gestation, months 1, 18, 24, 36 and 48 postpartum. The same procedure described for children is followed at all follow-ups.

Parenting styles. The Infant Feeding Style Questionnaire (IFSQ)[30] is applied to women when their child is 1, 3, 6, 9, 12, 18, 24, 36, and 48 months old. The IFSQ measures feeding beliefs

and behaviors among mothers of infants and young children. It identifies five feeding styles: laissez-faire, restrictive, pressuring, responsive and indulgent.[30]

Breast milk composition*:* A sample of breastmilk is collected using an electric pump one month postpartum, during the morning in our research facility. The total amount of milk extracted from one breast (approximately 35-50 mL) is collected in sterilized glasses and it is homogenized and stored at -80°C.[46 47] Leptin, adiponectin and nutrient composition of breastmilk is then measured.

Other variables: Child Characteristics

Sleep. Sleep duration Questionnaire at months 1, 3, 6, 9, 12, 18, 24 and the Brief Screening Questionnaire for Infant Sleep Problems[31] is applied to women to elicit information about their child's sleeping habits at 48 months. This instrument has been previously validated against objective sleep measurements.[31]

Morbidity. Morbidity information includes a list of infectious diseases and accidents collected at months 1, 3, 6, 9, 12, 18, 24, 36 and 48.

Other variables: Environment

Environmental and area-level data can be linked to participants by leveraging public use datasets and geographic information systems. Participant's households are georeferenced and area-level information regarding poverty, education, public services, built and food environment, and safety among others will be linked to participants' home addresses.

Patient and public involvement

Participants were not invited to comment on the study design of this cohort study. However, there is a Facebook group that participants can join to share their experience of participating

in the study. The results of the study will also be disseminated through this channel to ensure that participants benefit from the research.

Statistical analyses

We will conduct adjusted regression analyses to understand the associations between our exposure and our outcome variables at different time points. We will integrate all time points into a system of regressions with structural equation models to conduct a longitudinal, life-course analysis. This type of analyses will enable us to understand the effect at each follow-up period. Given that this age period is very dynamic in terms of growth and the feeding modes, we will perform longitudinal models that will capture these transitions. In addition, we will perform path analysis to identify the mediation role for some variables.

When the associations investigated require the use of numerous models, we will consider multiple comparison analyses, such a Bonferroni correction.

STRENGTHS AND LIMITATIONS OF THIS STUDY

The main strengths of this cohort lie in its intensive follow-up during pregnancy and the first 48 months postpartum, its thorough longitudinal collection of infant feeding information, ASR and anthropometry measures. The core project will allow us to answer a number of research questions related to the cohort aims which will significantly expand what is known on these topics. Looking ahead, we have managed to secure two additional grants, from CONACYT and Rio Arronte Foundation. These grants will allow for an extension of the follow-up period and to address new research questions related to complimentary feeding and sweetness preferences.

It is important to highlight that the cohort was not planned to be representative of the Mexican general population since we are recruiting from the social security system in one city. Our intention with this study is not to make inferences about the frequency and distribution of public health issues (we have nationally representative surveys for that purpose), but to investigate associations between biologic and behavioral exposures with health outcomes. In terms of limitations of this study, we are observing relatively low response rates compared to national surveys in Mexico and attrition rates which are comparable to other international cohorts. Up to January 2021 we have recruited 1152 women, and remain in contact with 690 who are at various stages of follow up. A thorough analysis of the implications of these limitations will be performed once the sample is complete. Selection into the sample may be associated with health consciousness, which can decrease the variability in both exposures and outcomes, and/or bias our results.

ETHICS AND DISSEMINATION

Written informed consent is obtained at study entry for each participant and her child. The study protocol, data collection instruments and consent forms and procedures were approved by the Institutional Review Boards of the National Institute of Public Health, and Mexican Social Security Institute in Mexico. Results will be disseminated through peer review studies and communication with local health authorities.

