Putting teeth into the developmental origins hypothesis:

A longitudinal study of early childhood malnutrition, enamel hypoplasia and adolescent health in Amazonian Bolivia

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Abstract

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Adult teeth may chronologically reflect early childhood experience because enamel on the permanent teeth calcifies incrementally during early childhood and is sensitive to physiological stress. Defects in the enamel do not repair after occurrence or during the life course, leaving a permanent biological mark of physiological insults that occurred during early childhood. These characteristics suggest enamel defects may serve as a useful biomarker of chronic malnutrition and thus predictor of long-term health. This dissertation sought to investigate associations between malnutrition-related early childhood exposures and dental enamel hypoplasia (EH) and to evaluate EH as a predictor of adolescent anthropometrics and biomarkers.

We conducted analyses using data from 349 Tsimane' adolescents in Amazonian Bolivia, collected between 2002-2010 and in 2015. In 2015, we examined EH in the permanent maxillary incisors and mandibular canines using digital photography from which the following measures of EH were abstracted: occurrence (any, none), extent of occurrence (<1/3, 1/3-2/3, >2/3 of the tooth surface) and estimated age at occurrence (1, 2, 3, 4 years of age). Data on malnutrition-related early childhood exposures (1-4 years of age) were collected between 2002 and 2010, including stunted growth (height-

for-age z-scores, HAZ), underweight (weight-for-age z-scores, WAZ), anemia (hemoglobin), immune activation (c-reactive protein) and parasitic gastrointestinal infection (hookworm infection). Adolescent outcomes (10-17 years of age) were collected in 2015 and included anthropometrics (height, weight, body mass index (BMI)) and biomarkers (hemoglobin (Hb), glycated hemoglobin (HbA_{1c}), white blood cell count (WBC) and blood pressure). First, we evaluated the reliability of EH measurement using digital photographs and the Modified DDE (developmental defects in the enamel) Index by investigating inter- and intra-rater reliability and evaluating the frequency of EH detection across examiners for systemic biases. Next, we investigated associations between several malnutrition-related childhood exposures and EH in the permanent central maxillary incisors using multivariate log-binomial and ordinal logistic regression as well as generalized estimating equations (GEE). Finally, we investigated EH in the permanent central maxillary incisors (as a marker of early childhood experience) in relation to anthropometrics and biomarkers in adolescence using multivariate linear regression. We further evaluated the accuracy of EH in the permanent central maxillary incisors as a marker of chronic malnutrition and utility as a predictor of subsequent health outcomes compared to growth stunting through sensitivity, specificity and receiver operating characteristics (ROC) analyses.

Our evaluation of the digital photographs revealed a rough, cobblestone-like EH pattern in the tooth surface that was particularly prevalent in the study sample (92.3%) and was thus the focus of this project. EH detection was most common on the central maxillary incisors (87%) compared to the lateral maxillary incisors (63%) and mandibular canines (26%). The intra-examiner reliability for detecting EH occurrence on the central maxillary incisors was very good (mean kappa=0.77) and greater than the lateral maxillary incisors (mean kappa=0.68) and mandibular canines (mean kappa=0.49). However, the inter-examiner reliability was fair to poor, though the inter-examiner reliability was also better for the maxillary incisors (mean kappa= 0.29) than the mandibular canines (mean kappa=0.17). The study sample had a high prevalence of childhood malnutrition, demonstrated by prevalence of stunted linear growth (75.2%), anemia (56.9%), elevated immune activation (39.1%), and gastrointestinal hookworm infection (49.6%) between 1 and 4 years of age. Results indicated an association between average childhood HAZ (PR=0.98, 95% CI: 0.95, 1.00), CRP levels (PR=1.01, 95% CI: 1.00, 1.03) and presence of gastrointestinal hookworm infection (OR=0.28, 95% CI: 0.08, 0.94 for <1/3 vs. >2/3 of the tooth

affected by EH) and EH, though some of the point estimates lacked statistical precision. Extent of the tooth surface affected by EH seemed to be an important measure of EH as it related to early childhood exposures, particularly for average HAZ and hookworm infection. Greater extent of EH on the tooth surface was also associated with adolescent outcomes, including shorter height (-0.14 HAZ, 95% CI: -0.24, -0.03 and -1.35 cm, 95% CI: -2.21, -0.50), lower weight (-0.98 kg, 95% CI: -1.73, -0.23), lower Hb (-0.36 g/dL, 95% CI: -0.59, -0.13), lower HbA_{1c} (-0.04 $\%A_{1c}$, 95% CI: -0.08, -0.00), and higher WBC count (0.74 10^9/L, 95% CI: 0.35, 1.14) but not BMI-for-age z-score or blood pressure. EH extent was associated with anemia (PR=1.08, 95% CI: 1.00, 1.18) and elevated WBC count (PR=1.12, 95% CI: 1.01, 1.26) based on public health and clinically-relevant thresholds. When evaluated against growth stunting as the "gold standard" (HAZ< -2.0: prevalence = 62.3% in childhood and 34.1% in adolescence), EH had high sensitivity (93% in childhood, 96% in adolescence) and low specificity (11% in childhood, 10% in adolescence). EH extent was a more accurate marker of childhood stunting (AUC 0.56) than EH occurrence (AUC 0.52) due to increased specificity (0.36 vs. 0.11). The addition of EH extent to a set of markers for childhood stunting (gender and adolescent HAZ) only slightly improved the AUC (0.77 vs. 0.76, p=ns). The AUC for adolescent WBC count was greater for EH extent (AUC 0.61) than for childhood HAZ (AUC 0.58, p=ns) and adolescent HAZ (AUC 0.59, p=ns). Addition of EH extent to sets of markers for adolescent health outcomes (age, gender, childhood HAZ and adolescent HAZ) improved the AUC for nearly all outcomes.

In conclusion, we detected an EH pattern that was nearly ubiquitous in the study sample, but the rough, cobblestone-like hypoplastic pattern does not fit the typical linear/grooved pattern described in the overwhelming majority of the malnutrition literature. The pattern does not provide evidence in support of a systemic cause. Intra-examiner reliability results suggest that digital photography is a reproducible method for capturing EH, particularly for the central maxillary incisors. The inter-examiner reliability results bring into question the reliability of the digital photography method, but may be explained by systemic biases between the examiners, the subjective measures included in the Modified DDE Index, insufficient examiner training, and the very high prevalence of EH in the study sample. Improvements in examiner training and the measurement index used to classify EH would likely improve inter-examiner reliability. We provided evidence in support of a relationship between early childhood chronic malnutrition

(HAZ), immune activation (CRP), parasitic infection (helminth infection) and EH and between EH extent and several adverse anthropometric and biomarker measures, including shorter height and lower weight, lower hemoglobin and greater WBC count. Given that chronic malnutrition and adverse health outcomes are associated with increased mortality, our findings are in line with the bioarchaeological findings. EH extent also seemed to capture a childhood exposure relevant to adolescent HAZ, hemoglobin and WBC count outcomes above and beyond that of childhood anthropometrics. Although not a strong proxy measure for chronic malnutrition, EH extent may be an important measure for predicting adolescent health outcomes. EH extent may serve as a useful proxy measure of childhood experience among adolescents in settings where childhood stunting data is not available. Furthermore, EH extent may capture childhood exposures relevant to adolescent health outcomes, particularly WBC count, that are not captured by childhood or adolescent HAZ and may thus be a useful addition to the "toolkit" of chronic malnutrition markers. This project makes a unique contribution to the existing literature because it prospectively recorded multiple early childhood exposures (beyond height or stunted growth) and had the minimum follow-up time necessary for full eruption of the permanent dentition to demonstrate an association between malnutrition-related childhood exposures, EH in the permanent dentition and adverse adolescent health outcomes. Subsequent work that builds on this project will be directed toward improving measurement of EH, including further characterization of the spectrum of enamel defects observed in the human dentition, systematically investigating EH etiology across populations and further developing EH as a useful predictor of long-term health by evaluating additional health outcomes, associations in more populations and employing advanced methodology.

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Introduction

In recent decades, research on the developmental origins of health and disease (DOHaD) has demonstrated the important role of early life experience on chronic disease risk later in life, primarily through study of birth weight and growth stunting as markers of early life experience.[1–4] Proposed biological mechanisms underlying these observations are based on human adaptation and plasticity during early life, and seek to make sense of seemingly contradictory relationships, such as that of undernutrition in childhood with overweight and chronic illness in adulthood.[5,6] This dissertation evaluates enamel hypoplasia (EH) in the permanent dentition as a retrospective, biological measure of early childhood experience that may improve understanding of these mechanisms by providing additional specificity to the measure of early childhood experience and enabling more widespread study of the DOHaD in low-resource settings.[4,7] Bioarchaeological findings consistently indicate that an association exists between EH in the permanent dentition and early mortality among skeletal remains.[8] However, few studies have evaluated adult health outcomes in relation to EH in living human populations.

The purpose of this dissertation is to investigate associations between malnutrition-related early childhood experience and EH and to evaluate EH as a predictor of long-term consequences of early childhood experience. Like a window into the past, adult teeth may chronologically reflect early childhood experience.[8] Dental enamel on the permanent teeth calcifies incrementally during early childhood and is sensitive to physiological stress (including micronutrient deficiency, gastrointestinal disturbance, and parasitic, bacterial and viral infections). Defects that result during the formation process do not repair during early childhood nor throughout the life course.[9–11] Few studies of human populations with a high prevalence of EH had the minimum follow-up time (early childhood through full eruption of the permanent dentition) required to prospectively study factors related to EH. Further, the evidence for associations between measures of physiological stress during early childhood and EH is inconsistent and has several methodological weaknesses, including little adjustment for confounding factors, use of different methods for measuring EH and limited longitudinal data.[8]

The primary objectives of this dissertation are to assess whether EH – detected using a validated digital photography method[12] – can be reliably measured, is a marker of prior malnutrition-related

childhood experiences, and is associated with adolescent anthropometrics and biomarkers in a living human population. This dissertation tests the central hypothesis that EH of permanent teeth marks a physiologically-stressful early childhood that is associated with unhealthy anthropometrics and health in adolescence. The rationale that underlies the investigation is that EH is potentially a reliable, retrospective biomarker of early childhood experience in living human populations that may advance insight into the DOHaD. This dissertation tests the central hypothesis by pursuing the following specific aims within an indigenous, rural Bolivian study sample, using a prospective cohort study design:[13]

Aim 1. To determine the reliability of a standardized digital photography method for detecting EH on the permanent maxillary incisors and mandibular canines in an adolescent population from rural Bolivia (Chapter 3)

Aim 2. To evaluate whether malnutrition-related early childhood experiences are associated with EH in the permanent dentition, specifically stunted growth (height-for-age z-scores, HAZ), underweight (weight-for-age z-scores, WAZ), anemia (hemoglobin, Hb), immune activation (c-reactive protein. CRP) and parasitic gastrointestinal infection (hookworm infection) (Chapter 4)

Aim 3. To investigate the relationships between EH and adolescent anthropometrics and biomarkers (Chapter 5)

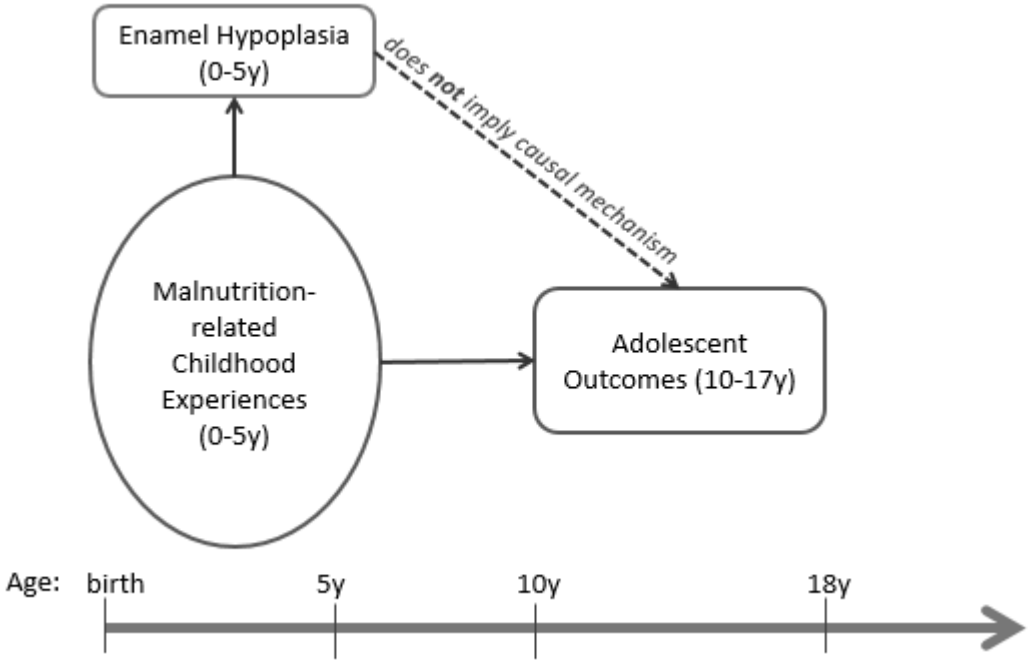
- **Sub aim (3a).** To investigate whether EH in the permanent dentition is associated with individual measures of adolescent anthropometrics and biomarkers, specifically height, weight, BMI, hemoglobin, glycated hemoglobin, white blood cell (WBC) count and blood pressure
- **Sub aim (3b).** To evaluate if associations between EH and measures of adolescent anthropometrics and biomarkers persist after adjustment for malnutrition-related early childhood exposures
- **Sub aim (3c).** To assess whether associations between EH and adolescent biomarkers persist after adjustment for adolescent anthropometrics

Aim 4. To evaluate the accuracy of EH as it relates to early childhood and adolescent growth stunting and to investigate the receiver operating characteristics (ROC) of EH as a classification tool to predict adolescent health risk (Chapter 6)

The findings from this dissertation were expected to positively impact the field of public health by enabling more widespread study of the DOHaD by using the permanent tooth enamel as an insightful exposure measurement tool. This project sought to do so by providing more confidence in interpretations of EH in the permanent dentition and by developing EH as a retrospective measure of early childhood experience that is related to health later in life.

This dissertation project presented a unique opportunity to integrate multiple disciplines, including clinical and social sciences, to test a novel hypothesis derived from the bioarchaeology literature (that EH is associated with adverse outcomes) in a living human population in rural Bolivia.[8] It also addresses calls in the existing DOHaD literature for the identification of better measures of early childhood experience, beyond birth weight and growth stunting, that will capture the source and timescale of specific early childhood exposures.[4,14,15] This dissertation follows the DOHaD conceptual framework and drew from scientific evidence generated by multiple fields of study, including clinical dentistry, epidemiology, and biological anthropology (Figure 1).

Figure 1.
Conceptual framework for the present study



Chapter 1. Background

The developmental origins of health and disease (DOHaD) – exposure assessment

In recent decades, David Barker and colleagues' work uncovered a deeper understanding of the influence of early life experience on chronic disease in adulthood, particularly with regard to critical periods of pre- and post-natal development.[1,2,4] In 1992, Hales and Barker first proposed the "thrifty phenotype" hypothesis to explain how biological mechanisms underlie and can make sense of seemingly contradictory relationships, such as that of undernutrition in childhood with overweight and chronic illness in adulthood and the rise of chronic disease prevalence in low-resource countries where infectious diseases remain endemic.[6,16–18] This model has been substantiated with evidence suggesting that systemic physiologic disruption related to under-nutrition during early life may result in increased risk for chronic disease through physiologic, structural, metabolic, immunologic and epigenetic pathways.[4,19,20] Low birth weight and growth stunting —childhood experiences associated with chronic, low-grade infection and poor dietary quality [21,22] — are associated with insulin resistance and type II diabetes, high blood pressure, and increased risk of cardiovascular disease and obesity in adulthood.[1,2,4,23] These findings diverted researchers' attention from "genetic and behavioral" causation of disease, thinking that predominated the 20th century, to consideration of the role of social and ecological factors that affect early childhood experience**.**

The ultimate goal of DOHaD research is to elucidate the complex ways in which multiple, integrated early life influences, including biological, ecological, psychological and social factors, operate together during the life course to affect adult health outcomes.[24] Several potential models for the "thrifty phenotype" hypothesis have emerged to explain how environmental cues during development may be permanently embodied in the "biologic make-up".[3,5,14,25–29] To better understand the mechanisms underlying the DOHaD, biomarkers, including anthropometric measures, are commonly relied on to indicate early childhood experiences.[21,22,24,30,31] Adult health outcomes potentially associated with early childhood influences that have been widely studied include obesity, hypertension, cardiovascular disease, type II diabetes, respiratory illness, and neuropsychiatric outcomes.[1,2,4,23] Potential biological mechanisms underlying these associations are biological adaptations during critical periods of development (i.e.,

"programming") in response to environmental influences that result in permanent structure and/or function of tissues, organs and/or systems, which increases susceptibility to disease in adulthood.[4,19,20,24,30]

However, the advancement of DOHaD research and a clearer understanding of other potential underlying mechanisms is hindered by issues related to characterizing early childhood experience.[4,7,15] First, the ability to study the DOHaD depends on decades of follow-up data from longitudinal studies since human memory tends to include error when recalling events that occurred many years in the past or during the early years of life. Second, even records of birth weight and stunted growth, commonly relied upon indicators of early childhood experience, are often unavailable in low-resource settings and are likely imperfect markers of more complex processes (including organ growth patterns and epigenetic programming).[14,32,33] Finally, it is generally accepted that age at exposure is an important measure for predicting adult health outcomes, but the extremely limited amount of data that reflects onset and duration of exposure has resulted in an ongoing debate around the timing of critical periods of development.[7,14,19] Therefore, the current DOHaD research community has identified the critical need to identify more accessible measures of early childhood experience that that will specifically capture the source and timescale of various early childhood exposures.[4,14,15]

Etiology of enamel defects

The enamel on the permanent dentition forms during two developmental stages that occur during early childhood in a process known as amelogenesis. First, the matrix is laid down. Then, the enamel matrix mineralizes or calcifies. Physiological disruption to this process leaves a permanent mark in the dental enamel. When these defects are observed clinically, they are classified as hypoplastic (a deficit in quantity of enamel) or opacities/hypomineralization (a defect in the quality of the enamel). Enamel hypoplasia (EH) is traditionally sub-categorized as linear hypoplasia (grooves in the enamel) or pitting (small holes or pits in the enamel).[34] Opacities are described as an opaque, chalky white discoloration of the enamel attributed to hypomineralization.[35]

Underlying these observation-based descriptions may be an indication of the stage of enamel formation/ameloblastic production when the physiologic disruption occurred. EH results from systemic and genetic factors that interrupt the enamel mineralization process during the development/first phase of

amelogenesis, when layers of the enamel matrix are being formed, which results in a reduction in the enamel thickness.[9,11,36] Opacities result from disruptions to the second stage of amelogenesis, which involves mineralization and calcification of the enamel.[35]

Enamel is formed chronologically in layers in a ring-like fashion, like a tree, which has made it possible to establish the rate of formation in 6-month intervals and thereby retrospectively estimate the age when defects developed based on location of the defect on the tooth surface.[37–39] While buried in the gums, before eruption, enamel formation begins at the tip/cervical end of the tooth and proceeds toward what will eventually be the gingival margin. Because of this, the duration of the physiologic stressor is thought to influence the clinical presentation of the defect. For example, if the entire stage of enamel formation is adversely affected, the enamel of the whole dentition will be thin/pitted/rough and mottled with white discoloration, whereas if the physiologic stressor only lasted a short time, linear bands of opacities or grooves in the tooth surface will be seen.[35]

Oral pathology textbooks tend to categorize etiology of enamel defects as systemic/environmental, local/traumatic or hereditary/genetic. Systemic or environmental factors include nutrition and gastrointestinal disturbances (hypocalcemia and vitamin A, C, or D deficiency), bacterial and viral infections, genetic disorders and syndromes/chronic conditions, excessive fluoride exposure, and/or prolonged use of medications.[9–11,36,40] Local factors include trauma, infections that affect the primary teeth, radiation exposure, and cleft lip and palate. Hereditary factors are referred to as “amelogenesis imperfecta”, which are also recognized as hypoplastic or hypomineralized nature, and are sub-classified into four main types and 15 subtypes.[35,41]

In the population health literature, EH tends to be interpreted as a non-specific indicator of childhood physiological stress, so the inferences that may be made from the dental enamel are limited.[42–45] The reported prevalence of EH in the permanent dentition varies greatly across and within populations, in both the past and present, but it tends to correlate with population-level measures of nutrition status, infectious disease prevalence and socioeconomic marginalization.[40] However, the evidence in the human population literature for particular causes of EH in the permanent dentition is inconsistent and has several methodological weaknesses, including little adjustment for confounding factors, use of different methods for measuring EH and limited longitudinal data.[8,46] Studies that have assessed malnutrition, measured by

growth stunting and absence of nutritional supplementation, however, tend to find children who are short for their age and who do not receive nutritional supplements to be significantly more likely to have EH than their well-nourished counterparts.[47–50]

A systematic review of EH in the primary dentition (1991) has been published.[51] The review recognized that the EH etiology remains poorly understood and called for long-term follow up of children with enamel anomalies.[51] This dissertation is the first study to our knowledge that has aimed to investigate specific early childhood experiences and EH in the permanent dentition using prospectively-collected, longitudinal data.