AUTHOR CONTRIBUTIONS

JRD and IRS were responsible for the conception of the study. JRD, IRS, CB, ACA, ABG, EZ, MS, MTO, ADS, RM, UR, LA and AC were responsible for the design of the study. JRD, IRS, ACA and CB obtained funding for initial and later follow ups. IRS, SB, ACA and

CPF were responsible for acquisition of data. CPF, IRS, ACA and MTO were responsible for drafting the manuscript. IRS, CPF, ACA, MTO, SB, CB, AC, MS, EZ, ABG, LA, UR, ADS, RM and JRD critically revised the manuscript for important intellectual content, approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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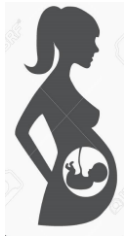
FIGURE LEGENDS

Figure 1. Conceptual framework of the determinants of appetite and satiety regulation in young children and their association with growth, adiposity and metabolic risk factors

Maternal Nutrition
- Nutrition status (Undernutrition/obesity)
- Unhealthy diet



Perinatally programmed appetite

Infant Feeding habits
- Breastfeeding
- Complementary feeding

Postnatally programmed appetite

Appetite and satiety dysregulation

Inadequate Eating Habits
(Dietary patterns and eating behaviors)

Rapid weight gain

Greater Adiposity

Genetic predisposition
Genes: FTO, Leptin
Adiponetin (polymorphisms)

Oxidative Stress

Cardiometabolic alterations

Prenatal stage

Early postnatal stage (0 – 12 months)

Later postnatal stage (Toddlers)

BMJ Open

Infant feeding, appetite and satiety regulation, and adiposity during infancy: Study design and protocol of the "MAS-Lactancia" birth cohort

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Infant feeding, appetite and satiety regulation, and adiposity during infancy: Study design and protocol of the "MAS-Lactancia" birth cohort

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ABSTRACT

Introduction. The prevalence of childhood obesity has risen dramatically in recent years. A proportion of this burden has been attributed to factors that occur during the first 1,000 days of life such as genetic predisposition, breastfeeding and complimentary feeding. Although the mechanisms by which these factors affect weight and adiposity are less well-understood, appetite and satiety regulation may be key to understanding them. This cohort study aims to investigate the role of appetite and satiety regulation as a mediator in the association between infant feeding practices and genetic polymorphisms with children's growth, adiposity and metabolic risk factors.

Methods and analysis. "MAS-Lactancia" is an open, ongoing, prospective birth cohort that began the enrollment in 2016 of mother-child pairs affiliated to the Mexican Social Security Institute and that live in the city of Cuernavaca, Mexico. Pregnant women between 16 and 22 weeks' gestation are followed during the second half of their pregnancies, at birth, and throughout their infant's first 48 months of life (at 1, 3, 6, 9, 12, 18, 24, 36, 48 months) at the clinic and at-home visits that include questionnaires, anthropometric measurements and biospecimen collection. The main exposure variables are infant feeding (breastfeeding and complementary feeding), and genetic polymorphisms (FTO, leptin and adiponectin). Outcome variables include infant's growth, adiposity and metabolic risk factors. We will conduct longitudinal models and path analyses to identify the potential mediating role of satiety and appetite indicators (leptin, adiponectin, insulin concentrations, appetite and satiety perception).

Ethics and dissemination. The study protocol, data collection instruments, consent forms and procedures were approved by the Institutional Review Boards of the National Institute of Public Health, and the Mexican Social Security Institute in Mexico. Findings will be

disseminated through conferences, peer-reviewed publications and meetings with stakeholders.

Keywords: Infant feeding, appetite, satiety, growth, adiposity, childhood obesity

ARTICLE SUMMARY

Strengths and limitations of this study

- This is the first study to investigate the role of appetite and satiety regulation in the development of obesity and chronic diseases in a middle-income-country undergoing a rapid nutrition transition.
- The study uses reliable and validated tools to collect information on a diverse range of factors including infant feeding, appetite and satiety regulation, anthropometry, biomarkers and related genetic polymorphisms.
- Intensive follow-up during pregnancy and the child's first 48 months will help to closely track growth and metabolic risk factors.
- Limitations include a low response rate in recruitment (affected by a strong earthquake in 2017 and the current COVID-19 pandemic) and attrition rates which are comparable to those in other birth cohort studies.

INTRODUCTION

Excess body fat (overweight or obesity) affects 41 million children under 5 years-old globally and is associated with increased metabolic risk factors later in life.[1] Obesity results from a positive energy balance which is modulated by environmental and genetic factors.[2] A proportion of the obesity burden has been attributed to factors that occur during the first 1,000 days of life (i.e. from conception to age 2 years) such as maternal nutrition, genetic predisposition, breastfeeding and complimentary feeding (CF).[3-5] However, the mechanisms by which these factors affect weight and adiposity are less well understood; appetite and satiety regulation (ASR) being one of the most promising. Alterations to these mechanisms during gestation and the first two years of life potentially increase susceptibility to develop obesity throughout the course of life (Figure 1).[6]

Early feeding practices and appetite and satiety regulation

Early feeding (< 6 months of age) is thought to be key for ASR because it occurs during a period of biological plasticity and of behavioral modeling, which can determine long-term eating habits, growth outcomes and future metabolic responses.[7] Further, breastmilk contains adipokines such as leptin and adiponectin which in turn have been associated with insulin sensitivity, body composition and ASR.[8-10] Breastfed babies self-regulate intake in a more efficient way than bottle-fed babies. In fact, bottle feeding has also been proposed as a driver of ASR. The complementary feeding period is known as the transition process between breastfeeding (or formula feeding) to the family diet. The age at which new foods are introduced, the parents' infant feeding styles, along with genetic predisposition, determine food preference patterns and consumption that may influence the ASR in childhood and throughout the life span.[11-13]

Genetic polymorphisms and appetite and satiety regulation

Several genes have been linked to obesity development including polymorphisms of leptin, adiponectin and FTO (Fat mass and Obesity-associated gene).[14, 15] Evidence shows that expression of these genes is particularly relevant in the hypothalamus, consistent with the hypothesis that these genes are involved in appetite and food intake regulation. Observational studies have shown that genetic susceptibility to childhood obesity seems to be partially explained by appetitive traits during infancy.[16, 17]

Appetite and satiety regulation, growth, adiposity and metabolic risk factors

Appetite traits such as emotional overeating and food responsiveness in early childhood have been associated with overeating, weight and metabolic risk factors in later life.[18, 19] Such traits can modify or even impair satiety signals, and along with other factors, can lead to overweight and obesity.[20, 21] This latter condition is characterized by subclinical chronic inflammation and oxidative stress that induce cellular and physiological responses contributing to cardiometabolic comorbidities.[22] The cellular phenotype in obesity modifies the function of adipocytes and influences their micro-environment, increasing the secretion of pro-inflammatory cytokines, reactive oxygen species (ROS) and a parallel response in adipokines production. Moreover, ROS can also decrease insulin sensitivity and damage B-cells in the pancreas that can lead to glucose intolerance and type II diabetes mellitus.[23] Once oxidative stress is established, a cascade of events is generated that can predict the rapid progression of the disease and the development of related complications. This suggests that oxidative stress could be used as an early indicator of the risk of cardio-metabolic alterations associated with obesity, with promising clinical relevance.

[insert Figure 1 about here]

There are still many gaps in the literature regarding the association of infant feeding and genetic polymorphisms with ASR and of ASR with growth, later-life adiposity and metabolic

risk factors in humans. To fill these gaps, a life-trajectory approach is key, considering the feeding history and growth of the child, while adjusting for important prenatal factors. Studies with reliable longitudinal infant feeding data and robust follow-up protocols are scarce in middle-income countries that are currently experiencing a nutrition transition and are facing a growing prevalence of overweight and obesity. Understanding the nature of the association of infant feeding and genetic polymorphisms with growth, adiposity and metabolic risk factors is crucial to design better and more efficient policies and programs for this stage of life, within and outside the health system.

Our birth cohort study addresses some of these gaps. The cohort is referred to as "MAS-Lactancia" (the first word means "more" and is also an acronym in Spanish for "Appetite and Satiety Mechanisms", the second word is "breastfeeding"). Our cohort is based in Mexico, an upper middle-income country that has experienced a fast nutrition transition, and is facing a large burden from obesity and associated chronic diseases.[24, 25] Further, the prevalence of breastfeeding is among the lowest in Latin America and complementary feeding does not comply with international recommendations.[26, 27] The overarching study aim is to establish whether ASR is a mediator between infant feeding (breastfeeding/formula, and CF) and genetic polymorphisms (FTO, leptin and adiponectin) with children's growth, adiposity, and metabolic risk factors while adjusting for important prenatal factors such as maternal nutrition.