Enamel hypoplasia as a tool for studying the DOHaD.

Logic follows that factors that lead to physiologic and systemic disruption to amelogenesis could simultaneously have deleterious effects on other developing biological systems. Whatever the physiologic disruption is that causes EH may simultaneously afflict other biological systems that are developing during the first five years of postnatal life, which could subsequently influence growth and metabolic function later in life.[3,8,19,52] The bioarchaeological literature provides evidence that health and survivorship is greater among those without EH,[8,43,44,53–55] but little more than discussions and calls to test this association in a contemporary population have been contributed by public health researchers.[8,56,57]

EH in the permanent dentition may be an available, but overlooked, retrospective biological measure of the occurrence and timing of physiological stressors that occurred during early childhood. It also may be readily measured in cross-sectional human population studies using digital photography.[12,58,59] Because enamel on the permanent maxillary incisors and mandibular canines calcifies incrementally during early childhood (0 through 5 years of age) and does not repair following disruptive physiological stress, EH on these teeth can be considered as a permanent physical embodiment of physiological stressors that occurred during early childhood. Since systemic physiologic disruption during early childhood causes defects on the developing permanent dentition, EH may capture a wider range of relevant and particular exposures (such as infectious disease episodes, fever, or periods of micronutrient deficiency) than commonly-used measures of early childhood experience (such as growth stunting or birth weight). Furthermore, the incremental calcification of dental enamel permits

retrospective estimation of the age at which an individual experienced a physiological stressor (in 6-month intervals) during early childhood, which may help to identify critical periods of development in early childhood.[37,38,60] Such improvements in the measurement of early childhood experience could advance DOHaD research by improving the study sensitivity, which would result in valid and consistent risk estimates.

However, the use of EH as a measure of early childhood experience in the study of living human populations is hindered for two main reasons: 1) few human population studies have been designed to assess the relationship between childhood experiences and EH in the permanent dentition, and 2) there is limited evidence for an association between EH and long-term health consequences in living human populations. This dissertation sought to fill these two gaps in the existing literature.

Chapter 2. Methods

This study extended a prospective cohort study initiated in 2002. It involved a sample of adolescents, aged 10-17 years, who were enrolled as young children (5 years and younger) in the 9-year Tsimane' Amazonian Panel Study (TAPS). The original TAPS sought to understand how modernization, increasing intensity of contact with the outside world and market exposure has affected the well-being of people in a remote rural society. The present study built on the TAPS by adding measures of enamel hypoplasia (EH) in the permanent dentition and follow-up measures of adolescent anthropometrics and biomarkers for a subset of the original study's child participants. It is important to note that, although the EH was measured in adolescence, these defects developed during early childhood, while the permanent tooth buds were still forming in the gums. The pre-existing 9-years of longitudinal TAPS data were merged with the new enamel defect and adolescent outcome data to test the central hypothesis.

Study design

This dissertation extended the TAPS (2002-2010) to include adolescent anthropometrics, health measures, and dental enamel data for a subset of individuals who were enrolled in the original study. TAPS was a 9-year prospective cohort study with annual follow-up conducted by Northwestern and Brandeis Universities (PIs: Drs. William Leonard and Ricardo Godoy). The study was designed to assess the impact of market economy integration on the general well-being of the Tsimané people, an Amerindian population of hunter-foragers and horticulturists who live in the Amazonian lowlands of Bolivia.[13] Seven years of pilot work preceded the 2002 initiation of the 9-year TAPS. A study sample of 2,549 individuals (an estimated 8-12% of the Tsimane' population), including all ages, who resided in 13 Amazonian communities in Bolivia were followed annually between 2002 and 2010. During the course of the TAPS, only 7.5% of the study participants permanently left the study but 53.8% left for at least one year of follow-up.[61] These villages were selected based on pilot data findings and on the basis of cost and safety. The selected villages extended from *Campo Bello* in the north, through the market town of *San*

Borja, south to the village of *Yaranda* (Figure 2). A cross-sectional survey of 58 Tsimane' villages was carried out and confirmed the generalizability of the TAPS study sample across the Tsimane' territory.

Figure 2.
Map showing study communities (Bolivia)*

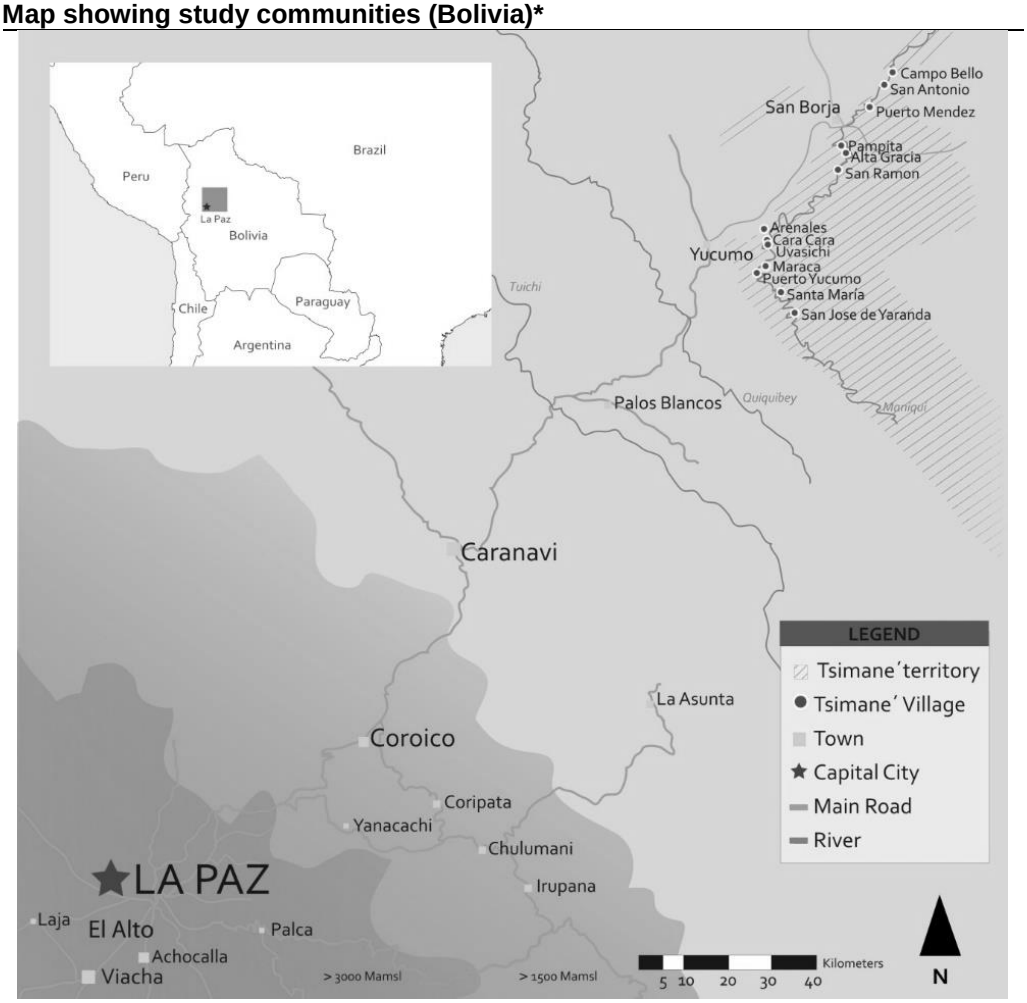


*figure compliments of Dr. William Leonard, Northwestern University[61]

The TAPS team collected detailed measurements of child growth and development, including anthropometric measurements, hemoglobin, immune activation, parasitic infection measures and household demographics. The TAPS dataset is unique because it longitudinally captures the human growth and development process with prospectively-collected data in a rural, low-income country setting. This cohort, which has been followed for more than 10 years, includes extensive systemic health data but – until this dissertation – had no dental component.

Study population

The Tsimane' are an Amerindian population of approximately 8,000 that resides in 80 small villages in the Beni Department of Bolivia, in the Amazonian lowlands. The villages are accessible by dirt road or river. The native language is Tsimané, although some individuals speak Spanish as a second language. Particularly relevant from a global health perspective, the TAPS dataset captures this population's transition process from a traditional hunting and horticultural diet and lifestyle to a market economy and a Western diet of processed food and simple carbohydrates.[62] The Tsimané suffer from persistent under-nutrition (growth stunting) in childhood through adulthood as well as a rising prevalence of overweight and obesity among adults.[63] It is estimated that 45% of Tsimané children are short for their age and 77% have a parasitic infection, yet approximately 23% of adults are overweight or obese (BMI ≥ 25).[21,64,65]

Human subjects protection

All new data collection activities were approved by the University of Washington's (UW) Human Subjects Division (HSD) and the local governing body, the *Gran Consejo Tsimane'* (GCT, Tsimane' Grand Council). Non-UW affiliated researchers were approved under the UW through individual investigator agreements. Consent was obtained from participants who were 16-17y and married, whereas for those not married and those less than 16y, assent and parental permission were obtained. Approval from the UW HSD was also obtained to carry out secondary analyses on the linked 2002-2010 TAPS data.

Data collection activities

Permission to carry out data collection activities in the Tsimane' Territory in 2015 was first obtained from the tribal governing body, the *GCT*. Eligible study participants were then identified by their study identification number from the existing TAPS data. The study team collaborated with a local non-profit research institute in San Borja, Bolivia – the *Centro Boliviano de Investigación y Desarrollo Socio-Integral* (*CBIDSI)*– to locate the eligible study participants. *CBIDSI* was established in collaboration with the TAPS team to implement and supervise projects in the Tsimané communities. The *CBIDSI* has successfully collaborated with the Tsimané communities for approximately 20 years.

Table 1.
Variables and their measures, samples sizes, data source and collection time point

Variable		Measure	Sample Size	Data Source	
				existing TAPS data 2002-2010	dissertation data collection 2015
Enamel Hypoplasia					
	occurrence	any, none	349		X
	extent	< 1/3, 1/3-2/3, >2/3 of tooth surface	349		X
	estimated age at occurrence	age, in 1-year intervals	349		X
Childhood Experience					
	stunted growth	height (cm) converted to sex- and age-standardized z-scores*	338 [1]	X	
	underweight	weight (kg) converted to sex- and age-standardized z-scores*	337 [2]	X	
	anemia	g/dL hemoglobin (Hb), dried bloodspot samples	79 [4]	X	
	immune activation	mg/L c-reactive protein (CRP) concentration, dried bloodspot samples [22]	72 [5]	X	
	parasitic infection	helminth-hookworm infection, fecal samples [64]	115 [3]	X	
Adolescent Outcomes					
Anthropometrics	height	(1) height (cm) and (2) converted to sex- and age- standardized z-scores*	349		X
	weight	weight (kg)			
	body mass	height (cm) /weight (kg)^2 converted to sex- and age-standardized z-scores*	349		X
Biomarkers	anemia	hemoglobin, Hb (g/dL), point-of-care	347 [6]		X
	type II diabetes	glycated hemoglobin, HbA_{1c} (% A_{1c}), point-of-care	331 [6]		X
	immune activation	white blood cell count, WBC (10^9/L), point-of-care	265 [6]		X
	hypertension	systolic and diastolic blood pressure (mm Hg), digital cuff [66]	348 [6]		X

* according to World Health Organization growth charts
[1] = 2002-2010; subsample of 1,006 child-years
[2] = 2002-2010; subsample of 964 child-years
[3] = 2003 and 2007 only; subsample of 115 child-years
[4] = 2002 and 2003 only; subsample of 95 child-years (2-4y)
[5] = 2002 and 2003 only; subsample of 90 child-years (2-4y)
[6] = due to point-of-care device errors in the field

All data collected are listed in Table 1. Data were recorded electronically in the field using KoBo Toolbox (Harvard - http://www.kobotoolbox.org/) and four ASUS MeMO Pad 7®s (ME70CX) running the Android 4.3 Jelly Bean® operating system. Each data collection station had a tablet in which the subject's study identification number and information were recorded. Data were subsequently uploaded and

merged using TAPS study identification numbers. Goal Zero®'s Nomad 7 solar panels and Yeti 150 battery were used to charge equipment in the field.

The field team was comprised of a local dental hygiene student, a local nurse, a lead CBIDSI researcher and two Spanish-Tsimane' translators from the *GCT*. E. Masterson and an undergraduate student from the United States completed the field team of seven. Team members were assigned specific roles, received training in human subjects research and in their particular role in the data collection activities by E. Masterson. Data collection activities were held in the Tsimane' communities (Appendix A). Upon arrival in each community, the Tsimane' translators presented a letter of support from the *GCT* and introduced the study to the community. They explained who was eligible for participation and asked those who were eligible if they were interested in participating. Consent or parental permission and assent were obtained per the approved UW HSD protocol.

In exchange for the communities' and participants' goodwill and time, the study team's hygienist and translators offered each community a dental education workshop in the native Tsimane' language that addressed dental caries etiology and prevention. School teachers were taught about oral hygiene routines and toothbrushes and toothbrush holders were left in each classroom. Incentives were also offered to individual study participants to compensate them for their time and their participation in the study. Data were collected in an assembly line fashion with multiple stations, and it took approximately 40 minutes to complete all data collection elements for each participant. Counselling regarding oral health, anthropometrics, and point-of-care test results were also offered to study participants and their parents by the study team nurse and dental hygienist.

On average, the field team was able to collect all data on 3-4 participants per hour. The team worked approximately 8-10 hours per day, though an extensive amount of time was spent in transit to arrive at the remote study communities, dealing with unexpected logistical challenges, and waiting in the communities for word to spread to eligible participants and then for them to arrive on foot at the central study site in the community. Data was collected on 349 participants in 7-8 weeks (30 days of work in the field). During this time, the 13 original TAPS communities were visited (318 participants enrolled), and then two "sweep" weeks were spent contacting and visiting additional communities that were feasible to access and where eligible participants were believed to have moved to (enrolled 31 more participants).

Study sample

A sample of adolescents, who had been enrolled in TAPS as children, were recruited for this study. Criteria for inclusion in the study sample were being 10-17 years old in 2015 and previous enrollment in the TAPS for at least one year at age five years or less. All of those who met the inclusion criteria were included in the study sample. In 2015, a portion of the study sample of interest was expected to have unhealthy anthropometric measurements and perhaps even exhibit early indicators of chronic disease risk.[65] Furthermore, by 10 years of age the permanent anterior dentition is typically fully erupt, meaning EH is visible to the naked eye and able to be captured in a digital photograph. 655 individuals in 13 communities were eligible for inclusion in the study.

The final study sample was comprised of 349 Tsimane' adolescents (53% of eligible individuals) who the study team were able to locate and enroll. Given the semi-nomadic lifestyle of the study population and that 5-13 years had passed since eligible study participants' last recorded TAPS enrollment record, this proportion was, anecdotally, greater than was anticipated by the TAPS investigators. The final study sample did not differ significantly from the eligible study sample in demographic or child growth characteristics (see Appendices B1 and B2).

Digital dental photography

Digital photographs were taken of all participants according to a "best tooth" assessment,[38,67] which includes images of the four permanent maxillary incisors and two mandibular canines (Figure 3), to detect EH according to a validated method.[12] Based on histologically-informed estimates of the timing of enamel development for these six teeth (birth through five years of age), this approach captures 95% of the information that could be obtained from all teeth (Figure 3).[37,38,67]

Figure 3.
A "best tooth" assessment of the permanent dentition

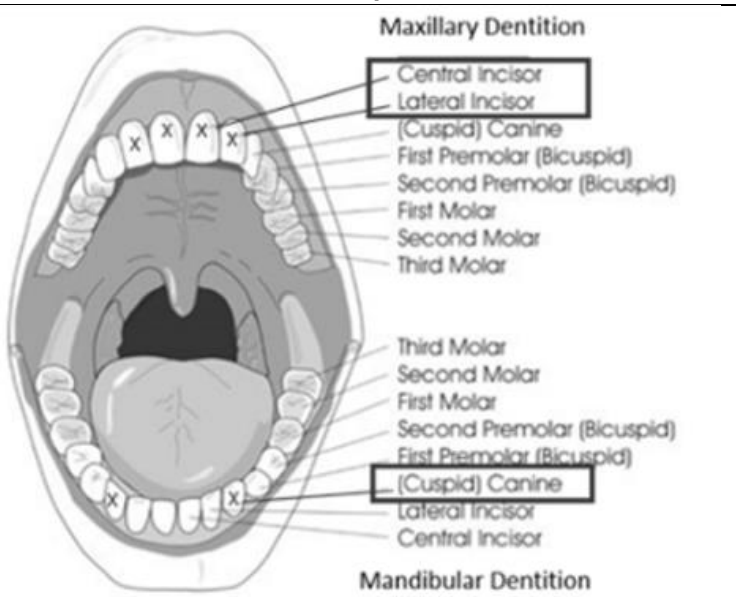
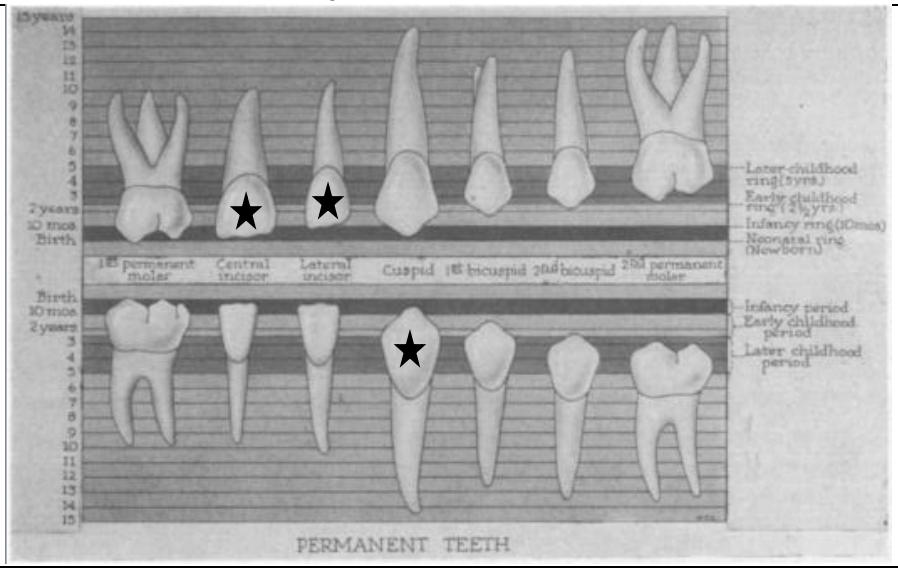


*figure adapted from dentalimplants-usa.com

It is important to note that although the EH was measured in adolescence (in 2015), the measure reflects events that occurred during early childhood (between 2002 and 2010 for the study sample), according to the biology of dental enamel development on the permanent teeth of interest (Figure 4).

Figure 4.
Age during enamel formation for the permanent dentition



Note: teeth with stars denote the "best tooth" assessment, adapted from Massler and Schour 1946[68]

A digital single lens reflex (SLR) camera with a 100mm macro lens and ring flash was used to take all dental photographs for evaluation of EH (Canon Rebel SL1 body, Canon EF 100mm f2.8 USM macro lens, Canon MR-14 EX Macrolite ring flash). Digital SLRs are superior to compact cameras because the SLR enables manual focus (and therefore consistent magnification) and captures a higher quality image with settings manually set to be comparable/consistent across shots. SLR cameras enable confident and accurate focusing on a specific point in the oral cavity. The 100mm prime telephoto macro lens enables shots of the oral cavity to be captures from about 100mm away from the study participant (opposed to only, say, 55mm away). The ring flash provides even and strong lighting to reduce shadows.

For all photos, the following camera settings were used: manual, shutter speed at 1/200, ISO at 100, image quality to save two files per photograph (a RAW and JPEG file), white balance as “daylight”, picture style as “neutral”, and aperture was adjusted depending on the photograph being taken. The macro lens was set to manual focus and the magnification on full. The ring flash was also set to manual focus. For anterior photos, capturing the maxillary incisors and canines and the mandibular canines, a 1:2 magnification and f29 aperture was used and only one flash set to half power was used so as to better capture the contours of the enamel surface. A second, close-up photo of the central maxillary incisors was captured with a 1:1 magnification, f29 aperture and only one flash set to half power.