We hypothesize that inadequate infant feeding practices (such as inadequate breastfeeding duration, early food introduction, and high-energy food consumption) and genetic polymorphisms will be associated with: (1) ASR in children from 3 to 48 months of age, (2) rapid weight gain, greater adiposity and metabolic risk factors in children from 3 to 48 months of age. Also, (3) the association between feeding patterns and genetic polymorphisms

with rapid weight gain, adiposity and metabolic risk factors will be partly mediated by ASR. In this manuscript we describe the design and data collection protocol for the ongoing MAS-Lactancia birth cohort study.

METHODS AND ANALYSES

Design and study population

The MAS-Lactancia birth cohort is an ongoing, open prospective cohort of mother-child pairs who are affiliated to the Mexican Social Security Institute (IMSS for its acronym in Spanish) and that live in the city of Cuernavaca, Morelos, Mexico. IMSS provides health care and social protection to private-sector formal employees and their families. It serves approximately 60% of the Mexican population. Pregnant women are enrolled in the cohort between 16 and 22 weeks' gestation. Cohort participants are followed during the second half of their pregnancies, at birth, and throughout the infant's first 48 months of life through a series of clinic and home visits that include questionnaires, anthropometric measurements and biospecimen collection.

The study aims to follow at least 400 mother-child pairs through the child's first 48 months. The target sample size (N=400) was calculated to be large enough to detect differences between breastfeeding modalities (exclusive and predominant breastfeeding, partial breastfeeding and no breastfeeding) and the primary outcome variables (weight gain, adiposity and appetite and satiety indicators). Based on 80% of power (1-β), with a sample of 400 participants (from which 15% or n= 60 are in the smallest breastfeeding modality group), we will be able to detect a 0.60 kg/m^2 difference in body mass index, 0.12 mm in sum of skinfold thicknesses, 0.30 mm in height and 0.40 mg/dL in insulin concentration between breastfeeding modalities at 95% confidence (α= 0.05, two – side). For the

polymorphisms, we estimated that the sample of 400 children had sufficient power (80%) to identify the presence of 11 polymorphisms: FTO (rs9930506, rs9922708, rs17817449, rs7206790, rs9939609, rs7185735), leptin (rs4731427, rs17151919) and adiponectin (rs266729, rs2241766, rs1501299) genes. Likewise, to identify the differences between explanatory (e.g. presence of polymorphisms) and outcome variables, this sample size can identify linear correlations of up to r=0.14 with a power of 80% and a 95% confidence level. We used the program GPower3 for all sample size calculations. For the mediation variables, we considered that a minimum sample of n=200 dyads will be sufficient to identify direct and indirect relationships between the main study variables according to Kline's methodology for structural equation models.[28]

Eligibility, enrollment and follow-up

Enrollment into the cohort began in March 2016 from IMSS clinics No.1 and No.20 that provide antenatal care for IMSS-affiliated women in Cuernavaca, Morelos. Recruitment has been interrupted twice for periods of up to one year, due to a strong earthquake in 2017 which damaged the clinics and due to the COVID-19 pandemic. During active recruitment periods, all pregnant women attending the clinics are invited to participate in the study. Women that accept to participate answer a screening questionnaire. If they meet the eligibility criteria, they are invited to read and sign the informed consent. Women are eligible if they are between 18 and 39 years of age, between 16 and 22 weeks pregnant, living in Cuernavaca's metropolitan area and with plans to remain there over the next three years, planning on giving birth at the local IMSS hospital (Hospital General No.1, IMSS), without previous diagnosis of hypertension, preeclampsia, renal, hepatic or cardiovascular diseases, accept to participate and sign the informed consent. Exclusion criteria are applied at the time of birth of the child

as follows: preterm birth (<37 weeks' gestation), multiple pregnancy, evidence of maternal substance abuse, intrauterine growth restriction (low birth weight for gestational age), congenital diseases which may affect appetite, feeding and growth (i.e. cleft lip and palate, food allergies) and physical malformations which may affect anthropometric measurements. All pregnant women enrolled in the study are offered breastfeeding counseling from week 34 of gestation onwards through a series of face-to-face sessions and printed materials. There are two reasons for offering counseling: 1) breastfeeding rates are very low in Mexico therefore, counseling is necessary to achieve a sufficient sample with adequate infant feeding practices. There is evidence that breastfeeding advice and counseling can increase exclusive breastfeeding rates up to 3.5 times during the neonatal period and up to 5.2 times at six months of age;[29] 2) breastfeeding counseling is an incentive for participation and retention. Table 1 presents an overview of the data and measurement collection of the cohort. Trained and standardized interviewers administer questionnaires, collect in-person measurements, and biospecimens (blood, breastmilk and saliva).