Study participants were asked to sit in a chair, use two separate metal cheek retractors to pull back their cheeks and lips so that all 12 anterior teeth were visible to the gum lines, and close their incisors edge to edge (then slightly open so all anterior teeth were completely visible). The anterior teeth were dried with cotton, but due to field conditions, it was not feasible for participants to brush their teeth prior to taking the photographs. Prior to taking the photograph, a photo of the participant’s study identification (written on a card) was taken to record and be able to identify which photo belongs to which study subject. Two photos were taken of each study participant’s teeth – one with 1:2 magnification of the “best tooth” assessment and another with 1:1 magnification of only the central maxillary incisors. Both photos were focused on the central maxillary incisors. Participants were asked to close their eyes so as to not be bothered by the bright ring flash. The procedure was repeated immediately if the photographic

quality was not acceptable. All photographs were saved on a field laptop and frequently backed up on two 128 GB USB memory sticks.

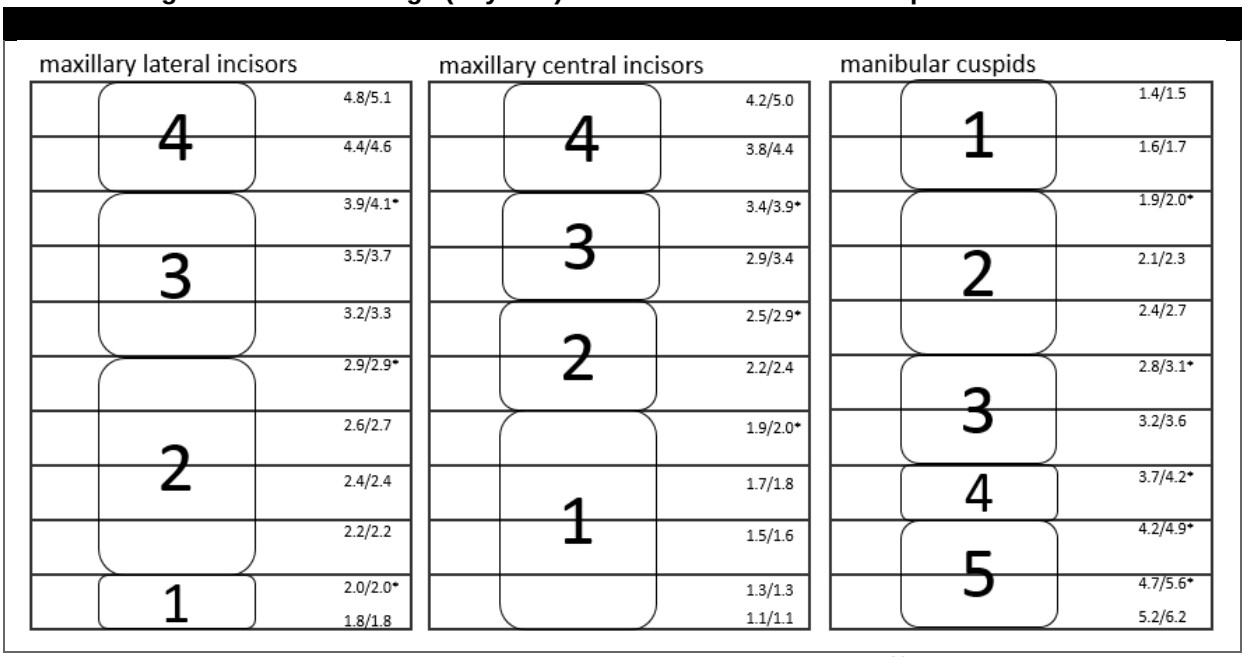
Evaluation of digital dental photographs

EH data was abstracted from digital photographs using Adobe Photoshop software. The full set of photographs for the whole study sample (n=349) was evaluated by E. Masterson to measure occurrence of EH (any, none), type of EH (grooves, pitting, missing or "cobblestone" pattern), extent of EH (< 1/3, 1/3-2/3, >2/3 of the tooth), and estimated age at occurrence of EH (by year of age) by tooth, for each of the six teeth of interest (central and lateral maxillary incisors and mandibular canines). Intra-examiner (two assessments by the same examiner – E. Masterson -- at two different points in time, 4.5 months apart) and inter-examiner (assessment by two different examiners – E. Masterson and W. Sabbah, DDS, PhD) reproducibility of EH measurement for each of the six teeth of interest was evaluated on a sub-sample (n=80). For each of the six teeth assessed, both examiners evaluated the tooth to (1) detect the occurrence of EH and (2) complete the data abstraction form to record type and extent of EH according to a pre-defined protocol [Appendices C and D]. E. Masterson also marked (using the Photoshop brush tool) EH accordingly on the affected teeth to (3) estimate the age at EH occurrence. Examiners were blinded to all study participant characteristics.

The occurrence, type and extent of EH were measured according to the standard, internationally-accepted Developmental Defects of the Enamel (DDE) Index and Modified DDE Index descriptions of enamel defects [Appendix E].[34,69] The estimated age at EH occurrence was based on histologically-informed estimates of age at the time of enamel development. Because enamel is formed on the tooth chronologically, the distance from the gingival margin to the enamel defect can be measured, and this distance can be used to estimate the age at development of EH.[37] The tooth-based estimates put forth by Reid and Dean were used for this project.[39] Comparisons of human enamel formation timing, the basis for these estimates, have been made across diverse populations (southern Africa and northern Europe) and the variation is found to be very small.[39] When there was a discrepancy between age cutoffs from African and European populations, the European cutoffs were used.

The estimated age at EH occurrence was measured using age estimates (by deciles) appropriate for each of the six teeth of interest. These age estimates were used to create grids in Microsoft PowerPoint (Figure 5).

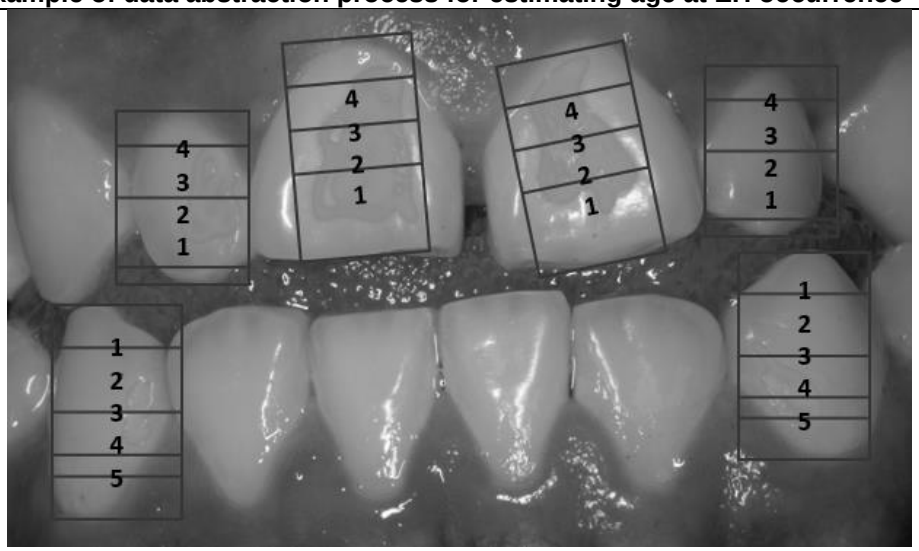
Figure 5.
Mean histological estimates of age (in years) at enamel formation in the permanent anterior teeth



*Note: age estimate on left (from African pop.) and on right (European pop.)[39]

The grid lines form zones to identify age in years during the enamel formation for each portion of the tooth surface. Each relevant grid was overlaid and adjusted on the close-up photograph of the participants' teeth, already marked for EH, so that each age zone could be classified as "yes/no" for EH occurrence on each tooth (Figure 6).[40]

Figure 6.
Example of data abstraction process for estimating age at EH occurrence



Adolescent anthropometrics and biomarkers

Anthropometric measurements were taken on study participants, according to standard procedures,[70] which included: height (cm), sitting height (cm), leg length from knee to heel (cm), weight (kg), body composition (percent body fat), and waist circumference (cm). Several biomarkers derived from blood samples (described below) were also measured on all study participants, including hemoglobin (Hb, in g/dL), glycated hemoglobin ($HbA_{1c,}$ as $\%A_{1c}$) and white blood cell count (WBC, in 10^9/L). Systolic and diastolic blood pressure (mmHg) were also measured.

To measure height, a stadiometer was placed on a flat surface against a wall. Participants were asked to stand with their heels, buttocks and scapula touching the stadiometer. The researcher adjusted his or her chin so that it was in line with the F plane before lowering the headboard, asked the participant to take a deep breath and stand tall. The headboard was then compressed through the participant's hair. Height was then recorded in centimeters. This procedure was repeated twice. If the two measurements were not within 1cm, height was measured a third time. To measure weight and estimate body composition (percent fat), a bioelectrical impedance scale (Tanita BF522W Body Fat Monitor/Scale) was placed on a flat surface and turned on. The age, sex and height of the participant was entered and the participant was then asked to stand on the scale, face the researcher with his or her hands at his or her

side, look straight ahead and stand still. The researcher then recorded the weight and percent body fat. Waist circumference was measured using a tape measure according to published methods.[71] The participant was asked to stand erect, feet together, facing the researcher with arms held away from the body. A tape measure was place around the waist ensuring it was level horizontally, touching but not compressing the soft tissue at the top of the hip bone. The participant was asked to take a deep breath and exhale. At the end of the exhalation, the tape measure was locked in place and the measurement recorded. This procedure was repeated twice. If the two measurements were not within 1cm, waist circumference was measured a third time. Sitting height and knee height were also measured with a tape measure. The participant was asked to sit upright in a chair. Sitting height was recorded as the measurement from the vertex of the head to the seated buttocks. Knee height was recorded as the measurement from the anterior surface of the thigh (above the condoyle of the femur and ~4cm above the patella) to the floor.

Point-of-care devices were used to obtain measurements from blood samples. Wearing gloves, the researcher cleaned the participant's index or ring finger with an alcohol wipe and allowed it to fully dry in the air. A lancet was used to prick the finger, off center but away from the nail bed. The first drop of blood was wiped away and three microcuvettes were filled with blood. The blood on the participant's finger was wiped away again and a band aid was applied. The three microcuvettes were inserted into their corresponding analyzer – the Hemocue WBC, Hemocue Hb 201+, or Bayer A1cNow. Lancets were disposed of in a sharps container and biohazard materials were collected in a separate bag and both were later disposed of according to local standards. To measure blood pressure, the Omron Digital Blood Pressure Monitor HEM-907XL was used. Participants were asked to sit in a chair with their legs not crossed and their feet on the ground. The appropriately-sized cuff was placed on the participant's right upper arm approximately ¼" above the elbow ensuring that no clothing was under the cuff. The participant was guided to slightly flex the elbow so the cuff was level with his or her heart. Systolic and diastolic blood pressure and pulse were recorded three times for each participant and the average was used for analytic purposes.

The primary equipment necessary to take these measurements – including the portable stadiometer, a plastic measuring tape, the bioelectrical impedance body fat analyzer/scale, the digital

blood pressure cuff, and the Hemocue hemoglobin and white blood cell count analyzers – were provided to E. Masterson free of charge, with several hours of laboratory training in their use, by the Center for Studies in Demography and Ecology's (CSDE) Biodemography Core at the UW. Cards with written test results were offered to study participants. The study team nurse was available to consult with participants and discuss their point-of-care test results.

Childhood exposure data

To evaluate whether EH was associated with malnutrition-related early childhood experiences in the study sample, including stunted growth (height-for-age z-scores, HAZ), underweight (weight-for-age z-scores, WAZ), anemia (hemoglobin, Hb), immune activation (c-reactive protein, CRP) and parasitic gastrointestinal infection (hookworm infection), the existing data collected by TAPS when study participants were young children were used to measure early childhood exposures of interest (see Table 1 for a description of these measures).[13]

Demographic and covariate data

Age, sex, household socioeconomic status (SES) and dietary consumption measures were used from the TAPS dataset. The fact that many Tsimane' are uncertain of their exact age presents an analytic challenge. The TAPS team developed an algorithm to correct inconsistencies in reported age during the TAPS. The present study utilized this age variable to select the eligible study sample and to determine appropriate informed consent procedures.[61] Household SES was measured by the TAPS team between 2002 and 2010 by total wealth, a locally-developed sum of animal, trade and modern wealth measured in the local currency (bolivianos, Bs).[72] Dietary consumption for one week was also measured on the household level by the TAPS team between 2002 and 2010 through annual interviews in participants' homes. Household sugar, plantain and manioc (*yuca*) consumption per week were measured in kilograms (kg).[72]

Chapter 3. Reliability of intraoral digital photography for detecting enamel hypoplasia in an Amerindian population from the Bolivian Amazon

Abstract

Objective: Enamel hypoplasia (EH) in the permanent dentition may serve as a useful retrospective marker of early childhood physiological stress and thus as a screening tool for general health risk in adolescence. Digital photography may facilitate measurement of EH, as has been suggested in recent literature. However, it has historically been argued that EH cannot be detected with the naked eye. Few studies have evaluated the reliability of the digital photograph method for measuring EH. The present study evaluated the reliability of EH measurement using digital photographs and the Modified DDE (developmental defects of the enamel) Index in a study sample from rural Bolivia.

Methods: This study was conducted within an Amerindian population in the Bolivian Amazon. Our study sample included 349 adolescents (10-17 years of age) who enrolled for at least one year in a longitudinal study during early childhood (1-4 years). We detected EH in the permanent maxillary incisors and mandibular canines using digital photography from which the following measures of EH were abstracted: occurrence (any, none), extent of occurrence (<1/3, 1/3-2/3, >2/3 of the tooth surface) and estimated age at occurrence (1, 2, 3, 4 years of age). The digital photographs were scored by two independent examiners. Intra- and inter-examiner reliability for abstracting EH measures from digital photographs by blinded examiners was quantified by concordance and kappa values. Frequency of EH detection across examiners was described and evaluated for systemic biases.

Results: The overall prevalence of a rough hypoplastic pattern of EH in the study sample was 92.3%. EH detection was most common on the central maxillary incisors (87%) compared to the lateral maxillary incisors (63%) and mandibular canines (26%). The intra-examiner reliability for detecting EH occurrence on the maxillary incisors (mean kappa=0.77) was very good and greater than on the lateral maxillary incisors (mean kappa=0.68) and mandibular canines (mean kappa=0.49). The inter-examiner reliability

was fair to poor, though EH occurrence detection on the maxillary incisors (mean kappa= 0.29) was also greater than on mandibular canines (mean kappa=0.17).

Conclusion: The present study detected a EH pattern that was nearly ubiquitous in the study sample, but the rough, cobblestone-like hypoplastic pattern does not fit the typical linear/grooved pattern described in the overwhelming majority of the malnutrition literature. The pattern does not provide evidence in support of a systemic cause. Intra-examiner reliability results suggest that digital photography is a reproducible method for capturing EH, particularly for the central maxillary incisors. More than one photo may be required to reliably measure EH with a “best tooth analysis”. Poor inter-examiner reliability may be explained by systemic biases between the examiners, the subjective measures included in the Modified DDE Index, insufficient examiner training, and the very high prevalence of EH in the study sample.

Introduction

Infectious illness (including fever) and micronutrient deficiency during early childhood may cause development of enamel hypoplasia (EH) through disruption of the enamel mineralization process during the development phase when layers of the enamel matrix are being formed, resulting in a reduction in the enamel thickness.[9,11,36]. Studies that have assessed malnutrition, growth stunting, or absence of nutritional supplementation during early childhood have consistently found children with poorer nutritional status to be significantly more likely to have EH in their permanent dentition than their well-nourished counterparts.[47–50] Enamel hypoplasia (EH) in the permanent dentition may thus serve as a useful retrospective marker of early childhood physiological stress and thus as a screening tool for general health risk in adolescence if it can be easily and reliably measured in individuals

Digital photography may facilitate unbiased measurement of EH, as has been suggested in recent literature.[12,58] However, it has historically been argued that EH cannot be detected with the naked eye.[73–75] Few studies have actually evaluated the reliability of the digital photograph method for measuring EH without error. The present study evaluated the reliability of EH measurement using digital photographs in a study sample from rural Bolivia.

Methods

A detailed account of all study methods are described in Chapter 2.

Study design and sample

This study extended a prospective cohort study that began in 2002. In 2015, it followed up a sample of adolescents, aged 10-17 years, who were enrolled as young children (5 years and younger) in the 9-year Tsimane' Amazonian Panel Study (TAPS).[72] The present study built on the TAPS by adding measures of enamel defects in the permanent dentition for the original study's child participants. Criteria for inclusion in the follow-up study included that an individual be 10-17 years of age at the time of data collection (so that the central maxillary incisors will have fully erupted), was previously enrolled in TAPS for at least one

year when aged 5 years or less, and resided in one of 15 communities visited by the data collection team during the 2015 follow-up study.

The study sample was derived from the Tsimane' population, an Amerindian group of approximately 8,000 people who live across more than 100 small, remote communities, accessible by dirt roads and/or canoe and river in Bolivia's Amazonian Basin. Although they are increasingly integrating into mainstream Bolivian society and exposed to the market economy and a Western diet, the Tsimane' maintain a traditional hunter-horticultural lifestyle and primarily speak their native language in their communities.[72] The original TAPS study included 1,453 people, 655 of who were eligible for the 2015 follow-up study. 349 participants were enrolled in the 2015 follow-up study and included in the present analysis. Approval for all data collection and use was obtained from the local tribal government, the *Gran Consejo Tsimane'* (*GCT,* Tsimane' Grand Council) and the University of Washington (UW) Human Subjects Division (HSD).

EH Measurement

The measurement of interest in these analyses was EH in the permanent dentition. EH was captured through digital intraoral photographs with a macro lens and ring flash, detected using Photoshop software, and quantified according to the internationally-accepted Developmental Defects of the Enamel (DDE) Index/Modified DDE Index to permit comparison to past and future studies among other populations.[34,69] EH was classified by occurrence (any, none), extent of tooth surface affected by EH (less than 1/3/ 1/3 to 2/3 / more than 2/3), and the estimated age at EH occurrence (by year of age).

Statistical Analyses

Characteristics of the reliability sub-sample were reported in comparison to the full study sample. Variation in the prevalence of EH occurrence and extent by each of the six teeth of interest (central and lateral maxillary incisors and mandibular canines) was also reported, based on the full study sample.

To determine the reliability of a standardized digital photography method for detecting EH on the permanent maxillary incisors and mandibular canines, we categorized photographs by subject's year of age at the time the photograph was taken (10-17 years, 8 age categories). We then used SAS's

SURVEYSELECT procedure to randomly select (without replacement) ten participants from each age category (80 participants selected). A sub-sample of 80 individuals was justified based on the precision for the half width of a kappa 95% confidence interval (CI) being 0.28 and 0.16 for a kappa value of 0.6 and 0.8, respectively.[76] Intra-examiner (two assessments by the same examiner – E. Masterson, MPH -- at two different points in time, 4.5 months apart) and inter-examiner (assessment by two different examiners – E. Masterson and W. Sabbah, DDS, PhD) reproducibility of EH measurement for each of the six teeth of interest for 80 participants was evaluated. Examiners were blinded to all study participant characteristics. Concordance and kappa (or weighted kappa) values and their 95% confidence intervals were reported for the occurrence and extent of EH for intra- and inter-examiner evaluations. Overall frequencies of EH detection by examiner was also reported to evaluate systemic biases between the two examiners.

Finally, a description of the prevalence and incidence (based on histologically-informed estimates of the age at enamel calcification[39]) of EH measures in the full 2015 study sample (n=349) were reported. Concordance of EH occurrence and extent by tooth were reported to evaluate symmetry across the dental arch. Analyses were completed with the PROC TTEST and PROC FREQ (with AGREE and ZEROS options) functions in SAS Version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Study sample characteristics

The full study sample was comprised of 349 adolescents. A rough, cobblestone-like pattern of EH on the tooth surface was particularly prevalent in the study sample (observed in 92.3%, Appendix F). The results that follow focus on this particular pattern of EH. A sub-sample of 80 individuals were randomly selected from the full study sample of 349 adolescents to evaluate the intra- and inter-examiner reliability for measuring enamel hypoplasia using digital photographs. The sub-sample was not statistically different from the full study sample by participant age, sex, EH occurrence or extent on the central maxillary incisors (Table 1).

Table 1.
Descriptions of the sub-sample and full study sample

	Sub-sample, n=80	Full study sample, n=349
Age, mean ± SD	13.5 ± 2.3	13.0 ± 2.2
Sex, % male	51.2%	51.3%
EH occurrence *	89.7%	92.3%
EH extent ł		
no EH	10.3%	7.7%
<1/3	23.4%	23.8%
1/3 – 2/3	49.4%	43.8%
> 2/3	16.9%	24.7%

*sub-sample n=78/ full sample n=337, based on non-missing/severely decayed central maxillary incisors
ł sub-sample n=77/ full sample n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)

EH measures varied in prevalence by tooth surface in the study sample. Overall, EH occurrence was detected on the central maxillary incisors the most and the mandibular canines the least. Similarly, on average, the central maxillary incisors were affected with the greatest extent of EH (Table 2).

Table 2.
Variation in EH measures by tooth and based on full study sample (n=349)

	Central maxillary incisor		Lateral maxillary incisor		Mandibular Canine	
	Right (n=332)	Left (n=328)	Right (n=339)	Left (n=336)	Right (n=322)	Left (n=331)
EH occurrence	86.2%	87.2%	68.9%	56.3%	27.2%	24.7%
EH extent						
no EH	13.8%	12.8%	31.1%	43.7%	72.8%	75.3%
<1/3	24.3%	22.3%	41.3%	41.1%	26.6%	23.8%
1/3-2/3	38.0%	41.0%	21.9%	10.9%	0.6%	0.3%
> 2/3	23.9%	23.9%	5.7%	4.3%	0.0%	0.6%

Intra-examiner reliability

To estimate intra-examiner reliability, the primary investigator (E. Masterson) evaluated a random sub-sample of 80 photographs 4.5 months after the initial evaluation of the full study sample. When detecting EH occurrence, concordance was greatest for the central maxillary incisors, but when detecting the extent of EH, concordance was greatest for the mandibular canines (Table 3). When extent of EH classification off by one level of measurement was considered concordant (e.g., <1/3 ≈ 1/3-2/3, 'none' was only considered concordant with 'none'), the agreement for the extent of EH measurement was greater,

especially for the maxillary incisors (89% and 93% for the right and left central incisor, respectively, and 83% and 84% for the right and left lateral incisor, respectively).

Table 3.
Concordance and kappa values of intra-examiner reproducibility by tooth (4.5 months, n=80)

	Central maxillary incisor		Lateral maxillary incisor		Mandibular Canine	
	Right	Left	Right	Left	Right	Left
EH occurrence						
concordance	93.8%	95.1%	84.0%	84.8%	83.8%	77.5%
kappa	0.75	0.79	0.65	0.70	0.54	0.44
& 95% CI	(0.54, 0.96)	(0.59, 0.99)	(0.48, 0.82)	(0.54, 0.85)	(0.33, 0.76)	(0.22, 0.66)
EH Extent						
concordance	65.4%	50.6%	65.4%	69.6%	83.8%	77.5%
weighted kappa	0.60	0.33	0.56	0.53	0.54	0.44
& 95% CI	(0.46, 0.74)	(0.14, 0.52)	(0.41, 0.70)	(0.38, 0.68)	(0.33, 0.76)	(0.22, 0.66)

Kappa values indicated that the intra-examiner reliability for detecting EH occurrence on all four of the maxillary incisors was "substantial" (average kappa=0.72) according to conventional guidelines.[77] The intra-examiner reliability for detecting EH occurrence on the central maxillary incisors, in particular, was nearly considered "almost perfect" or kappa > 0.80 (average kappa=0.77). The intra-examiner reliability for detecting EH occurrence on the mandibular canines was only "moderate" (average kappa=0.49). Weighted kappa values indicated the intra-examiner reliability for detecting EH extent varied from "fair" to "moderate" (average kappa = 0.50).

The best reliability overall was for the central maxillary incisors. For the right and left central maxillary incisors, the observed concordance for EH occurrence exceeded that expected by chance by 75% (95% CI: 54%, 96%) and 79% (95% CI: 59%, 99%), respectively, of the potential improvement beyond chance.

Inter-examiner reliability

To estimate inter-examiner reliability, a clinical investigator (W. Sabbah) evaluated a random sub-sample of 80 study participants' photographs. For EH occurrence, concordance across examiners was similar for

all six teeth evaluated (mean: 70.0%). For EH extent, concordance was greatest for the mandibular canines (Table 4).

Table 4.
Concordance and kappa values of inter-examiner reproducibility by tooth (v. clinician, n=80)

	Central maxillary incisor		Lateral maxillary incisor		Mandibular Canine	
	Right	**Left**	**Right**	**Left**	**Right**	**Left**
EH occurrence						
concordance	73.8%	69.1%	67.9%	68.8%	70.9%	69.4%
kappa	0.31	0.26	0.36	0.37	0.19	0.14
& 95% CI	(0.10, 0.53)	(0.07, 0.45)	(0.16, 0.56)	(0.17, 0.58)	(-0.04, 0.43)	(-0.09, 0.38)
EH Extent						
concordance	33.8%	29.6%	50.6%	56.3%	70.9%	70.4%
weighted kappa	0.18	0.14	0.19	0.25	0.19	0.14
& 95% CI	(0.07, 0.28)	(0.05, 0.22)	(0.08, 0.30)	(0.12, 0.38)	(-0.04, 0.43)	(-0.09, 0.38)

Kappa values indicated that the inter-examiner reliability for detecting EH occurrence on the maxillary incisors was “fair” (average kappa=0.29), but only “slight” for the mandibular canines (average kappa=0.17). Weighted kappa values indicated the inter-examiner reliability for detecting EH extent varied from “poor” to “fair” (average kappa=0.18).

The mandibular canines had the worst reliability overall, with the lower bound of the 95% confidence interval suggesting the agreement was equal or worse than chance alone could produce. For the right and left mandibular canine, the observed concordance exceeded that expected by chance by only 19% (95% CI: -4%, 43%) and 14% (-9%, 38%), respectively, of the potential improvement beyond chance.

The poor inter-examiner reliability was due, in part, to the tendency of the first examiner to detect EH more often and with greater extent than the second examiner (Table 5). Spearman rank correlation coefficient indicates a linear relationship between examiner measurements of EH extent ($r=0.46$, $p<0.001$). Thus, when extent of EH classification off by one level of measurement was considered concordant (e.g., <1/3 ≈ 1/3-2/3, ‘none’ was only considered concordant with ‘none’), the agreement across examiners for the extent of EH measurement was greater, particularly for the central maxillary incisors (65% instead of 34% for the right side and 61% instead of 30% for the left side).

Table 5.
EH detection for all teeth, by examiner

	Examiner 1, first evaluation (n=480)	Examiner 1, second evaluation (n=480)	Examiner 2 (n=480)
EH occurrence	52.7%	59.5%	46.2%
EH extent			
no EH	47.3%	40.5%	53.8%
<1/3	26.4%	22.0%	43.7%
1/3 – 2/3	20.5%	28.2%	2.5%
> 2/3	5.8%	9.3%	0%

Description of EH in the study sample

The descriptive EH results that follow were based on observations made on the central maxillary incisors, which had the best overall reliability, of the full study sample (n=349). 337 (96.6%) participants had at least one non-missing and not severely decayed central maxillary incisor by which we could measure EH. We classified EH based on the left central maxillary incisor in 328 participants (94.0% of the full study sample). Nine more participants (2.6% of the full study sample) who did not have a non-missing and not severely decayed left central maxillary incisor had a right central maxillary incisor by which we classified their EH status. Childhood and demographic characteristics between those who were included in the description and those who were not, due to absence of both central maxillary incisors, differed in average age, childhood average anthropometrics, but not in sex, adolescent anthropometrics (Appendix H).

Variation in the extent (portion of tooth surface affected by EH) and incidence of EH by year of age (1-4 years) was observed across the study sample (Table 6). Ninety-two point three percent of the study sample had EH. EH affected 1/3 to 2/3 of the tooth surface for most of those with EH. The incidence of EH was similar for age 1, 2, 3 years (average 89.7/100 child-years), but was much lower for age 4 years (62.9/100 child-years). EH was not associated with sex or age in adolescence, at the time of measurement (Appendix G).

Table 6.
Description of enamel hypoplasia in the full study sample, overall and by central maxillary incisor

	Right (n=332)	Left (n=328)	Overall (per person) (n=337)
EH occurrence	299 (90.1%)	304 (92.7%)	311 (92.3%)
EH extent*			
no EH	33 (9.9%)	24 (7.3%)	26 (7.7%)
< 1/3	82 (24.7%)	79 (24.1%)	80 (23.8%)
1/3 to < 2/3	132 (39.8%)	142 (43.6%)	147 (43.8%)
≥ 2/3	84 (25.6%)	82 (25.0%)	83 (24.7%)
Incidence of EH, per 100 child-years*			
occurrence at age 1 year	83.7	90.8	90.2
2 years	88.5	91.7	91.1
3 years	84.6	88.4	87.8
4 years	64.4	62.1	62.9

* left n=327, right n=331, combined n=336 due to one participant without fully erupted central maxillary incisors (only teeth with all enamel zones present are included)

If the cause of the EH pattern observed in this study population was systemic (genetic or environmental), symmetry in the EH would have been expected across the dental arch (i.e., right and left sides). For example, it would have been expected that both central maxillary incisors (left and right) had the same extent of EH and that the age 2 years zone on the central maxillary incisor corresponded to the age 2 years zone on the lateral maxillary incisor. On the other hand, a local cause could have resulted in asymmetric defect patterns. Among the 323 study participants that had both the left and right central maxillary incisors present (not severely decayed or missing), there was 93.4% agreement (302/323) across both central maxillary incisors for EH occurrence (Table 6). The concordance for right and left central incisors for the extent of EH measurement (a 4-level, ordinal measure: no EH, < 1/3, 1/3-2/3, > 2/3) was 65.8% (212/322). When measures off by one level of measurement were considered concordant (e.g., <1/3 ≈ 1/3-2/3, 'none' was only considered concordant with 'none'), the agreement across both central incisors for the extent of EH measurement was 95.7% (308/322). The concordance across both central incisors for the incidence of EH at age 1 year, 2 years, 3 years, and 4 years was 89.4 (288/322), 93.4 (301/322), 92.2 (297/322), and 84.5 (272/322) per 100 child-years, respectively. However, the concordance for EH occurrence across the same age zones on all six teeth was only 9.4% for one year of age, 13.5% for two years, 14.6% for three years and 25.5% for four years.

We evaluated EH incidence by EH occurrence and extent to determine if EH incidence captured a pattern in the dentition that was not captured by EH occurrence or extent (Table 7). Among those with EH, 60% (203/336) were affected during all four years of the childhood period of interest (1-4 years). The total number of years affected was correlated with the measure of EH extent ($r=0.70$, $p < 0.0001$). When evaluated year by year, the distribution of EH occurrence and extent for those with EH incidence at year 4 was distinct from those with incidence at years 1, 2 and 3 years. Fewer individuals were affected at 4 years than at 1, 2, or 3 years. However, those who were affected at age 4 years seemed to have a greater extent of EH than those affected during age 1, 2 and 3 years. Finally, we classified individuals by EH pattern, where each individual could only be in one category. For example, "EH1" indicates EH incidence *only* at age 1 year and "EH1234" indicates EH incidence all four years. These patterns revealed that EH incidence at age 4 years was the primary source of variation with regards to EH incidence. Almost all participants were affected by EH at age 1, 2 or 3 years, but only 62% of participants with EH were affected at 4 years of age. 101 participants (33% of those with EH) had EH occurrence at 1 year of age but not at 4 years of age, but only 6 participants (2% of those with EH) had EH occurrence at 4 years of age, but not at 1 year of age.

Table 7.
Description of EH incidence by occurrence and extent (n=336), n (%)

	EH occur. (n=310)	no EH (n=26)	EH Extent		
			< 1/3 (n=80)	1/3 – 2/3 (n=147)	> 2/3 (n=83)
Total number of years with EH					
0	0	26 (100)	0	0	0
1	1	0	1 (100)	0	0
2	15	0	12 (80)	3 (20)	0
3	91	0	41 (45)	40 (44)	10 (11)
4	203	0	26 (13)	104 (51)	73 (36)
EH incidence					
at 1y	304	32 (10)	75 (22)	146 (43)	83 (25)
at 2y	307	29 (9)	77 (23)	147 (44)	83 (25)
at 3y	296	40 (12)	69 (21)	144 (43)	83 (25)
at 4y	209	127 (38)	31 (9)	105 (31)	73 (22)
EH incidence patterns					
EH1	1	--	1 (100)	0	0
EH12	13	--	10 (77)	3 (23)	0
EH123	87	--	38 (44)	39 (45)	10 (11)
EH1234	203	--	26 (13)	104 (51)	73 (36)
EH234	4	--	3 (75)	1 (25)	0
EH34	2	--	2 (100)	0	0

Discussion

EH among the Tsimane'

The present study detected a EH pattern that was nearly ubiquitous in the study sample, but the rough, cobblestone-like hypoplastic pattern does not fit the typical linear/grooved pattern described in the overwhelming majority of the malnutrition literature. The hypoplasia observed among the Tsimane' may better fit an early description (from 1746 France) of what would eventually be regarded as EH: "*Its [the tooth's] exterior surface sometimes becomes uneven and rough, pierced by many small holes, somewhat like a rasp in form; but spaced more irregularly…*".[73] Given the acceptance of linear EH (horizontal grooves) as a marker of undernutrition, particularly in the bioarchaeology literature,[73] the absence of this particular EH pattern in this study sample is curious. Furthermore, the pattern also does not provide

evidence in support of a systemic cause despite the strong concordance across the dental arch in the maxillary incisors.

To our knowledge, this was the second study to record enamel defects in an Amerindian Amazonian population. The extremely high prevalence of EH observed in the permanent anterior teeth of the Tsimane' is in line with that observed in another Amerindian Amazonian population (98.7% of the Tupí-Mondé in Brazil have EH classified as linear or missing enamel).[49] Also similar between the Tsimane' and Tupí-Mondé is that the lowest frequency of EH occurred age 4 years in the central maxillary incisors and that there was little difference in EH between sexes. Like the Tsimane', the Tupí-Mondé also have a high protein-energy malnutrition and anemia prevalence, are burdened with endemic infectious and parasitic disease, have been in contact with national society for nearly 100 years, and their overall health status reflects an inadequate diet, poor sanitation and a shortage of health care services.[49]

Reliability of the digital photography method

Our study was one of the few that has empirically evaluated the reliability of the digital photograph method for measuring EH. Our intra-examiner reliability results suggested that digital photography is a reproducible method for capturing and detecting EH, particularly for the central maxillary incisors. On the other hand, inter-examiner reliability results suggest detection of EH with digital photographs is not reproducible across examiners. Poor inter-examiner reliability may be explained by systemic biases between the examiners, the subjective measures included in the Modified DDE Index, insufficient examiner training, and the very high prevalence of EH in the study sample. Inter-examiner reliability results, however, also suggest EH detection on the maxillary incisors is best, though extensive examiner training would be needed to produce reliable measures of EH with this method.

High reliability in the central maxillary incisors may be due to the fact that these two teeth were in focus and directly perpendicular to the camera, making the EH easier to detect on them than on the other four teeth of interest. The mandibular canines had the lowest reliability scores, but also the lowest frequency of EH occurrence and of EH extent > 1/3 of the tooth surface. This supports anecdotal reports by examiners that the mandibular canine tooth surface was at an angle and thus not fully visible in the photograph, making it difficult to detect EH on a portion of the tooth. The "best tooth analysis" may best

be coupled with multiple photos taken perpendicular to each tooth surface of interest, especially in individuals with a narrow dental arch.

The Modified DDE Index

Our inter-examiner reliability results may be suggestive of a difference in recognition of and training in the definitions and characteristics of enamel defects between examiners. The Modified DDE Index, last updated more than 25 years ago, in 1989, offers little description to classify types of DDEs and subtypes of EH, particularly relative to the boundless variation that is observed in human enamel.[34] Furthermore, the index is based on some subjective measures that are vulnerable to error in measurement, such as EH extent. Estimation of the area of an irregularly-shaped tooth surface affected by what tended, in this study sample, to be a round EH area in the middle proved to be imprecise and not highly reproducible by intra- nor inter-examiner comparison. Digital photography and photography software developed since the last modification of the DDE Index may enable further characterization and expansion of the patterns described in the Modified DDE Index to improve classification of the variation observed in the human dental enamel and perhaps help to narrow in on pathogeneses unique to each pattern. Furthermore, the rough, cobblestone-like EH pattern observed in this study did not fit any of the EH sub-categories included on the Modified DDE Index, so the unfamiliar EH pattern likely contributed to the poor inter-examiner reliability. Our low inter-examiner reliability results are suggestive of a meaningful amount of measurement error that may attenuate observed associations when EH is evaluated in relation to other measures.

There seems to be a lack of consensus within the scientific and clinical communities whether EH can be detected with the naked eye and so also by macro digital photography.[73,74,78] The more recent literature seems to suggest that digital photography with a macro lens facilitates unbiased measurement of EH.[12,58] Our findings contribute to this body of literature, suggesting that the digital photography method for detecting EH has the potential to be reproducible, but that examiner training is extremely important to produce reliable measures of EH.

Limitations and methodological weaknesses

One of the limitations of this study was that it was not feasible for the participants to brush their teeth prior to taking the digital photographs. It was thus difficult to decipher plaque on the tooth surface from EH in some cases and in other cases was difficult to detect EH on the tooth surface due to plaque, especially near the gingival margin. A methodological weakness was the extremely high prevalence of EH in the study sample, which made for unstable kappa value estimates. Even a small departure from perfect agreement in detection of EH occurrence likely decreased the kappa values greatly. Finally, children who were missing both central maxillary incisors in adolescence and thus excluded from these analyses were taller and heavier as children and older during 2015 data collection, which may bias the results of analyses evaluating childhood malnutrition, EH and adolescent health toward the null.

Chapter 4. Malnutrition-related early childhood exposures and enamel hypoplasia in the permanent dentition

Abstract

Objective: The enamel on the permanent dentition calcifies incrementally during early childhood and is sensitive to physiological stress. Defects in the enamel do not repair after occurrence or during the life course, leaving a permanent biological mark of physiological insults that occurred during early childhood. Enamel hypoplasia (EH), a deficit in the quantity of enamel on the surface of the tooth, may be caused by local or systemic factors that occur during early childhood or by genetics. Few human studies, especially among populations with a high prevalence of EH, have been prospectively conducted to examine factors related to EH in the permanent dentition (birth through full eruption of the permanent dentition, at approximately 10 year of age). The present study investigated several malnutrition-related early childhood exposures and EH in the permanent dentition using prospectively-collected, longitudinal data that spans childhood through adolescence.

Methods: This study was conducted within an Amerindian population in the Bolivian Amazon. Our study sample included 349 adolescents (10-17 years of age) who enrolled for at least one year in a longitudinal study during early childhood (1-4 years). We detected EH, our primary outcome of interest, in the permanent central maxillary incisors using digital photography. The outcomes evaluated included three measures of the prevalent rough, cobblestone-like EH pattern in the study sample: occurrence (any, none), extent of occurrence (<1/3, 1/3-2/3, >2/3 of the tooth surface) and estimated age at occurrence (1, 2, 3, 4 years of age). Childhood exposures of interest included the following malnutrition-related measures: stunted growth (height-for-age z-scores, HAZ), underweight (weight-for-age z-scores, WAZ), anemia (hemoglobin, Hb), immune activation (c-reactive protein, CRP) and parasitic gastrointestinal infection (hookworm infection). We used log-binomial and ordinal logistic regression, as well as generalized estimating equations (GEE) to estimate associations between malnutrition-related childhood exposures and various EH measures.

Results: The overall prevalence of a rough hypoplastic pattern of EH in the study sample was 92.3%. The study sample also had a high prevalence of stunted linear growth (75.2%), anemia (56.9%), elevated immune activation (39.1%), and gastrointestinal hookworm infection (49.6%) between 1 and 4 years of age. Results indicated an association between average childhood HAZ (PR=0.98, 95% CI: 0.95, 1.00), CRP levels (PR=1.01, 95% CI: 1.00, 1.03) and presence of gastrointestinal hookworm infection (OR=0.28, 95% CI: 0.08, 0.94 for <1/3 vs. >2/3 of the tooth affected by EH) and EH, though some of the point estimates lacked statistical precision. Extent of the tooth surface affected by EH seemed to be an important measure of EH as it related to early childhood exposures, particularly for average HAZ and hookworm infection. A greater average childhood HAZ was associated with greater odds of having no EH compared to EH on >2.3 of the tooth surface (OR=1.54, 95% CI: 1.05, 2.29) and presence of hookworm infection was associated with lower odds of having EH on < 2/3 of the tooth surface compared to >2/3 of the tooth surface (OR=0.31, 95% CI: 0.11, 0.86).

Conclusions: The present study provided evidence in support of a relationship between early childhood chronic malnutrition (HAZ), immune activation (CRP), parasitic infection (helminth infection) and a rough, cobblestone-like hypoplastic pattern on the surface of the permanent central maxillary incisors, though some of the point estimates lacked precision. These findings are unique among the existing EH human population literature, because they detected an association between particular measures of malnutrition in childhood (beyond stunted growth) using prospectively-recorded data.