Table 1. Data collection in the MAS-Lactancia Cohort.

	Pregnancy (weeks)		Postnatal time points (months)										Data source
	16-22	34	Birth	1	3	6	9	12	18	24	36	48	
Maternal variables													
Sociodemographic and demographic	X												Questionnaire
Employment and family composition	X											X	Questionnaire
Medical history	X	X											Questionnaire & MR
Diet	X	X		X				X	X	X	X	X	24-hour dietary recall
Anthropometry	X	X		X				X	X	X	X	X	Measured
Smoking	X	X		X				X	X	X	X	X	Questionnaire
Leptin in breastmilk				X									Lab analysis
Adiponectin in breastmilk				X									Lab analysis
Nutrition composition of breastmilk				X									Lab analysis
Parenting styles[1]				X	X	X	X	X	X	X	X	X	Parenting styles questionnaire
Infant variables													
Birth weight and length			X										Measured/MR
Breastfeeding practices and complementary feeding				X	X	X	X	X	X	X			Questionnaire
Diet				X	X	X	X	X	X	X	X	X	24-hour dietary recall
Sleep duration/sleeping habits [2]				X	X	X	X	X	X	X	X	X	Questionnaire
Appetitive traits[3]				X	X	X	X	X	X	X	X	X	BEBQ and CEBQ
Primary caregivers[4]				X	X	X	X	X	X	X	X	X	Questionnaire

Infant morbidity				X	X	X	X	X	X	X	X	X	Questionnaire
Physical Activity												X	Questionnaire
Weight				X	X	X	X	X	X	X	X	X	Measured
Length				X	X	X	X	X	X	X	X	X	Measured
Skinfolds				X	X	X	X	X	X	X	X	X	Measured
Abdominal circumference				X	X	X	X	X	X	X	X	X	Measured
Head circumference			X	X	X	X	X	X	X	X	X	X	Measured
Leptin concentrations					X			X				X	Lab analysis
Adiponectin concentrations					X			X				X	Lab analysis
Glucose concentrations					X			X					Lab analysis
Insulin concentrations					X			X				X	Lab analysis
Malondialdehyde												X	Lab analysis
Presence of polymorphisms in obesity related genes (FTO, leptin and adiponectin)								X	X	X	X	X	Lab analysis
Body composition									X	X	X	X	Dual Energy X ray Absorptiometry (DXA)

MR: Medical record
[1]Infant Feeding Style Questionnaire (IFSQ) [30]
[2]Brief Screening Questionnaire for Infant Sleep Problems [31]
[3]Through maternal perception, Child Eating Behavior Questionnaire (CEBQ) and Baby Eating Behavior Questionnaire (BEBQ) [32, 33]
[4]Questionnaire applied to the mother to identify principal caregivers.

Primary outcomes

Appetite and satiety regulation

Operationalizing ASR is complex because the physiological regulation of appetite and satiety includes a network of interacting mechanisms (signaling cascades that include hormones and effectors). Therefore, we do not consider ASR as an individual variable that categorically identifies a state of satiety or appetite. Rather, we use a series of variables that complement each other and will allow us to understand, at least in part, the regulation of ASR: 1) scales of maternal perception of child's appetite and satiety; 2) biomarkers in child's blood; 3) child's diet.