Introduction

The dental enamel on the permanent dentition mineralizes postnatally, beginning shortly after birth.[1] Developmental defects of the enamel (DDEs) in the permanent dentition result from local, systemic and genetic factors that interrupt the enamel formation processes. Enamel hypoplasia (EH), one particular type of DDE, results from disruption to the early, secretory phase of enamel formation when layers of the enamel matrix are being formed, which results in a reduction in enamel thickness.[9,11,36] EH is sub-classified based on its appearance, which may be linear or horizontal grooves, pits and missing enamel in the tooth surface.[34] Furthermore, enamel is formed chronologically in a ring-like fashion, making it possible to establish the rate of formation and retrospectively estimate the age at which defects developed.[37–39] The mineralization process is highly-sensitive to environmental alterations and physiological stress and is not repaired during the life course.[9–11] For this reason, teeth have been considered "a snapshot into the past" for scientific study.[2]

The prevalence of EH in the permanent dentition varies greatly across and within populations, in both the past and present. EH prevalence tends to correlate with population-level measures of nutrition status, infectious disease prevalence and socioeconomic disempowerment, [40] and reported prevalences also suggest a disproportionate burden of enamel hypoplasia is shouldered by marginalized, low-income populations.[40]

Systemic factors thought to cause EH include nutrition deficiencies (hypocalcemia and vitamin A, C, or D deficiency), gastrointestinal disturbances, bacterial and viral infections, genetic disorders, syndromes/chronic conditions, excessive fluoride exposure, and/or prolonged use of medications.[9–11,36,40] EH is thus generally interpreted as a non-specific indicator of metabolic disruption and physiological stress during early childhood.[42–45] The evidence supporting this comes from animal studies carried out as early as the 1920s, clinical case studies and population studies that have evaluated EH in the *primary* dentition (for which the etiologically relevant exposure period is time *in utero*).[51,75]

Few human studies, among populations with a high prevalence of EH, had the follow-up time required to prospectively study factors related to EH in the *permanent* dentition (birth through full eruption of the permanent dentition, at approximately 10 years of age). So, the evidence from human population studies for an association between specific physiological stresses during early childhood and EH in the

permanent dentition is limited.[8,46] In general, studies that have assessed malnutrition during early childhood, measured by growth stunting and absence of nutritional supplementation, tend to conclude that children who are short for their age and who do not receive nutritional supplements to be significantly more likely to have EH in their permanent dentition than their well-nourished counterparts.[47–50] The present study applied 13 years of prospectively-collected longitudinal data to investigate the relationships between early childhood growth stunting and EH, but also to investigate the influence of more specific malnutrition-related exposures, including weight, hemoglobin, immune activation and parasitic infection on EH in the permanent dentition.

Methods

A detailed account of all study methods are described in Chapter 2.

Study design and sample

This study extended a prospective cohort study that began in 2002. In 2015, it followed up a sample of adolescents, aged 10-17 years, who were enrolled as young children (5 years and younger) in the 9-year Tsimane' Amazonian Panel Study (TAPS).[61] The present study built on the TAPS by adding measures of enamel defects in the permanent dentition for the original study's child participants. Criteria for inclusion in the follow-up study include that an individual be 10-17 years of age at the time of data collection (so that the central maxillary incisors will have fully erupted), was previously enrolled in TAPS for at least one year when aged 5 years or less, and resided in one of 15 communities visited by the data collection team during the 2015 follow-up study.

The study sample was derived from the Tsimane' population, an Amerindian group of approximately 8,000 people who live across more than 100 small, remote communities, accessible by dirt roads and/or canoe and river in Bolivia's Amazonian Basin. Although they are increasingly integrating into mainstream Bolivian society and exposed to the market economy and a Western diet, the Tsimane' maintain a traditional hunter-horticultural lifestyle and primarily speak their native language in their communities. The original TAPS study included 1,453 people, 655 of who were eligible for the 2015

follow-up study. 349 participants were enrolled in the 2015 follow-up study. The sample was further reduced to 337 due to 12 participants with missing or severely decayed central maxillary incisor by which EH could not be measured. Of these participants, 327 were included in the present analysis because at least one year of childhood anthropometric (height and weight) data that correlated with the etiologically-relevant exposure period for the study outcome (ages 1 through 4 years) was available. Sample size varied by each childhood exposure evaluated based on available data (Hb, n=72, CRP, n=64, hookworm infection, n=113). Approval for all data collection and use was obtained from the local tribal government, the *Gran Consejo Tsimane'* (*GCT*, Tsimane' Grand Council) and the University of Washington (UW) Human Subjects Division (HSD).

Outcome

The outcome of interest in these analyses was EH in the permanent central maxillary incisors. EH was captured through digital intraoral photographs with a macro lens and ring flash, detected using Photoshop software, and quantified according to the internationally-accepted DDE Index/Modified DDE Index.[34,69] EH was measured on the two central maxillary incisors because these teeth were determined to be the most reliable for abstracting enamel defect data from digital photographs in this study sample (see Chapter 3). The results of this analysis focused on the most prevalent EH pattern observed in the study sample, a rough, cobblestone-like pattern. EH was classified by occurrence (any, none), extent of tooth surface affected by EH (less than 1/3/ 1/3 to 2/3 / more than 2/3), and the estimated age at EH occurrence (by year of age, 1 through 4 years). These measures of EH were recorded based on the left central maxillary incisor or – if the left was missing or severely decayed– on the right central maxillary incisor. There was 93.4% concordance across the two central maxillary incisors for EH occurrence.

Exposures

Based on histological evidence,[39] the etiologically-relevant exposure period for defects in the permanent central maxillary incisors is from one year through four years of age, so childhood measures evaluated were limited to this period of early childhood. Childhood data were collected annually by the TAPS study team between 2002 and 2010.[61] The primary exposure of interest was stunted growth (an indicator of

chronic malnutrition) during early childhood. Additional malnutrition-related measures from early childhood included underweight (an indicator of acute malnutrition) and anemia, immune activation, and parasitic gastrointestinal infection (indicators of general health and infection). Growth stunting was measured on a continuous scale using mean height-for-age z-scores (HAZ) between 1 and 4 years of age. Similarly, underweight was measured on a continuous scale using mean weight-for-age z-scores (WAZ) between 1 and 4 years of age. Both HAZ and WAZ were age- and sex- standardized according to World Health Organization (WHO) reference growth charts. Also, according to WHO standards, a threshold of <-2.0 was used to dichotomize stunted vs. normal average height and underweight vs. normal average weight during early childhood.[79] Anemia was measured on a continuous scale by measuring hemoglobin (Hb; in g/dL) using dried blood spots (in 2002 and 2003) for a sub-sample of 79 participants.[22] Immune activation was measured on a continuous scale by measuring c-reactive protein (CRP; in mg/L) using dried blood spots for a sub-sample of 72 study participants (in 2002-2003). Standard age- and sex-based reference values for clinical practice were used to determine thresholds for anemia and elevated CRP classification.[80] Finally, parasitic infection was determined based on assessment of fecal samples from a sub-sample of 115 study participants (in 2003 and 2007).[64,81] Children were classified as infected vs. not by presence or absence of any hookworm in fecal samples.

Covariates

Covariate measures from the TAPS 2002-2010 dataset were used and included sex (male/female), age (measured in years), household socioeconomic status (SES) and household dietary consumption. Household SES was measured by total wealth, a locally-developed sum of animal, trade and modern wealth measured in the local currency (bolivianos, Bs).[72] Dietary consumption for one week was measured through interviews in participants' homes and included traditional food (plantain and manioc (*yuca*)) as well as processed, market-based food (sugar) consumption per week, measured in kilograms (kg).[72]

Statistical Analyses

Descriptions of the prevalence and incidence (based on histologically-informed estimates of the age at enamel calcification[39]) of EH measures in the study sample were reported. Malnutrition-related childhood experiences were summarized by (1) EH measures and (2) year of age, since there were repeated measures for each child.

To test whether the early childhood experiences described above were associated with EH, we evaluated the relationship between each of the malnutrition-related childhood exposures and (a) EH occurrence, (b) EH extent, and (c) estimated age at EH occurrence (from 1 through 4 years of age). To assess whether malnutrition-related early childhood exposures were associated with occurrence of EH, log-binomial regression with robust standard errors was used to estimate the prevalence ratio of EH occurrence associated with each childhood exposure measured. We used ordinal logistic regression to estimate the odds ratio between each early childhood experience and the extent of EH**.** To evaluate whether each early childhood exposure was associated with EH occurrence by year of age at occurrence, we used a generalized estimating equation (GEE) model with robust standard errors and the log link and Poisson distribution for prevalence ratio estimation, accounting for clustering by study participant.

Child age and sex at the time of the childhood exposure measure were adjusted for in all analyses as confounders. For all statistical tests described, the null hypothesis was that the prevalence and odds ratio estimates would be equal to 1 for each of the exposures assessed, indicating no difference in the influence of the exposure on the outcome. Statistically significant estimates at the $p< 0.05$ level are bolded in the tables of results. Confidence intervals were calculated based on the standard error of the point estimates. Analyses were completed with the PROC TTEST, PROC MEANS, PROC FREQ, PROC GLM, PROC LOGISTIC and PROC GENMOD functions in SAS Version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Study sample description

The most prevalent EH pattern in the study sample was a rough and cobblestone-like tooth surface (prevalence = 92.4%). Most (44%) participants with EH had 1/3 to 2/3 of the tooth surface affected, and

the remainder were evenly split between <1/3 and >2/3 of the tooth surface affected. The incidence of EH decreased with age during early childhood. The incidence of EH per 100 children per year at age 1y, 2y, 3y and 4y was: 91.9, 90.8, 88.3, and 61.9, respectively. The prevalence of EH was significantly different across years of age at occurrence (chi-square, $p<0.05$), but not across sex.

Overall, the study sample was approximately half male, contributed 3.1 ± 1.1 child-years to the analyses and were nearly three years of age on average at the time of HAZ and WAZ measurement (Table 1). The study sample had a high prevalence of stunted linear growth (75.2%), anemia (56.9%), elevated immune activation (39.1%), and gastrointestinal hookworm infection (49.6%) between 1 and 4 years of age. On average, those with EH had lower SES, lower household consumption of sugar and greater household consumption of plantains and manioc. Those with EH also had a greater prevalence of stunting, anemia, higher risk CRP levels and hookworm gastrointestinal infection. The difference in mean CRP was statistically significant between those with and without EH ($p=<0.01$). A linear trend across EH extent was observed in mean family SES, household sugar, plantain and manioc (*yuca*) consumption, hemoglobin and CRP.

The average HAZ was approximately -2.2 for each year of age between 1 and 4 years, indicating the average child's growth was stunted throughout early childhood years. The HAZ standard deviations suggested the distribution of the study sample was as expected (1.10-1.30) compared to the WHO reference population, except for age 1y (wider). The average WAZ increased from -1.4 at 1 year of age to -0.9 at 4 years. The WAZ standard deviations suggested that the spread of data in the study sample may have been narrower (a more homogenous population) than that of the WHO reference distribution (1.0-1.2). According to WHO cut-offs for prevalence of public health significance (and beyond the 2.3% expected prevalence), the Tsimane' had a "very high" prevalence of stunting in all ages 1-4 years and a "medium" prevalence of underweight among 1 year old children. The prevalence of underweight in 2-4 years olds was considered "low" (Table 2).[79] Statistical tests indicated a statistically significant difference in WAZ across years of age (ANOVA F-test, $p< 0.05$). The difference in mean HAZ and WAZ across sex were also statistically meaningful (t-test, $p < 0.05$); on average, males had a lower mean HAZ (-2.3 ± 1.5) and mean WAZ (-1.1 ± 0.7) compared to females (HAZ= -2.1 ± 1.2, WAZ = -0.9 ± 0.8).

Table 1.
Description of childhood demographics and malnutrition-related measures, by EH measures

MEASURE	no EH (n=25)	EH occur. (n=302)	EH Extent			Overall (n=327)
			< 1/3 (n=77)	1/3 – 2/3 (n=144)	> 2/3 (n=81)	
	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD
sex, % male	36.0%	53.5%	48.1%	56.9%	51.9%	52.0%
age, in years	2.9 ± 0.5	2.8 ± 0.6	2.8 ± 0.6	2.8 ± 0.5	2.9 ± 0.7	2.8 ± 0.6
household wealth (Bs)	3,957 ± 2,852	3,388 ± 2,308	3,634 ± 2,899	3,280 ± 2,144	3,327 ± 1,948	3,425 ± 2,344
household diet (kg/week)						
sugar	1.5 ± 1.0	1.4 ±1.0	1.7 ± 1.0	1.4 ± 0.9	1.2 ± 1.0	1.5 ± 1.0
plantain	43.7 ± 16.4	49.8 ± 21.1	47.4 ±18.3	49.9 ± 21.1	51.9 ± 23.3	49.7 ± 22.4
manioc	8.0 ± 6.5	10.7 ± 9.1	10.1 ± 9.8	10.8 ± 9.0	11.1 ± 8.7	10.5 ± 9.1
height-for-age z-score	-1.8 ± 1.2	-2.3 ± 1.3	-2.3 ± 1.5	-2.2 ± 1.0	-2.4 ± 1.4	-2.2 ± 1.3
% stunted (< -2.0)	64.0%	76.2%	73.7%	73.6%	82.7%	75.2%
weight-for-age z-score	-1.0 ± 0.8	-1.0 ± 0.7	-1.0 ± 0.8	-1.0 ± 0.6	-1.1 ± 0.7	-1.0 ± 0.7
% underweight (<-2.0)	16.0%	17.9%	22.1%	13.4%	21.0%	17.5%
hemoglobin* (g/dL)	10.9 ± 1.2	10.7 ± 1.5	10.5 ± 1.9	10.7 ± 1.4	10.8 ± 1.3	10.8 ± 1.4
% with anemia	44.6%	55.4%	62.5%	51.7%	55.0%	56.9%
c-reactive protein* (mg/L)	1.0 ± 0.8	2.9 ± 4.0	3.3 ± 3.2	3.2 ± 4.9	2.2 ± 2.9	2.6 ± 3.7
% "higher risk"	28.6%	40.4%	58.3%	40.0%	30.0%	39.1%
hookworm infection*	42.9%	50.0%	48.0%	43.1%	63.3%	49.6%

*hemoglobin n=72, c-reactive protein n=64, hookworm infection n=113

Although limited to 2-4 years old children, the prevalence of anemia was high, between 52% and 74%. Similarly, there was a high prevalence of elevated CRP, indicating high risk of or actual acute inflammation. The prevalence of gastrointestinal hookworm infection also increased with age from 1 to 4 years, from 11% to nearly 70%, a statistically meaningful difference (chi square, $p<0.05$). There was no statistical evidence of a difference in average Hb, CRP or hookworm prevalence between males and females.

Table 2.
Description of malnutrition-related early childhood experiences, by year of age

MEASURE	n	1 year (n=187)	2 years (n=245)	3 years (n=269)	4 years (n=305)	Overall
height-for-age (z-score)**,** mean ± SD	327	-2.2 ± 1.9	-2.3 ± 1.3	-2.2 ± 1.2	-2.2 ± 1.1	-2.2 ± 1.3
% stunted (< -2.0)		60.4%	61.2%	59.9%	55.1%	74.6%
weight-for-age (z-score)**,** mean ± SD	327	-1.4 ± 0.8	-1.1 ± 0.8	-0.9 ± 0.7	-0.9 ± 0.7	-1.0 ± 0.7
% underweight (<-2.0)		21.5%	10.7%	4.1%	3.3%	17.2%
hemoglobin (g/dL), mean ± SD	72	--	10.8 ±	10.7 ±1.5	10.7 ±1.6	10.8 ±
% with anemia		--	52.0%	73.5%	58.3%	59.3%
c-reactive protein (mg/L), mean ± SD	64	--	2.7 ± 5.7	2.6 ±4.6	2.8 ±3.1	2.6 ± 3.7
% “higher risk”		--	21.4%	29.0%	36.8%	37.0%
hookworm infection, %	113	11.1%	47.2%	42.9%	68.6%	49.6%

Multivariate analyses (by child)

Our results indicated that childhood HAZ, CRP and hookworm infection are associated with occurrence and extent of EH (Table 3). For a one unit difference in childhood HAZ, the prevalence of EH was diminished by 0.98 (95% CI: 0.95, 1.00), given that age and sex were held constant, though this did not quite reach the 0.05 statistical significance level ($p=0.0531$). For a one unit difference in HAZ, the odds of having no EH, compared to having >2/3 of the tooth surface affected by EH, was significantly higher (OR=1.54, 95% CI: 1.05, 2.29). Those with a lower HAZ were more likely to have EH. Similarly, for a one unit difference in childhood CRP, the prevalence of EH was increased by 1.01 (95% CI: 1.00, 1.03), given that age and sex were held constant ($p=0.04$), but no statistically significant relationship was observed between childhood CRP and EH extent. Finally, the association between hookworm infection and EH occurrence was not statistically significant, but for those with hookworm, compared to those without, the odds of having only <1/3 or 1/3-2/3 of the tooth surface affected by EH was significantly less than having

>2/3 of the tooth surface affected (OR=0.28, 95% CI: 10.08, 0.94 and OR=0.31, 95% CI: 0.11, 0.86, respectively).

Table 3.
Early childhood experience and EH regression results (1 record/child; n=327) (prevalence or odds ratios and 95% CIs reported)*

	EH occurrence	EH extent			
	prevalence ratio	no EH odds ratio	< 1/3 odds ratio	1/3-2/3 odds ratio	> 2/3 (ref.)
avg. height-for-age (z-score)	**0.98**	**1.54**	1.05	1.11	--
	(0.95, 1.00)	**(1.05, 2.29)**	(0.82, 1.34)	(0.90, 1.39)	
avg. weight-for-age (z-score)	0.99	1.32	1.19	1.40	--
	(0.95, 1.04)	(0.70, 2.48)	(0.77, 1.84)	(0.94, 2.08)	
avg. c-reactive proteinł (mg/L)	**1.01**	0.80	1.14	1.10	--
	(1.00, 1.03)	(0.45, 1.42)	(0.91, 1.43)	(0.91, 1.33)	
avg. hemoglobinł (g/dL)	0.99	1.11	0.93	0.97	--
	(0.95, 1.03)	(0.56, 2.21)	(0.59, 1.44)	(0.64, 1.47)	
hookworm infectionł (v. not)	1.01	0.40	**0.28**	**0.31**	--
	(0.92, 1.10)	(0.06, 2.53)	**(0.08, 0.94)**	**(0.11, 0.86)**	

* adjusted for mean age at childhood measure and sex
ł hemoglobin n=72, c-reactive protein n=64, hookworm infection n=113
bolded results indicate statistically significant estimates at p< 0.05

Multivariate analyses (by child-year)

We also assessed whether childhood exposures were associated with EH by estimated year of age at EH occurrence. The results indicated that childhood HAZ and CRP levels may be associated with EH occurrence by estimated year of age at EH occurrence (Table 4). The results suggested the prevalence of EH, per one unit difference in HAZ, is diminished by 0.98 (95% CI: 0.96, 1.00), across all observations in all clusters and holding all other covariates in the model constant. This estimate did not reach the 0.05 statistical significance level (p = 0.0547). Similarly, results indicated that the prevalence of EH, per one unit difference in CRP, is 1.01 times greater (95% CI: 1.00, 1.03), though this did not reach the 0.05 statistical significance level either (p=0.0808).

Table 4.
Early childhood experience and EH regression results (1 record/ child-year; n=1,006) (odds ratios and 95% CIs reported)*

	EH occurrence, by year of age
	prevalence ratio
height-for-age (z-score)	**0.98 (0.96, 1.00)**
weight-for-age (z-score)	1.02 (0.98, 1.06)
c-reactive proteinǂ (mg/L)	**1.01 (1.00, 1.03)**
hemoglobinǂ (g/dL)	1.06 (0.91, 1.23)
hookworm infectionǂ (v. not)	0.92 (0.75, 1.12)

* adjusted for age at childhood measure and sex
ǂ hemoglobin n=97, c-reactive protein n=91, hookworm infection n=113
bolded results indicate statistically significant estimates at p< 0.05

Therefore, the prevalence of EH, per a two-unit difference in HAZ (for example, for 'normal stature', 0.00, compared to those at the threshold for 'stunted', -2.0, by WHO standards), is diminished by 0.96 (95% CI: 0.93, 1.00), across all observations in all clusters and holding all other covariates in the model constant. Similarly, the prevalence of EH, for a 9-unit difference in CRP (for example, for normal CRP, 1.0, to the threshold for acute inflammation, 10.0), is increased by 1.11 times (95% CI: 0.99, 1.26).

The role of household socioeconomic status and diet

We carried out a posthoc analysis to evaluate the role of household SES and dietary consumption in the relationship between malnutrition-related childhood exposures and EH. The statistical relationships between childhood stunting and hookworm infection and EH occurrence and extent were not changed when adjusted for household SES nor household dietary consumption (Appendix I). However, household SES and plantain consumption may confound the relationship between CRP and EH. The same observations were made when childhood exposures and EH were assessed by year of age basis.

Discussion

The present study provided evidence in support of a relationship between early childhood chronic malnutrition (HAZ), immune activation (CRP), parasitic infection (helminth infection) and a rough, cobblestone-like hypoplastic pattern of EH on the surface of the permanent central maxillary incisors.

Given the high prevalence of EH and limited sample size, analyses may have lacked the statistical power to detect significant relationships between other childhood exposures and EH.