We apply the Baby Eating Behavior Questionnaire (BEBQ) at months 1,3, 6 and 9 and the Child Eating Behavior Questionnaire (CEBQ) at months 12, 18, 24, 36 and 48.[32-34] The CEBQ is a parent-report measure comprised of 35 items, each rated on a five-point Likert scale that ranges from never to always. It includes eight scales: food responsiveness, emotional over-eating, enjoyment of food, desire to drink, satiety responsiveness, slowness in eating, emotional under-eating, and food fussiness. The BEBQ was derived from the CEBQ and consists of 18 items that identify 4 distinct appetitive constructs: enjoyment of food, food responsiveness, slowness in eating and satiety responsiveness.[32] Both instruments are standardized measures of infant appetite designed to characterize appetitive traits that have been associated to excess weight gain.[35] Both questionnaires were validated for the study. The validation involved the translation to Spanish and the retranslation to English to assess consistency, adaptation, clarification test and psychometric analysis. After the clarification test, the adapted Spanish version of the instruments was submitted to the institutional review board (IRB) for approval.

Infant's leptin, adiponectin and insulin blood concentrations are measured at months 3 (5mL sample) and 12 (7.5 mL sample). Blood samples are collected by a trained phlebotomist using standard pediatric venipuncture protocols. These hormones are associated with appetite regulation, energy balance and adiposity. Serum, and whole blood are stored at -80°C until analyzed at The National Institute of Medical Science and Nutrition Salvador Zubirán in Mexico City. Hormones are determined by ELISA using commercial kits (Merk-Millipore). Child's diet information is collected to complement the above indicators using a standardized 24-hr dietary recall applied to the mother using an automated software.[36] We follow an iterative multi-step procedure to increase recall accuracy.[37] In the first step, a preliminary list of foods consumed in the last 24 hours is recorded, the interviewer then prompts the mother about commonly omitted foods (e.g. added sugar), this is followed by recalling the time and situation when the food was consumed; finally, the interviewer reviews the responses and makes final additions or revisions. The 24-hr dietary recall is collected at 1, 3, 6, 9, 12, 18, 24, 36 and 48 months using the same protocol; it allows estimation of total energy, macronutrient and food group intakes to complement information from the questionnaires and biomarkers.

Infant growth and adiposity

Child's anthropometric measurements: weight, length, height, arm, head and abdominal circumference, and skinfold thicknesses are taken at birth, 1, 3, 6, 9, 12, 18, 24, 36 and 48 months of age using standardized procedures.[38, 39] A portable electronic pediatric scale (Tanita BABY MOMMY model 1582) with a precision to the nearest 10g is used to measure weight. A wooden infantometer (Schorr) with a precision of 1mm is used to measure length. Standing height is measured using a Schorr stadiometer accurate to the nearest 1mm. Circumferences are measured using a fiberglass tape accurate to 1mm and skinfold thickness

using a Langer caliper accurate to 1mm. All measurements are taken twice and averaged, and skinfold thicknesses are measured in triplicate. We use the WHO-Anthro software (v.3.2.2, 2011) to estimate Z-scores of weight-for-height (WHZ) and Z-score of height for age (HAZ). Birth weight is obtained from two sources: 1) from the medical record and 2) measured by the cohort personnel within 48 hours of birth. Body composition (fat and lean mass) is measured using dual-energy X-ray absorptiometry (DXA scan) using a General Electric Lunar Prodigy model at months 18, 24, 36 and 48. The DXA scan provides an accurate measure of body composition,[40] is noninvasive and has been previously used in infants and children.[41, 42]

Metabolic risk factors

At 48 months of age, unstimulated saliva samples will be obtained from children to determine leptin, adiponectin and insulin that will serve both as biochemical indicators of ASR and metabolic risk factors, as well as malondialdehyde as an oxidative stress biomarker. We will use the thiobarbituric acid reactive substances (TBARS) method described by Richard *et al.[43]* The remaining saliva samples will be stored and adequately preserved for future determinations of other metabolic risk factors such as inflammatory cytokines.

Primary exposures

Infant feeding including breastfeeding and complimentary feeding

We use a questionnaire previously used in the National Nutrition and Health Surveys of Mexico to elicit information on breastfeeding and complimentary feeding practices.[44] It enquires about feeding practices the day prior to the interview. Questions related to breastfeeding include: if and when breastfeeding was initiated after birth, type of milk fed to infant, frequency, duration, mode (breast/bottle) and reasons for breast/bottle feeding. The

complimentary feeding questionnaire includes questions related to: type of foods consumed during the prior-to-interview day, and age at which certain foods were introduced i.e. water, milk, sugar sweetened beverages, cereals, pulses, vegetables, animal protein and dairy. This questionnaire is applied at child's 1, 3, 6, 9,12, 18, and 24 months of age.