This study was unique among the existing EH human population literature, because it prospectively recorded multiple early childhood exposures (beyond stunted growth) and had the minimum follow-up time necessary (to full eruption of the permanent dentition, at approximately 10 years of age) to evaluate these childhood exposures in relation to EH in the permanent dentition. There is evidence in the existing literature that childhood growth stunting and EH are related, and early childhood infection and micronutrient deficiencies have been posited as possible causal mechanisms for EH in the permanent dentition.[48–50,82] We thus expected to observe an inverse association between EH and HAZ, WAZ and Hb, and a positive association between EH and CRP and hookworm infection. Our study findings were in line with the existing literature, suggesting that EH is inversely associated with HAZ and positively associated with CRP and hookworm. EH has a complex etiology with multiple pathways.[9–11,36,40] Given the weak associations observed in our results, it is also possible that the primary pathway leading to EH in this study sample was not captured in our childhood measures (e.g., primary central maxillary incisor infection).

Upstream factors that were presumed to have a meaningful influence on malnutrition-related exposures during early childhood were intentionally excluded from initial analyses. These factors included socioeconomic status and poverty, sanitation and hygiene practices, water contamination and source, repeated and/or chronic infection and diet (particularly with regard to micronutrients). Adjustment for these factors in the analyses would have blinded us to the EH that was attributed to the factors adjusted for, which was not the intent of the investigation. However, posthoc analyses revealed that the relationship between childhood stunting and hookworm infection and EH were not explained by our measures of household SES or dietary consumption, yet the CRP-EH association may have been confounded by these factors.

Limitations and methodological weaknesses

The present study could rule out the possibility that chronic or acute malnutrition nor infection are associated with EH. Furthermore, the present analyses cannot rule out the possibility that there was a

causal mechanism at work (e.g., primary tooth caries) for EH that was not available for evaluation in the existing TAPS data. First, given the high prevalence of EH and limited sample size, the analysis may have lacked the statistical power to detect a significant relationship between childhood exposures and EH. This seems particularly plausible in relation to childhood anthropometric exposures based on the two analyses reported in Table 3. Second, non-differential measurement error in EH may have attenuated the point estimates in these analyses. It was not feasible for study participants to brush their teeth prior to taking the intraoral photographs, making detection and measurement of the occurrence, extent and estimated age at EH occurrence less precise (due to plaque on the tooth surface), resulting in non-differential misclassification and attenuation of any true association. If plaque obscured detection of EH, those with less plaque would be more likely to have EH, which may have biased the results away from the null if a high sugar/carbohydrate diet and access to the market economy is related to less infection and taller height. On the other hand, plaque may also have been misinterpreted as EH, making those who have access to the market economy and sugars/carbohydrates in their diets seem more likely to have EH, which would have biased the results toward the null. Similarly, the estimated age at EH occurrence was based on enamel formation estimated from a European population. If inter-population differences in enamel formation timing is great enough, the sensitivity of the results in Table 4 would have been low, reducing the likelihood of detecting an association if one truly exists. It may also be that the rough, generalized hypoplastic pattern prevalent on the Tsimane' dentition made it unlikely to detect defects (i.e., linear/horizontal groove) that would otherwise be expected from acute childhood exposures (i.e., episodes of low WAZ and bouts of infectious illness). Finally, children who were missing both central maxillary incisors in adolescence and thus excluded from these analyses were taller and heavier as children. Taller and heavier children were less likely to have been exposed to childhood malnutrition and less likely to have EH, so the reported point estimates for childhood exposures and EH may be underestimated due to their exclusion.

Chapter 5. Enamel hypoplasia in the permanent dentition and adolescent anthropometric and biomarker outcomes

Abstract

Objective: Adult teeth may chronologically reflect early childhood experience because enamel on the permanent teeth calcifies incrementally during early childhood and is sensitive to physiological stress. Defects in the enamel caused by physiological insults that occurred during early childhood do not repair after occurrence or during the life course, leaving a permanent biological mark of physiological insults that occurred during early childhood. Evidence from studies of the developmental origins of health and disease (DOHaD) shows early life experience influences disease risk throughout the life course, and bioarchaeological findings have indicated enamel hypoplasia (EH) is associated with early mortality.[8] We therefore investigated EH in the permanent dentition as a marker of early childhood experience in relation to subsequent anthropometrics and biomarkers to evaluate the utility of EH as an indicator of health risk in adolescence.

Methods: This study was conducted within an Amerindian population in the Bolivian Amazon. Our study sample included 349 adolescents (10-17 years of age) who had enrolled for at least one year in a longitudinal study during early childhood (1-4 years of age). We detected EH in the permanent central maxillary incisors using digital photography from which the following measures of EH were abstracted: occurrence (any, none), extent of occurrence (<1/3, 1/3-2/3, >2/3 of the tooth surface) and estimated age at occurrence (1, 2, 3, 4 years of age). Adolescent outcomes included anthropometrics (height, weight, body mass index (BMI)) and biomarkers (hemoglobin (Hb), glycated hemoglobin (HbA_{1c}), white blood cell count (WBC) and blood pressure). We used multiple linear regression to estimate associations between EH and study outcomes. To identify independent associations of EH with biomarkers, we adjusted for adolescent anthropometrics and for prospectively-collected malnutrition-related childhood exposures (e.g. stunted growth).

Results: Nearly all (92.3%) adolescents had a rough hypoplastic pattern on the tooth surface. Greater extent of EH on the tooth surface was associated in unadjusted models with shorter height (-0.14 HAZ, 95% CI: -0.24, -0.03 and -1.35 cm, 95% CI: -2.21, -0.50), lower weight (-0.98 kg, 95% CI: -1.73, -0.23), lower Hb (-0.36 g/dL, 95% CI: -0.59, -0.13), lower HbA_{1c} (-0.04 $\%A_{1c}$, 95% CI: -0.08, -0.00), and higher WBC count (0.74 10^9/L, 95% CI: 0.35, 1.14) but not BMI-for-age z-score or blood pressure. EH extent was associated with anemia (PR=1.08, 95% CI: 1.00, 1.18) and elevated WBC count (PR=1.12, 95% CI: 1.01, 1.26) based on public health and clinically-relevant thresholds. The EH extent-Hb and EH extent-WBC count associations persisted after adjustment for adolescent anthropometrics (height, HAZ, weight), but the EH extent-HbA_{1c} relationship was attenuated. Similarly, EH extent was significantly associated with adolescent HAZ, Hb and WBC count after adjustment for childhood anthropometrics, but the EH extent-HbA_{1c} relationship was attenuated.

Conclusion: Results indicated that EH extent is consistently associated with several adverse anthropometric and biomarker measures among an Amerindian adolescent study sample from rural Bolivia. Greater extent of EH was associated with shorter height and lower weight, lower hemoglobin and greater WBC count. Interestingly, greater EH extent was also associated with lower glycated hemoglobin, a healthy outcome. Adolescent body size did not account for the relationship between EH extent and hemoglobin or WBC count, but did seem to play an important role in the EH extent-glycated hemoglobin association. EH extent also seemed to capture a childhood exposure relevant to adolescent HAZ, hemoglobin and WBC count outcomes above and beyond that of childhood anthropometrics.

Introduction

Enamel hypoplasia (EH) in the permanent dentition may serve as a window into the past and thus serve as an underutilized retrospective source of information about early childhood experience.[8] Permanent teeth may record early childhood experience because dental enamel calcifies on the central maxillary incisors incrementally during early childhood (1 through 4 years of age), is sensitive to physiological stress and thus susceptible to defects (such as EH), and defective enamel does not repair during the life course.[9–11] Although EH formed during early childhood cannot be measured until the permanent teeth have fully erupted (from as early as approximately 10 years of age, but varies by tooth), the defects develop during early childhood, while the permanent tooth buds are forming within the gums.

Research on the developmental origins of health and disease (DOHaD) has demonstrated the important role of early life experience (such as malnutrition) on chronic disease risk later in life.[1–4] Proposed biological mechanisms underlying these observations are based on human adaptation and plasticity during early life, and make sense of seemingly-paradoxical relationships, such as the dual burden of under- and over-nutrition and rise of chronic disease prevalence in low-resource countries where infectious diseases remain endemic.[6,16–18] This model has been substantiated with evidence suggesting that systemic disruption by under-nutrition during early life may result in increased risk for chronic disease through physiologic, structural, metabolic, immunologic and epigenetic pathways.[4,19,20] Low birth weight and growth stunting —childhood experiences associated with chronic, low-grade infection and poor dietary quality— [21,22] are associated with insulin resistance and type II diabetes, high blood pressure, and increased risk of cardiovascular disease and obesity in adulthood.[1,2,4,23]

DOHaD research has had to primarily rely on non-specific measures of exposure (such as birth weight and growth stunting), particularly for studies of populations from low-resource settings.[1–4] Calls in the existing DOHaD literature have been made for the identification of better measures of early childhood experience (beyond birth weight and growth stunting) that that will capture the source and timescale of specific early childhood exposures.[4,14,15] EH has the potential to be a more sensitive and specific measure of childhood exposures relevant to DOHaD research (e.g., infectious illness, micronutrient deficiencies, etc.) than commonly-used proxy measures which aggregate heterogeneous exposures, decreasing the study sensitivity (and chance of determining the true result) in DOHaD research.[83] The potential

advantages to using EH as a biomarker of early childhood experience include that EH can be measured in a field setting using digital photography methods,[12] EH may be available when/where childhood medical records are not, EH is an objective measure that does not rely on fallible human memory to recall events that occurred in the distant past, EH may capture the *timing* of childhood experiences -- permitting retrospective estimation of the age at which an individual experienced a physiological stressor which could inform knowledge about critical periods of development -- and EH may be measured cross-sectionally in adult populations, eliminating the need to follow populations for decades to study the DOHaD.

The disruptions that cause EH may simultaneously afflict other biological systems that are concurrently developing during early childhood, including but not limited to the musculoskeletal, central nervous, gastrointestinal, immunologic and respiratory systems.[22] Disruption during the development of these organs and systems could influence growth and metabolic function in adulthood, resulting in increased risk for disease and premature death.[1,7,13,23] Bioarchaeological findings consistently indicate EH in the permanent dentition is associated with early mortality among skeletal remains.[8,43,44,53–55] This evidence reinforces the idea that EH may be an available, but overlooked, biological marker of physiological stressors that occurred during early childhood and which could enable more widespread study of the DOHaD in low-resource settings. However, the relationship between EH and adolescent anthropometrics and health outcomes has not been thoroughly evaluated in living, human populations. We, therefore, investigated whether EH in the permanent dentition was associated with individual measures of adolescent anthropometrics and health in an Amerindian population in Bolivia.

Methods

A detailed account of all study methods are described in Chapter 2.

Study design and sample

This study extended a prospective cohort study. It followed up a sample of adolescents, currently aged 10-17 years, who were enrolled as young children (5 years and younger) in the 9-year Tsimane'

Amazonian Panel Study (TAPS).[61] The present study built on the TAPS by adding measures of enamel defects in the permanent dentition and follow-up measures of adolescent anthropometrics and biomarkers for the original study's child participants. Criteria for inclusion in the follow-up study include that an individual be 10-17 years of age at the time of data collection (so that the central maxillary incisors will have fully erupted), was previously enrolled in TAPS for at least one year when aged 5 years or less, and resided in one of 15 communities visited by the data collection team during the follow-up study.

The study sample was derived from the Tsimane' population, an Amerindian group of approximately 8,000 people who lives across more than 100 small, remote communities, accessible by dirt roads and/or canoe and river in Bolivia's Amazonian Basin. Although the Tsimane' are increasingly integrating into mainstream Bolivian society and exposed to the market economy and a Western diet, they still maintain a traditional hunter-horticultural lifestyle and primarily speak their native language in their communities. The original TAPS study included 1,453 people, 655 of who were eligible for the 2015 follow-up study. 349 participants were enrolled in the 2015 follow-up study, but the final analytic sample for the present analyses consisted of 337 individuals due to 12 participants with missing or severely decayed central maxillary incisors by which EH could not be measured. Sample size varied by adolescent outcome according to missing values due to point-of-care device errors in the field; those with (n=265) and without (n=81) WBC count data did not significantly differ by age, hemoglobin or glycated hemoglobin levels, but those missing WBC count data were more likely to be male, to have higher blood pressure and to have EH to a lesser extent than those with WBC count data ($p<0.05$). Approval for all data collection and use was obtained from the local tribal government, the *Gran Consejo Tsimane'* (*GCT*, Tsimane' Grand Council) and the University of Washington (UW) Human Subjects Division (HSD).

Outcomes

The outcomes of interest included anthropometric measurements and biomarker outcomes measured in adolescence (10-17 years). The adolescent anthropometric outcomes are (a) height and height-for-age z-score (HAZ), (b) weight, and (c) body mass index (BMI)-for-age z-scores (BAZ).[84] Height and BMI measures were converted to age- and sex- standardized z-scores based on WHO growth reference data for 5-19 years of age to permit comparison across age and sex in the study sample. Meaningful public

health and clinical thresholds for anthropometric measurements were based on World Health Organization (WHO) guidelines.[84] BAZ less than -2SD was classified as "thinness" and greater than +1 was considered "overweight" (equivalent to BMI ≥ 25 kg/m^2 at 19y). HAZ less than -2SD was classified as "short stature" or "stunted growth". Though additional anthropometrics were measured in the study sample, those included in the analyses were limited to height, HAZ, weight and BAZ to (1) reduce multiple testing and the likelihood of type I errors and (2) because these measures could be age- and sex-standardized using the WHO's international reference growth charts. Within this study sample, HAZ was strongly correlated with measures of leg length in centimeters ($r=0.44$, $p<0.0001$) and knee height in centimeters ($r=0.47$, $p<0.0001$). BAZ was correlated with body composition (percent fat) ($r=0.55$, $p<0.0001$), waist circumference in centimeters ($r=0.55$, $p<0.0001$) (Appendix J).

The biomarker outcomes were: (d) hemoglobin (Hb, measured in g/dL), (e) glycated hemoglobin (HbA_{1c}, measured as $\%A_{1c}$), (f) white blood cell count (WBC, measured in 10^9xL), and (g) systolic and diastolic blood pressure (measured in mm Hg). A stadiometer was used to measure height; the average of two measurements was used in analyses and for BMI calculations. A bioelectric impedance scale was used to measure weight for the BMI calculation. Biomarker outcomes were collected in the field using blood from a finger prick and point-of-care devices and a digital blood pressure cuff charged by solar technology. Point-of-care measure thresholds were based on standard clinical care for adolescents. American Diabetes Association thresholds were used to classify HbA_{1c} measures as "pre-diabetic" and "type II diabetes".[85] Standard age- and sex-based reference values for clinical practice were used to determine thresholds for anemia based on Hb and elevated WBC count.[80] National Institute of Health's age and sex specific parameters for blood pressure were used to classify "hypertension" and "prehypertension".[86] An index (0-3) was created to indicate an additional outcome of interest, (h) "poor metabolic health" by summing overweight, prediabetes/type II diabetes and prehypertension/hypertension status.

Exposure

The exposure of interest in these analyses was EH in the permanent central maxillary incisors, a biomarker of early childhood malnutrition. EH was captured through digital intraoral photographs with a

macro lens and ring flash, detected using Photoshop software, and quantified according to the internationally-accepted DDE Index/Modified DDE Index.[34,69] EH was measured on the two central maxillary incisors because these teeth were determined to be the most reliable for abstracting enamel defect data from digital photographs in this study sample (see Chapter 3). The results of this analysis focused on the most prevalent EH pattern observed in the study sample, a rough cobblestone-like pattern. EH was classified by occurrence (any, none), extent of tooth surface affected by EH (less than 1/3/ 1/3 to 2/3 / more than 2/3), and the estimated age at EH occurrence (by year of age, 1 through 4 years). These measures of EH were recorded based on the left central maxillary incisor or – if the left was missing or severely decayed– on the right central maxillary incisor. There was 93.4% concordance across the two central maxillary incisors for EH occurrence.

Covariates

Covariates include age (measured in years) and sex (male/female), recorded in 2015. Measures of malnutrition-related early childhood experiences, household socioeconomic status (SES) and household dietary consumption were derived from the 2002-2010 TAPS dataset and included childhood (1-4 years of age) HAZ, WAZ, Hb, immune activation (c-reactive protein, CRP) and hookworm gastrointestinal (GI) infection. Household SES was measured by total wealth, a locally-developed sum of animal, trade and modern wealth measured in the local currency (bolivianos, Bs).[72] Dietary consumption for one week was measured through interviews in participants' homes and included traditional food (plantain and manioc (*yuca*)) as well as processed, market-based food (sugar) consumption per week, measured in kilograms (kg).[72]

Statistical Analyses

Overall descriptions of EH, adolescent anthropometrics and biomarkers in the study sample were provided. Descriptions of adolescent outcomes were also stratified by EH status.

To test whether EH, as a biomarker of malnutrition-related early childhood experience, was associated with adolescent anthropometrics and health, multiple linear regression analyses were used to evaluate whether each outcome of interest was associated with (a) the occurrence of EH, (b) the extent to

which the EH affects the tooth surface, and (c) whether or not EH occurred at 4 years of age (the incidence-based pattern that is not captured by EH occurrence or extent, demonstrated in Chapter 3). Adjusted log-binomial regression was used to estimate the prevalence ratio for adolescent outcomes in the study sample based on public health and clinically-meaningful thresholds for disease status. These initial models did not include early childhood experiences because the analysis intended to assess EH as a marker of each of these events.

In a second analysis, we aimed to evaluate whether associations between EH and adolescent biomarkers persisted after adjustment for adolescent anthropometrics. Due to the possibility that EH may be an indicator of adolescent health (Hb, HbA1c, WBC count, blood pressure) due to the relationship between body size and health, multiple linear regression analyses were used to evaluate the relationships between (1) adolescent anthropometrics and each biomarker outcome, and (2) EH and each biomarker outcome, adjusted for adolescent anthropometrics. In doing so, we sought to evaluate the EH-adolescent health outcomes relationships above and beyond the role of adolescent anthropometrics.

In a third analysis, we aimed to evaluate whether EH captures a relevant exposure for adolescent outcomes above and beyond that of each direct measure of malnutrition-related early childhood experiences. We used multiple linear regression to evaluate the relationships between (1) malnutrition-related childhood exposures and adolescent outcomes, and (2) EH and each adolescent outcome, adjusted for relevant childhood exposures one-by-one. The childhood exposures we evaluated included stunted growth (HAZ), underweight (WAZ), anemia (Hb), immune activation (CRP) and parasitic gastrointestinal infection (hookworm infection). In doing so, we sought to evaluate whether associations between EH and adolescent anthropometrics and biomarkers persist above and beyond malnutrition-related early childhood experiences captured in the TAPS dataset.

Age and sex at the time of the adolescent outcome measurements were adjusted for in all analyses for statistical precision (Appendix K). For all statistical analyses described, the null hypothesis was that the difference in means was equal to 0 and the prevalence and odds ratio estimates were equal to 1 for each of the exposures assessed, indicating no difference in the influence of the exposure on the outcome. Statistically significant estimates at the $p< 0.05$ level are bolded in the tables of results. Confidence intervals were calculated based on the standard error of the difference in means, prevalence

and odds ratio estimates. Analyses were completed with the PROC FREQ, PROC MEANS, PROC TTEST, PROC REG, PROC GLM and PROC GENMOD functions in SAS Version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Study sample description

The analytic study sample included 337 participants, aged 10-17 years. The sample was approximately half male and participants were an average age of 13 years. Ninety-two point three percent of the study sample (311/337) had a rough, cobblestone-like pattern of EH on the tooth surface. Variation in the extent (portion of tooth surface affected by EH) and incidence of EH by year of age (1-4 years) was observed across the study sample. EH affected 1/3 to 2/3 of the tooth surface for most (47%) of those with EH. Approximately 26% had <1/3 of the tooth surface affected and another 27% had >2/3 of the tooth surface affected. The incidence of EH was similar for age 1, 2, 3 years (average 89.7/100 child-years), but was much lower for age 4 years (62.9/100 child-years). EH was not associated with sex or age at the time of measurement in adolescence (Appendix G).

On average, and according to WHO age- and sex-standardized growth reference charts, the study sample had a normal BMI and below average vertical stature in adolescence. Only one participant was classified as “thin” but approximately 16% were classified as “overweight” (WAZ > 1.0). The average HAZ in this study sample (-1.7) was nearly at the threshold for “short stature” or “stunted growth” (< -2.0). Approximately one third of the study sample had stunted vertical growth.

The average Hb measure and WBC count were on the threshold for unhealthy values. Based on age- and sex-appropriate hemoglobin thresholds, over half of the sample (74%) had anemia. Age- and sex-appropriate reference values indicated that 66% of the sample had an elevated WBC count. The average HbA_{1c} and blood pressure measures were in the normal range. Only three participants had type II diabetes ($\%A_{1c}$ > 6.5), and 9.7% had prediabetes ($\%A_{1c}$ between 5.7 and 6.5). 5.7% had prehypertension and 3.7% had stage 1 hypertension. An index was created for “poor metabolic health” by summing overweight, prediabetes/type II diabetes and prehypertension/hypertension status. 26.7% of all participants had at least one of these indicators of poor metabolic health (Table 1; Appendix L).

Table 1.
Description of adolescent demographics, anthropometrics and biomarkers by EH

MEASURE	no EH (n=26)	EH occur. (n=311)	EH Extent [‡]			Overall (n=337)
			< 1/3 (n=80)	1/3 – 2/3 (n=147)	> 2/3 (n=83)	
	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD
Sex, % male	38.5%	53.1%	47.5%	56.5%	51.8%	51.3%
Age (years)	13.1 ± 2.2	13.0 ± 2.2	13.1 ± 2.1	12.8 ± 2.0	13.2 ± 2.4	13.0 ± 2.2
ANTHROPOMETRICS						
height (cm)	145 ± 13	142 ± 12	143 ± 12	142 ± 11	141 ± 12	142 ± 12
height-for-age (z-score)	-1.4 ± 0.7	-1.7 ± 0.9	-1.7 ± 0.9	-1.6 ± 0.9	-2.0 ± 1.0	-1.7 ± 0.9
short stature (< -2)	19.2%	35.4%	36.3%	31.3%	42.2%	33.2%
weight (kg)	43 ± 12	40 ± 11	40 ± 12	39 ± 10	39 ± 11	40 ± 11
BMI-for-age (z-score)	0.42 ± 0.8	0.23 ± 0.8	0.2 ± 0.8	0.26 ± 0.8	0.22 ± 0.7	0.25 ± 0.8
overweight (> +1)	30.8%	14.2%	15.0%	12.9%	15.7%	15.8%
thin (< -2)	3.9%	0%	0%	0%	0%	<1%
HEALTH OUTCOMES						
hemoglobin (g/dL)	11.2 ± 2.0	11.1 ± 1.9	11.5 ± 1.8	11.3 ± 2.0	10.4 ± 1.7	11.1 ± 1.9
anemia	72.0%	75.5%	73.8%	69.9%	86.8%	74.4%
glycated hemoglobin (%A_{1c})	5.39 ± 0.3	5.3 ± 0.3	5.35 ± 0.3	5.31 ± 0.3	5.26 ± 0.3	5.31 ± 0.3
prediabetes	21.7%	8.8%	9.1%	9.9%	6.5%	9.7%
type II diabetes	0%	3.1%	2.6%	0.7%	0%	1.0%
white blood cell count (10^9/L)	10.0 ± 2.8	11.0 ± 2.8	10.0 ± 2.8	10.9 ± 2.8	11.8 ± 2.8	10.9 ± 2.9
"elevated"	52.9%	66.5%	57.9%	64.6%	76.1%	66.0%
systolic blood pressure (mmHg)	115 ± 11	111 ± 10	111 ± 11	111 ± 10	112 ± 9	112 ± 10
diastolic blood pressure (mmHg)	65 ± 8	63 ± 8	63 ± 9	63 ± 7	65 ± 9	63 ± 8
prehypertension	8.0%	8.3%	5.0%	6.1%	4.8%	5.7%
stage 1 hypertension	8.0%	3.5%	3.8%	2.7%	4.8%	3.7%

poor metabolic health**						
	0	46.2%	71.4%	70.0%	72.1%	71.1%	69.4%
	1	42.3%	25.4%	25.0%	25.2%	26.5%	26.7%
	2	11.5%	2.9%	5.0%	2.0%	2.4%	3.6%
	3	0%	0.3%	0%	0.7%	0%	0.3%

ł n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)

* reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

** count of overweight, prediabetes/type II diabetes, prehypertension/stage 1 hypertension

Bivariate evaluation of EH and adolescent outcomes

Based on chi-square tests and two-tailed t-tests, as appropriate, no significant difference existed between those with and without EH with regard to age or sex. Those with no EH were significantly more likely to be overweight (p=0.04) and to have at least one indicator of poor metabolic health (p=0.01) than those with EH. Otherwise, no differences were observed in adolescent anthropometric or biomarker outcomes between those with and without EH. When observed across EH extent, mean adolescent outcomes followed a linear trend for height, weight, Hb, HbA_{1c} and WBC count.

On average, those who had EH at age 4 years had lower hemoglobin (p=0.05), lower glycated hemoglobin (p=0.03) and higher systolic and diastolic blood pressure (p=0.04 and p <0.01, respectively) in adolescence than those who had EH, but not at 4 years of age (Appendix M). However, those who had EH at age 4 years were older at the time of adolescent outcome measurement (p<0.01) than those who had EH, but not at 4 years of age, which may explain differences in blood pressure, but unlikely to explain differences in hemoglobin or glycated hemoglobin (Appendix M).

Multivariate evaluation of EH and adolescent outcomes

Adjusted linear regression analyses were carried out to evaluate the relationships between EH and adolescent anthropometrics and biomarkers, holding the influence of age and sex constant. The results indicated that EH occurrence was consistently inversely related HAZ and height (shorter), lower BAZ (thinner) and weight (lighter), though some of the point estimates lacked statistical precision (Table 2). A linear trend by EH extent was observed in relation to HAZ (p=0.02), height (p< 0.01) and weight (p=0.2) (Appendix N). The average difference in HAZ and height per a 1/3 difference in tooth surface affected by EH was -0.14 standard deviations (95% CI: -0.24, -0.03) and -1.35 cm (95% CI: -2.21, -0.50 cm), respectively. Those with a greater portion of tooth surface affected by EH averaged a lower HAZ and height. Compared to those with >2/3 of the tooth surface affected by EH, those with no EH were nearly 5 cm taller on average. Similarly, the average difference in weight per a 1/3 difference in tooth surface affected by EH was -0.98 kg (95% CI: -1.73, -0.23). Those with a greater portion of tooth surface affected by EH averaged a lower weight. Compared to those with >2/3 of the tooth surface affected by EH, those with no EH were nearly 4 kg heavier on average.

Compared to participants who had EH occurrence at an estimated 4 years of age, those who had EH between one and three years, but not at 4 years, were 1.83 cm taller (95% CI: 0.14, 3.49) on average in adolescence. Those who had no EH at any age were 3.28 cm taller (95% CI: 0.41, 6.14) and 2.90 kg heavier (95% CI: 0.38, 5.42) on average than those who had EH at 4 years of age.

Table 2.
EH and adolescent anthropometrics linear regression results (n=337)
(difference in means and 95% CIs reported)

	Height (cm) ł	**Adolescent height-for-age (z-score) ***	**Weight (kg) ł**	**Adolescent BMI-for-age (z-score) ***
EH occurrence (v. no EH)	-2.69	-0.29	**-2.66**	-0.19
	(-5.52, 0.14)	(-0.65, 0.07)	**(-5.13, -0.19)**	(-0.50, 0.11)
EH extent ** (per 1/3 of the	**-1.35**	**-0.14**	**-0.98**	-0.03
tooth surface affected)	**(-2.21, -0.50)**	**(-0.24, -0.03)**	**(-1.73, -0.23)**	(-0.12, 0.06)
Estimated age at EH occurrence **				
EH at age 4 years (ref.)	--	--	--	--
EH, but NOT at age 4	**1.83**	0.19	0.74	-0.06
years	**(0.14, 3.49)**	(-0.02, 0.40)	(-0.73, 2.22)	(-0.24, 0.12)
no EH	**3.28**	0.35	**2.90**	0.17
	(0.41, 6.14)	(-0.01, 0.72)	**(0.38, 5.42)**	(-0.14, 0.48)
type III sum of squares	p =0.02	p = 0.06	p = 0.07	p = 0.37

ł adjusted for covariates (sex and age) in adolescence
* age- and sex- standardized, by World Health Organization reference charts
** n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)
bolded results indicate statistically significant estimates at p< 0.05

The data also suggest that EH occurrence was consistently associated with lower hemoglobin, lower glycated hemoglobin, higher white blood cell count and lower blood pressure, though these estimates were not statistically significant (Table 3). A linear trend was observed for hemoglobin (p<0.01), glycated hemoglobin (p=ns) and white blood cell count (p<0.01) (Appendix N). The average difference in hemoglobin, glycated hemoglobin, and WBC count per a 1/3 difference in tooth surface affected by EH was -0.36 g/dL (95% CI: -0.59, -0.13), -0.04 %A_{1c} (95% CI: -0.08, 0.00) and 0.74 10^9/L (95% CI: 0.35, 1.14), respectively. Those with a greater portion of tooth surface affected by EH averaged a lower level of hemoglobin, a lower level of HbA_{1c} and a higher WBC count. There was no statistical evidence for an association between EH and systolic or diastolic blood pressure (Table 3).

Compared to participants who had EH occurrence at an estimated 4 years of age, those who had EH between one and three years, but not at 4 years, had higher hemoglobin (0.50 g/dL, 95% CI: 0.05, 0.95), higher glycated hemoglobin (0.09 $\%A_{1c}$, 95% CI: 0.01, 0.17), lower WBC count (-0.79 10^9/L, 95% CI: -1.58, -0.00) and lower diastolic blood pressure (-2.66 mm Hg, 95% CI: -4.62, -0.70) on average in adolescence.

Table 3.
EH and adolescent biomarker linear regression results (n=337)ł
(difference in means and 95% CIs reported)*

	Hemoglobin* (g/dL)	**Glycated Hemoglobin*** ($\%A_{1c}$)	**White Blood Cell count*** (10^9/L)	**Systolic blood pressure*** (mmHg)	**Diastolic blood pressure*** (mmHg)
EH occurrence (v. no EH)	-0.09 (-0.87, 0.69)	-0.07 (-0.21, 0.07)	0.92 (-0.50, 2.33)	-3.27 (-6.97, 0.48)	-1.65 (-4.98, 1.78)
EH extent** (per 1/3 of the tooth surface affected)	**-0.36 (-0.59, -0.13)**	**-0.04 (-0.08, 0.00)**	**0.74 (0.35, 1.14)**	-0.41 (-1.54, 0.71)	0.19 (-0.82, 1.21)
Estimated age at EH occurrence**					
EH at age 4 years (ref.)	--	---	--	--	--
EH, but NOT at age 4 years	**0.50 (0.05, 0.95)**	**0.09 (0.01, 0.17)**	**-0.79 (-1.58, -0.00)**	-1.17 (-3.35, 1.02)	**-2.66 (-4.62, -0.70)**
no EH	0.25 (-0.54, 1.04)	0.10 (-0.04, 0.24)	-1.17 (-2.60, 0.26)	2.87 (-0.92, 6.67)	0.75 (-2.65, 4.16)
type III sum of squares	p = 0.09	p = 0.05	p = 0.06	p = 0.13	p = 0.02

ł adjusted for covariates (sex and age) in adolescence
* reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336
** n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)
bolded results indicate statistically significant estimates at $p< 0.05$

Log-binomial regression was used to estimate the prevalence ratio for adolescent anthropometric and health outcomes, based on meaningful public health and clinical thresholds (Table 4). Due to so few participants with BAZ less than -2, with type II diabetes or stage 1 hypertension, we did not estimate prevalence ratios for “thinness” and we evaluated prediabetic and diabetic participants together (compared to those with non-prediabetic and non-diabetic HbA_{1c} levels) as well as prehypertension and hypertension participants together (compared to those with non-prehypertension and non- hypertension

levels). “Poor metabolic health” was defined as any or none of the following dichotomous outcomes: overweight, prediabetes/type II diabetes, and prehypertension/hypertension.

Those with EH were *more* likely to be short, anemic, and have an elevated WBC count compared to those without EH at the time of the study. Those with EH were *less* likely to be overweight, prediabetic or diabetic, have prehypertension or stage 1 hypertension and thus to have overall poor metabolic health compared to those without EH at the time of the study. With the exception of overweight status and poor metabolic health, these point estimates lacked statistical precision (Table 4).

When we evaluated dichotomous outcomes by EH extent, a linear trend was observed for prevalence of overweight (p=ns), prediabetes/diabetes (p=ns), elevated white blood cell count (p=ns), and prehypertension/hypertension (p=ns). Compared to those with >2/3 of the tooth surface affected by EH, those with no EH were more than three time as likely to have prediabetes/diabetes (PR=3.18, 95% 1.01, 10.06) (Appendix N). The prevalence ratio of WBC count, per a 1/3 difference in tooth surface affected by EH, was 1.12 (95% CI: 1.01, 1.26) (Table 4). Although a linear trend was not observed in relation to anemia, compared to those with >2/3 of the tooth surface affected by EH, the prevalence of anemia was significantly greater for those with <1/3 (PR=0.86, 95% CI: 0.73, 1.00) and 1/3-2/3 of the tooth surface affected (PR= 0.80, 95% CI: 0.70, 0.92) (Appendix N).

Compared to participants who had EH occurrence at an estimated 4 years of age, those who had EH between one and three years, but not at 4 years, were more likely to have prediabetes/diabetes (PR=2.29, 95% CI: 1.14, 4.60) on average in adolescence. Those who had no EH at any age were more likely to be overweight (PR=1.91, 95% CI: 1.01, 3.61), have prediabetes/diabetes (PR=2.91, 95% CI: 1.16, 7.37), and to have poor metabolic health overall (PR=1.76, 95% CI: 1.18, 2.64) on average than those who had EH at 4 years of age.

Table 4.
EH and adolescent biomarkers, log-binomial regression results (n=337)ł
(prevalence ratios and 95% CIs reported)

	Short stature (height-for-age z-score <-2)	**Overweight** (BMI-for-age z-score >+1)	**Anemic*ł±**	**prediabetes or diabetes±** (%A_{1c} > 5.7)	**elevated WBC count*±**	**prehyper-tension or stage 1 hyper-tension*±**	**poor metabolic health**±**
EH occurrence (vs. no EH)	1.85 (0.83, 4.12)	**0.49 (0.26, 0.90)**	1.05 (0.81, 1.35)	0.48 (0.21, 1.13)	1.18 (0.75, 1.88)	0.54 (0.20, 1.44)	**0.57 (0.39, 0.84)**
EH extent *** (per 1/3 of the tooth surface affected)	1.16 (0.98, 1.38)	0.83 (0.63, 1.09)	**1.08 (1.00, 1.18)**	0.73 (0.51, 1.02)	**1.12 (1.01, 1.26)**	0.90 (0.61, 1.33)	0.84 (0.71, 1.01)
Estimated age at EH occurrence ***							
EH at age 4 years (ref.)	--	--	**--**	--	**--**	--	--
EH, but NOT at age 4 years	0.86 (0.61, 1.20)	0.76 (0.40, 1.43)	0.98 (0.85, 1.12)	**2.29 (1.14, 4.60)**	0.88 (0.72, 1.08)	0.67 (0.29, 1.55)	1.01 (0.69, 1.48)
no EH	0.52 (0.23, 1.15)	**1.91 (1.01, 3.61)**	0.95 (0.73, 1.22)	**2.91 (1.16, 7.37)**	0.81 (0.51, 1.29)	1.64 (0.60, 4.45)	**1.76 (1.18, 2.64)**
type III likelihood ratio	p = 0.14	p = 0.10	p = 0.88	p = 0.02	p = 0.33	p = 0.34	p=0.06

ł adjusted for covariates (sex and age) in adolescence

* sex and age specific thresholds for disease status

** count of overweight, prediabetes/type II diabetes, prehypertension/stage 1 hypertension

± reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

*** n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)

bolded results indicate statistically significant estimates at p< 0.05

EH – adolescent biomarkers above and beyond adolescent body size

To evaluate whether associations between EH extent and adolescent biomarkers were associated above and beyond adolescent body size, we adjusted for adolescent anthropometrics in our linear regression analyses of EH extent and adolescent biomarkers. First, we confirmed that the expected association was observed between body size and biomarkers in this study sample. When the relationship between adolescent anthropometrics and biomarkers were evaluated without regard to dental enamel, adolescent HAZ, height, and weight were associated with hemoglobin, glycated hemoglobin and white blood cell count, but did not appear to be related to blood pressure (Appendix O). There was no statistical evidence that adolescent BAZ was related to any of the adolescent biomarkers measured. The average difference in hemoglobin, glycated hemoglobin and WBC count per a 2 SD difference in adolescent HAZ (for example, -2.0, the WHO definition of 'stunted' growth, and 0.00, 'normal' growth) was 0.72 g/dL, 0.10 $\%A_{1c}$ and -1.36 10^9/L, respectively, with those who were taller averaging a higher level of hemoglobin, a higher level of HbA_{1c} and a lower WBC count. Similarly, the average difference in hemoglobin, glycated hemoglobin and WBC count per a 1 kg difference in adolescent weight was 0.04 g/dL, 0.01 $\%A_{1c}$ and -0.08 10^9/L, respectively, with those who were heavier also averaging a higher level of hemoglobin, a higher level of HbA_{1c} and a lower WBC count.

The statistically significant relationship between EH extent and hemoglobin and between EH extent and WBC count were slightly attenuated, but persisted after adjustment for adolescent height, HAZ and weight. The relationship between EH extent and glycated hemoglobin was also attenuated and was no longer statistically significant after adjustment for adolescent height, HAZ and weight. Adjustment for adolescent anthropometrics did not influence the relationship between age 4years at EH occurrence and blood pressure, but attenuated the relationships with hemoglobin, glycated hemoglobin and WBC count differently. Adjustment for height attenuated the association between all of these outcomes so they were not longer statistically significant. Adjustment for HAZ attenuated the associations with hemoglobin and WBC count, and weight attenuated the relationship with weight.

EH – adolescent biomarkers above and beyond childhood exposures

To evaluate whether EH detected an underlying mechanism relevant for the adolescent biomarkers included in these analyses above and beyond that of the direct childhood measures, we used linear regression to investigate the association between EH extent and adolescent outcomes, adjusted one-by-one for direct malnutrition-related measures of early childhood experience. The particular childhood exposures considered included HAZ, WAZ, immune activation, hemoglobin and GI infection during age 1 through 4 years of age. First, childhood HAZ and WAZ were significantly associated with higher adolescent HAZ and BAZ. Childhood HAZ was also related to lower adolescent WBC count (Appendix P). Children whose average HAZ was > -2.0 between 1 and 4 years of age were more than twice as likely to be overweight (PR=2.11), to have prediabetes/type II diabetes (PR=2.18) and to have poor metabolic health overall (PR=1.87) in adolescence compared to those whose growth was stunted in early childhood (Appendix Q). Children whose average HAZ in early childhood was stunted were nearly four times as likely to also have stunted growth in adolescence (PR=3.65).

When adjusted for average childhood HAZ, the relationships between EH extent and adolescent HAZ and glycated hemoglobin were attenuated and no longer statistically significant, but the relationships between EH extent and adolescent hemoglobin and WBC count persisted (Appendix P). When adjusted for average childhood WAZ, the relationship between EH extent and glycated hemoglobin was attenuated and no longer significant, but the relationships between EH extent and adolescent HAZ, hemoglobin and WBC count persisted. Regarding the estimated age at EH occurrence, when adjusted for childhood HAZ and WAZ, the relationship between no EH occurrence at age 4y compared to EH at age 4y and adolescent WBC count was attenuated and no longer significant, but the associations with hemoglobin and glycated hemoglobin persisted (Appendix P).

The role of household socioeconomic status and diet

A posthoc analysis was carried out to evaluate the role of household socioeconomic status and dietary consumption in the relationships between EH and adolescent outcomes. First, we evaluated the relationship between household SES (in local currency, bolivianos (Bs) and household 1-week sugar consumption (in kg) and EH and adolescent outcomes (Appendix R). On average, those with EH had

lower SES, consumed less sugar and more plantains and manioc (*yuca*). These differences were not statistically significant. A linear trend was observed across EH extent for all household measures of SES and dietary consumption. After adjusting for age and sex in adolescence, household sugar consumption was significantly associated with a difference in average height (0.98 cm, 95% CI: 0.18, 1.78), weight (0.86 kg, 95% CI: 0.16, 1.56), hemoglobin (0.24 g/dL, 95% CI: 0.02, 0.45), glycated hemoglobin (0.06 $\%A_{1c}$, 95% CI: 0.02, 0.09) and WBC count (-0.37, 95% CI: -0.74, -0.00). Manioc consumption (1 kg household consumption per week) was associated with a greater diastolic blood pressure (0.11 mm Hg, 95% CI: 0.01, 0.21). We otherwise did not observe evidence for a relationship between household SES or other household dietary measures and adolescent outcomes.

Next, we evaluated the relationship between each measure of EH and adolescent outcomes adjusted separately for household SES and household sugar consumption (Appendix S). After adjustment for household SES and sugar consumption, EH occurrence was no longer statistically associated with adolescent weight. The EH extent – adolescent HbA_{1c} association was attenuated and no longer statistically significant when adjusted for household SES and for household sugar consumption, but SES and sugar consumption did not otherwise influence the EH extent- adolescent outcome relationships.

Similarly, the relationships between no EH at 4 years of age and adolescent outcomes, compared to EH at 4 years of age, were influenced differently by consideration of the influences of SES and sugar consumption. When adjusted for household SES, the difference in adolescent WBC count between those without and with EH at 4 years of age was no long significantly different, but the differences in height, hemoglobin, glycated hemoglobin and diastolic blood pressure remained. When adjusted for household sugar consumption, the difference in adolescent height, hemoglobin, glycated hemoglobin and WBC count were no longer statistically significant, but the difference in diastolic blood pressure persisted.