Breastfeeding is classified according to WHO recommendations as: 1) exclusive, when only breast milk is fed to the infant; 2) predominant, when in addition to breastmilk, the infant is given water, unsweetened tea and other beverages excluding formula milk; 3) partial, when in addition to breastmilk the infant is given formula, solid foods or other milks; and 4) not breastfeeding.

Adequate complimentary feeding is defined as follows: the child's diet must include at least one food from each food group (fruits and vegetables; pulses, eggs and meats; dairy; cereals), it must not include more than 12% of total energy from the total of the following groups: sugar sweetened beverages, milk or formula with added sugar, processed foods with >15% added sugars or >13% saturated fat or >275 kcal/100g. Additionally, consumption of estimated added sugar must not exceed 5% of total energy intake.[45]

Adequate infant feeding is defined as follows: exclusive or predominant breastfeeding up to 6 months of age, some breastfeeding and adequate complimentary feeding between 6 and 11 months of age, and adequate complimentary feeding with or without breastfeeding from 12 to 18 months of age.

Polymorphisms in obesity-related genes

Using infant blood drawn at month 12 (or 18, 24, 36 or 48 moths) we will genotype polymorphisms: LEP: rs4731427 and rs17151919, ADIPOQ: rs266729, rs2241766 and rs1501299, and FTO: rs9930506, rs9922708, rs17817449, rs7206790, rs9939609 and

rs7185735. Blood samples are stored at -70°C. Once we achieve the study sample size, we will extract DNA using commercial kits (Quiagen).

Other variables: Maternal characteristics

Demographics. At enrollment, information on marital status, mother and father's education, place of birth, occupation, household income and family composition is collected. Household characteristics and asset ownership such as TVs or vehicles are also recorded.

Medical history. Women are asked about previous diseases, medicines taken and nutrition supplements. Pre-pregnancy weight and height are self-reported. First day of last menstrual period, bleeding during pregnancy, diagnosed chronic diseases are obtained from clinical records.

Anthropometric measurements. Weight, height, arm and waist circumference and skinfold thickness are measured at recruitment, 34 weeks' gestation, 1, 12, 18, 24, 36 and 48 months postpartum. All anthropometric measurements are taken using "Lohman's Anthropometry Manual" as reference.[39] Personnel were trained and standardized at the beginning of the study to ensure consistent and accurate measurements.[38] Maternal weight is measured on a Tanita scale, model 1582 accurate to the nearest 10g. Standing height is measured using a Schorr stadiometer accurate to the nearest 1mm. Circumferences are measured using a fiberglass tape accurate to 1mm and skinfold thickness using a caliper accurate to 1mm. All measurements are taken twice and averaged.

Diet. A 24-hr recall is applied at 34 week's gestation, months 1, 18, 24, 36 and 48 postpartum. The same procedure described for children applies to maternal diet at all follow-ups.

Parenting styles. The Infant Feeding Style Questionnaire (IFSQ) is applied to women when their child is 1, 3, 6, 9, 12, 18, 24, 36, and 48 months old.[30] The IFSQ measures feeding

beliefs and behaviors among mothers of infants and young children. It identifies five feeding styles: laissez-faire, restrictive, pressuring, responsive and indulgent.[30]

Breast milk composition. A sample of breastmilk is collected using an electric pump at one month postpartum, in our research facility (during the morning). The total amount of milk extracted from one breast (approximately 35-50 mL) is collected in sterilized glasses, it is homogenized and stored at -80°C.[46, 47] Leptin, adiponectin and nutrient composition of breastmilk is then analyzed at The National Institute of Medical Science and Nutrition Salvador Zubirán in Mexico City.

Other variables: Child Characteristics

Sleep. The Brief Screening Questionnaire for Infant Sleep Problems is applied to women to elicit information about their child's sleeping habits at months 1, 3, 6, 9, 12, 18, 24 and 48.[31] This instrument has been previously validated against objective sleep measurements.[31]

Morbidity. Morbidity information includes a list of infectious diseases and accidents collected at months 1, 3, 6, 9, 12, 18, 24, 36 and 48.

Other variables: Environment

Environmental and area-level data can be linked to participants by leveraging public use datasets and geographic information systems. Participant's households are georeferenced and area-level information regarding poverty, education, public services, built and food environment, and safety among others will be linked to participants' home addresses.

Patient and public involvement

Participants were not invited to comment on the study design of this cohort study. However, there is a Facebook group that participants can join to share their experience of participating in the study. The results of the study will also be disseminated through this channel to ensure that participants benefit from the research.

Statistical analyses

We will conduct adjusted regression analyses to understand the associations between our exposure and our outcome variables at different time points. We will integrate all time points into a system of regressions with structural equation models to conduct a longitudinal, life-course analysis. This type of analyses will enable us to understand associations at each follow-up period. Given that this age period is very dynamic in terms of growth and feeding modes, we will perform longitudinal models that will capture these transitions. In addition, we will perform path analysis to identify the mediation role of some variables.

When the associations investigated require the use of numerous models, we will consider multiple comparison analyses, such as the Bonferroni correction.

STRENGTHS AND LIMITATIONS OF THIS STUDY

The main strengths of this cohort lie in its intensive follow-up during pregnancy and the first 48 months postpartum, its thorough longitudinal collection of infant feeding information, ASR and anthropometry measures. The core project will allow us to answer a number of research questions related to the cohort aims which will significantly expand what is known on these topics. Looking ahead, we have managed to secure two additional grants, from the Mexican Council for Science and Technology (CONACYT) and the Rio Arronte Foundation.

These grants will allow for an extension of the follow-up period and to address new research questions related to complimentary feeding and sweetness preferences.

It is important to highlight that the cohort was not planned to be representative of the Mexican general population since we are recruiting from the IMSS system and in only one city. Our intention with this study is not to make inferences about the frequency and distribution of public health issues (we have nationally representative surveys for that purpose), but to investigate associations between biologic and behavioral exposures with health outcomes. In terms of limitations of this study, we are observing relatively low response rates compared to national surveys in Mexico and attrition rates that are comparable to other international cohorts. Up to January 2021 we have recruited 1,152 women, and remain in contact with 690 who are at various stages of the follow-up. A thorough analysis of the implications of these limitations will be performed once the sample is complete. Selection into the sample may be associated with health consciousness, which can decrease the variability in both exposures and outcomes, and/or bias our results.

ETHICS AND DISSEMINATION

Written informed consent is obtained at study entry for each participant and her child. The study protocol, data collection instruments and consent forms and procedures were approved by the Institutional Review Boards of the National Institute of Public Health, and Mexican Social Security Institute in Mexico. Results will be disseminated through peer review studies and communication with local health authorities.

AUTHOR CONTRIBUTIONS

JAR and IRS were responsible for the conception of the study. JAR, IRS, CB, ACA, ABG, EZ, MS, MTO, ADS, RM, UR, LA and AC were responsible for the design of the study. JAR, IRS, ACA and CB obtained funding for initial and later follow ups. IRS, SB, ACA and CPF were responsible for acquisition of data. CPF, IRS, ACA and MTO were responsible for drafting the manuscript. IRS, CPF, ACA, MTO, SB, CB, AC, MS, EZ, ABG, LA, UR, ADS, RM and JAR critically revised the manuscript for important intellectual content, approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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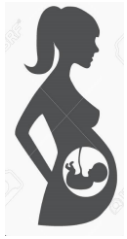
FIGURE LEGENDS

Figure 1. Conceptual framework of the determinants of appetite and satiety regulation in young children and their association with growth, adiposity and metabolic risk factors

Maternal Nutrition
- Nutrition status (Undernutrition/obesity)
- Unhealthy diet



Perinatally programmed appetite

Infant Feeding habits
- Breastfeeding
- Complementary feeding

Postnatally programmed appetite

Appetite and satiety dysregulation

Inadequate Eating Habits
(Dietary patterns and eating behaviors)

Rapid weight gain

Greater Adiposity

Genetic predisposition
Genes: FTO, Leptin
Adiponetin (polymorphisms)

Oxidative Stress

Cardiometabolic alterations

Prenatal stage

Early postnatal stage (0 – 12 months)

Later postnatal stage (Toddlers)