Discussion

This was the first study we are aware of to evaluate the relationships between EH and adolescent anthropometrics and biomarkers in a living human population. Our results indicated that EH extent was consistently associated with several adverse anthropometric and biomarker measures among an

Amerindian adolescent study sample from rural Bolivia. Greater extent of EH was associated with shorter height and lower weight, lower hemoglobin and greater WBC count. Interestingly, greater EH extent was also associated with lower glycated hemoglobin, a healthy outcome. The role of adolescent body size did not account for the relationship between EH extent and hemoglobin or WBC count, but did seem to play an important role in the EH extent-glycated hemoglobin association. EH extent also seemed to capture a childhood exposure relevant to adolescent HAZ, hemoglobin and WBC count outcomes above and beyond that of childhood anthropometrics.

Given that chronic malnutrition and adverse health outcomes are associated with increased mortality, our findings are thus consistent with bioarchaeological evidence that has repeatedly observed an association between EH and early mortality.[8] Human population studies have also demonstrated an association between EH and stunted growth (HAZ) in late childhood and early adolescence.[48,87] Evidence suggests that systemic physiologic disruption related to under-nutrition during early life may result in increased risk for chronic disease through physiologic, structural, metabolic, immunologic and epigenetic pathways.[4,19,20] It is through these proposed mechanisms that low birth weight and growth stunting — childhood experiences like EH that are associated with chronic, low-grade infection and poor dietary quality [21,22] — are thought to be associated with insulin resistance and type II diabetes, high blood pressure, and increased risk of cardiovascular disease and obesity in adulthood.[1,2,4,23] So, the EH-adolescent outcome associations we observed could be indicative of early life influences on subsequent health, but may have also been due to persistent exposures (common causes) or susceptibility throughout the life course.

Regardless, EH may serve as a useful screening tool for patients as young as 10 years of age (or when the permanent dentition erupts) to alert clinicians to underlying disease risk that may not otherwise present until later in adulthood.[88,89] An example of its utility in the existing literature is that enamel defects have been recognized as an early oral manifestation of celiac disease.[88] Our results further support its utility for screening for more widespread general health risk.

Upstream factors that were presumed to have a meaningful influence on malnutrition-related exposures during early childhood as well as on adolescent health were intentionally excluded from initial analyses. These factors included socioeconomic status and poverty, sanitation and hygiene practices,

water contamination and source, repeated and/or chronic infection and diet (particularly with regard to micronutrients). Adjustment for these factors in the analyses would have blinded us to the EH that was attributed to the factors adjusted for, which was not the intent of the investigation. Instead, we sought to first evaluate EH as a potential indicator of and screening tool for adolescent disease risk due to its presumed relationship with physiological stress in early childhood. If a clinician observed the teeth of a patient and noticed EH, should this alert him or her to a potential underlying general health risk for the patient? Posthoc analyses revealed that participants from households with lower SES and that lived more traditionally (less sugar consumption, more plantains and manioc (*yuca*)) were more likely to have EH. However, SES and sugar consumption only seemed to explain the relationships between EH extent and adolescent weight and HbA_{1c}. These findings may suggest that EH could also serve as a retrospective marker of SES in the absence of records of direct measures.

Limitations

Limitations to this study included the multiple testing done in these analyses, which may have produced spurious and misleading findings. The analyses were intended to be exploratory, as suggested by the presentation of confidence intervals without p-values. Additionally, potential measurement error of EH relating to the participants not having brushed their teeth prior to the photographs (due to feasibility in field conditions) and the unique pattern of EH observed in this study sample may have prevented us from detecting stronger associations. Similarly, imprecise measurement of childhood exposures may also have produced misleading findings. Finally, adolescents who were missing both central maxillary incisors and thus excluded from these analyses were older at the time of 2015 data collection. If their lack of central incisors was due to EH, the reported point estimates for EH-adolescent outcomes may be underestimated due to the positive association between age and adverse adolescent health.

Study findings should not be generalized to populations outside Amerindian populations living in the Amazonian Basin and in the midst of a dietary and lifestyle transition like the Tsimane'. Measurement of EH and the varying patterns in which it presents, its etiology and a better understanding of the mechanisms underlying the relationship between childhood malnutrition and subsequent outcomes and

the role of time-varying confounding factors of these relationships in these populations are necessary before EH can be reliably used as an indicator of or clinical screening tool for disease risk.

Chapter 6. Accuracy of enamel hypoplasia in the permanent dentition as a biomarker of chronic malnutrition and predictor of adolescent health

Abstract

Objective: Adult teeth may chronologically reflect early childhood experience because enamel on the permanent teeth calcifies incrementally during early childhood and is sensitive to physiological stress. Defects in the enamel caused by physiological insults that occurred during early childhood do not repair after occurrence or during the life course, leaving a permanent biological mark of physiological insults that occurred during early childhood. Evidence from studies of the developmental origins of health and disease (DOHaD) shows early life experience influences disease risk throughout the life course. Childhood growth stunting and birthweight are commonly used proxy measure for a physiologically stressful early childhood in DOHaD literature, but such birth and childhood records are often not available in low-resource settings. Bioarchaeological findings indicate enamel hypoplasia (EH) is associated with early mortality, suggesting the teeth may be a useful retrospective measure of childhood experience in the absence of childhood health records. We therefore investigated the accuracy of EH as a biomarker of chronic malnutrition and compared its performance in predicting adolescent health to that of growth stunting in a study sample from rural Bolivia.

Methods: This study was conducted within an Amerindian population in the Bolivian Amazon. Our study sample included 349 adolescents (10-17 years of age) who had enrolled for at least one year in a longitudinal study during early childhood (1-4 years of age). We detected EH in the permanent central maxillary incisors using digital photography from which the following measures of EH were abstracted: occurrence (any, none), extent of occurrence (<1/3, 1/3-2/3, >2/3 of the tooth surface) and estimated age at occurrence (1, 2, 3, 4 years of age). Chronic malnutrition was measured by growth stunting in childhood and adolescence using height-for-age z-scores (HAZ, where HAZ < -2.0 indicates stunted growth). Adolescent health outcomes were measured with biomarkers, including hemoglobin (Hb,

anemia), glycated hemoglobin (HbA_{1c}, type II diabetes), white blood cell count (WBC, immune activation) and blood pressure (hypertension). The validity of EH as a marker of chronic malnutrition during early childhood and adolescence was assessed using sensitivity, specificity and receiver operating characteristics (ROC). Cumulative AUCs were reported (a) to compare the performance of adolescent HAZ and EH extent as proxy measures of childhood stunting and (b) to comparatively evaluate EH extent, adolescent and childhood HAZ as predictors of adolescent health.

Results: The overall prevalence of a rough hypoplastic pattern of EH in the study sample was 92.4%. When evaluated against growth stunting as the "gold standard" (HAZ< -2.0: prevalence =62.3% in childhood and 34.1% in adolescence), EH had high sensitivity (93% in childhood, 96% in adolescence) and low specificity (11% in childhood, 10% in adolescence). EH extent was a more accurate marker of childhood stunting (AUC 0.56) than EH occurrence (AUC 0.52) due to increased specificity (0.36 vs. 0.11). The addition of EH extent to a set of markers for childhood stunting (gender and adolescent HAZ) only slightly improved the AUC (0.77 vs. 0.76, p=ns). The AUC for adolescent WBC count was greater for EH extent (AUC 0.61) than for childhood HAZ (AUC 0.58, p=ns) and adolescent HAZ (AUC 0.59, p=ns). Addition of EH extent to sets of markers for adolescent health outcomes (age, gender, childhood HAZ and adolescent HAZ) improved the AUC for nearly all outcomes.

Conclusion: Although not a strong proxy measure for chronic malnutrition, EH extent may be an important measure for predicting adolescent health outcomes. EH extent may serve as a useful proxy measure of childhood experience among adolescents in settings where childhood stunting data is not available. Furthermore, EH extent may capture childhood exposures relevant to adolescent health outcomes that are not captured by childhood or adolescent HAZ and thus may be a useful clinical screening tool and/or addition to the "toolkit" of markers of chronic malnutrition by which to identify high-risk adolescents.

Introduction

Enamel hypoplasia (EH) in the permanent dentition may serve as a window into the past and thus serve as an underutilized retrospective source of information about early childhood experience.[8] Permanent teeth may record early childhood experience because dental enamel calcifies on the central maxillary incisors incrementally during early childhood (1 through 4 years of age), is sensitive to physiological stress and thus susceptible to defects (such as EH), and defective enamel does not repair during the life course.[9–11] Infectious illness (including fever) and micronutrient deficiency during early childhood may cause development of enamel hypoplasia (EH) through disruption of the enamel mineralization process during the development phase when layers of the enamel matrix are being formed, resulting in a reduction in the enamel thickness.[9,11,36]. Studies that have assessed malnutrition, growth stunting, or absence of nutritional supplementation during early childhood have consistently found children with poorer nutritional status to be significantly more likely to have EH in their permanent dentition than their well-nourished counterparts.[47–50]

Research on the developmental origins of health and disease (DOHaD) has demonstrated the important role of early life experience (such as malnutrition) on chronic disease risk later in life.[1–4] The ultimate goal of developmental origins of health and disease (DOHaD) research is to elucidate the complex ways in which multiple, integrated early life influences, including biological, ecological, psychological and social factors, operate together during the life course to affect adult health outcomes.[24] To better understand the mechanisms underlying the DOHaD, biomarkers, including anthropometric measures, are commonly relied on to indicate early childhood experiences.[21,22,24,30,31] Adult health outcomes potentially associated with early childhood influences that have been widely studied include obesity, hypertension, cardiovascular disease, type II diabetes, respiratory illness, and neuropsychiatric outcomes.[1,2,4,23] However, the advancement of DOHaD research and a clearer understanding of other potential underlying mechanisms is hindered by issues related to characterizing early childhood experience.[4,7,15] First, the ability to study the DOHaD depends on decades of follow-up data from longitudinal studies since human memory tends to include error when recalling events that occurred many years in the past or during the early years of life. Second, even records of birth weight and stunted growth, commonly relied upon indicators of early childhood experience, are often unavailable in low-

resource settings and are likely imperfect markers of more complex processes (including organ growth patterns and epigenetic programming).[14,32,33] Therefore, the current DOHaD research community has identified the critical need to identify more accessible measures of early childhood experience that that will specifically capture the source and timescale of various early childhood exposures.[4,14,15]

As a measure of physiological disruption that occurred during enamel calcification of the permanent dentition, EH may serve as a biomarker for chronic malnutrition during early childhood, as measured by stunted growth. The validity of EH as a biomarker of chronic malnutrition exposure measure may vary by population (by severity of exposures and causal pathways for EH at play) and depends on the choice of gold standard measurement used to define the true "early childhood experience". Biomarkers may serve as useful measurement tools because they could help decrease measurement error of the exposure of interest, particularly in attempts to characterize exposure retrospectivley.[90,91] In doing so, they increase the power of studies to detect health effects of adverse exposures.[83] The validity of a measure is determined by its ability to correctly determine the presence/absence and/or value of an underlying characteristics, i.e. sensitivity and specificity. Though growth stunting is not considered a perfect measure of particular causes of chronic malnutrition (e.g., repeated bouts of infection, diet, weaning practices, poor sanitation and hygiene, etc.), it is important and strategic to evaluate EH against stunted growth since stunted growth is a widely and historically relied upon retrospective indicator of childhood chronic malnutrition. The present study therefore evaluated the accuracy of EH as a biomarker of chronic malnutrition in a study sample from rural Bolivia.

Methods

A detailed account of all study methods are described in Chapter 2.

Study design and sample

This study extended a prospective cohort study. It followed up a sample of adolescents, currently aged 10-17 years, who were enrolled as young children (5 years and younger) in the 9-year Tsimane' Amazonian Panel Study (TAPS).[61] The present study built on the TAPS by adding measures of enamel defects in the permanent dentition and follow-up measures of adolescent anthropometrics and biomarkers for the original study's child participants. Criteria for inclusion in the follow-up study include that an individual be 10-17 years of age at the time of data collection (so that the central maxillary incisors will have fully erupted), was previously enrolled in TAPS for at least one year when aged 5 years or less, and resided in one of 15 communities visited by the data collection team during the follow-up study.

The study sample was derived from the Tsimane' population, an Amerindian group of approximately 8,000 people who live across more than 100 small, remote communities, accessible by dirt roads and/or canoe and river in Bolivia's Amazonian Basin. Although the Tsimane' are increasingly integrating into mainstream Bolivian society and exposed to the market economy and a Western diet, they still maintain a traditional hunter-horticultural lifestyle and primarily speak their native language in their communities. The original TAPS study included 1,453 people, 655 of who were eligible for the 2015 follow-up study. 349 participants were enrolled in the 2015 follow-up study. The sample was reduced to 337 due to 12 participants with missing or severely decayed central maxillary incisor by which EH could not be measured. Of these participants, 327 were included in the present analysis because at least one year of childhood anthropometric (height and weight) data that correlated with the etiologically-relevant exposure period for the study outcome (ages 1 through 4 years) was available. Sample size also varied by each childhood exposure evaluated based on available data and by adolescent outcome according to missing values due to point-of-care device errors in the field. Approval for all data collection and use was obtained from the local tribal government, the *Gran Consejo Tsimane'* (*GCT*, Tsimane' Grand Council) and the University of Washington (UW) Human Subjects Division (HSD).

Enamel hypoplasia

The potential biomarker of interest in these analyses was rough, cobblestone-like hypoplastic pattern in the enamel of the permanent central maxillary incisors, considered as a biomarker of early childhood malnutrition during age 1 year through 4 years. EH was captured through digital intraoral photographs with a macro lens and ring flash, detected using Photoshop software, and quantified according to the internationally-accepted Modified Developmental Defects of the Enamel (DDE) Index.[34,69] EH was measured on the two central maxillary incisors because these teeth were demonstrated to be the most reliable for abstracting enamel defect data from digital photographs in this study sample (see Chapter 3). EH was classified by occurrence (any, none), extent of tooth surface affected by EH (less than 1/3/ 1/3 to 2/3 / more than 2/3), and the estimated age at EH occurrence (by year of age, 1 through 4 years). These measures of EH were recorded based on the left central maxillary incisor or – if the left was missing or severely decayed– on the right central maxillary incisor. There was 93.4% concordance across the two central maxillary incisors for EH occurrence.

Growth stunting as a gold standard measure of chronic malnutrition

Childhood growth stunting data were collected by the TAPS study team between 2002 and 2010, when children were between 1 and 4 years of age.[61] Adolescent growth stunting data were collected during the 2015 follow-up study, when TAPS child participants were 10-17 years of age. A stadiometer was used to measure height; the average of two measurements was used in analyses. For the early childhood period, mean HAZ between 1 and 4 years of age was used to determine stunting status. Growth stunting was measured on a continuous scale using HAZ. HAZs were age- and sex- standardized according to World Health Organization (WHO) reference growth charts for children and adolescence. Also, according to WHO standards, HAZ less than -2SD were classified as “short stature” or “stunted growth”.[79]

Outcomes

The biomarker outcomes were: (a) Hb, measured in g/dL, (b) HbA_{1c}, measured as $\%A_{1c}$, (c) WBC, measured in 10^9xL, and (d) systolic and diastolic blood pressure, measured in mm Hg. Biomarker outcomes were collected in the field using blood from a finger prick and point-of-care devices and a digital

blood pressure cuff charged by solar technology. Point-of-care measure thresholds were based on standard clinical care for adolescents. American Diabetes Association thresholds were used to classify HbA_{1c} measures as "pre-diabetic" and "type II diabetes".[85] Standard age- and sex-based reference values for clinical practice were used to determine thresholds for anemia based on Hb and elevated WBC count.[80] National Institute of Health's age and sex specific parameters for blood pressure were used to classify "hypertension" and "prehypertension".[86] An index (0-3) was created to indicate (e) "poor metabolic health" by summing overweight, prediabetes/type II diabetes and prehypertension/hypertension status.

Covariates

Covariates of primary interest included age (measured in years) and sex (male/female), recorded in 2015.

Statistical Analyses

Overall descriptions of EH, childhood and adolescent growth stunting and biomarkers in the study sample were provided. Descriptions of growth stunting and adolescent biomarkers were also stratified by EH status.

Stunted growth was considered the "gold standard" measurement for chronic childhood malnutrition (measured without error). To evaluate the validity of EH for reflecting chronic malnutrition, we carried out sensitivity, specificity and receiver operating characteristics (ROC) analyses for growth stunting in early childhood and in adolescence (separately). First, EH was dichotomized as any occurrence or none ('diseased' status was indicated by EH occurrence). Sensitivity and specificity were reported. Then, a range of thresholds for disease status, as measured by EH extent, were evaluated. The sensitivity and specificity of EH extent thresholds were reported and ROC analyses were carried out to further evaluate EH as a biomarker of stunted growth. The areas under the curve (AUC) and corresponding 95% CIs were reported.

To compare EH extent and adolescent growth stunting as proxy measures of childhood stunting, AUCs were compared. To compare EH extent to childhood and adolescent stunting as predictors of adolescent health biomarkers, we compared the AUCs of cumulative sets of biomarker predictors. AUCs and corresponding 95% confidence intervals are reported numerically and displayed in bar graphs. Age

and sex at the time of the adolescent outcome measurements were considered in AUC comparisons for statistical precision (Appendix K). Analyses were completed with the PROC FREQ, PROC MEANS and PROC LOGISTIC (with ROC option) functions in SAS Version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Study sample description

Most of the study sample (62.3%) were stunted (HAZ < -2.0) during childhood (1-4 years of age) and 34.1% had stunted growth in adolescence (10-17 years of age) (Table 1). A greater proportion of children were stunted among those with EH than those without EH in both early childhood and adolescence, yet no obvious trends emerged regarding EH extent and stunting in childhood or adolescence. Children who were stunted in childhood were 3.65 times more likely to be stunted as adolescents (Appendix Q). More than half (52%) of the children who were stunted during childhood were no longer stunted in adolescence, but very few children (5.0%) with normal growth subsequently had stunted growth in adolescence.

Table 1.
HAZ in adolescence and childhood, overall and by EH occurrence and extent

MEASURE	no EH (n=26)	EH occur. (n=311)	EH Extent ł			Overall (n=337)
			< 1/3 (n=80)	1/3 – 2/3 (n=147)	> 2/3 (n=83)	
	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD	% or mean ± SD
GROWTH STUNTING						
Childhood (1-4 years)*						
mean height-for-age (z-score, HAZ)	-1.8 ± 0.8	-2.3 ± 1.3	-2.3 ± 1.5	-2.2 ± 1.0	-2.4 ± 1.4	-2.2 ± 1.3
mean HAZ < -2.0	46.2%	63.7%	65.0%	59.2%	69.9%	62.3%
Adolescence (10-17 years)						
HAZ	-1.4 ± 0.7	-1.7 ± 0.9	-1.7 ± 0.9	-1.6 ± 0.9	-2.0 ± 1.0	-1.7 ± 0.9
HAZ < -2.0	19.2%	35.4%	36.3%	31.3%	42.2%	34.1%
ADOLESCENT HEALTH±						
anemia	72.0%	75.5%	73.8%	69.9%	86.8%	74.4%
pre-/type II diabetes	21.7%	11.9%	11.7%	10.6%	6.5%	10.7%
elevated white blood cell count	52.9%	66.5%	57.9%	64.6%	76.1%	66.0%
pre-/stage I hypertension	16.0%	11.8%	8.8%	8.8%	9.6%	9.4%
poor metabolic health**	53.8%	28.6%	30.0%	27.9%	28.9%	30.6%

ł n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)
* reduced sample (n=327) due to limited availability of at least one year of childhood height data between ages 1 through 4 years
± reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336
** count of overweight, prediabetes/type II diabetes, prehypertension/stage 1 hypertension

Validity of EH as a biomarker for stunted growth during early childhood

We first evaluated EH occurrence on the central maxillary incisors as a classification tool to capture stunted growth during early childhood years that corresponded to enamel formation on the central maxillary incisors (1-4 years). We estimated the accuracy of EH as a retrospective diagnostic of chronic malnutrition during age 1-4 in childhood using three childhood mean HAZ thresholds as gold standards: HAZ < -1.0 (at risk for growth stunting), -2.0 (the WHO threshold for "stunted growth"), and HAZ < -2.5 (the best threshold for predicting adolescent growth stunting; Appendix T). Overall, EH occurrence had a high sensitivity but low specificity (Table 2, Appendix T). When stunted growth in childhood was present, according to the WHO threshold for growth stunting, EH occurrence detected it 93% of the time. However, when stunted growth in childhood was absent, EH occurrence only indicated no stunted growth 11% of the time. The area under the curve (AUC) of EH occurrence for predicting stunted growth (HAZ < -2.0) was 0.52 (95% CI: 0.49, 0.56).

Table 2.
Sensitivity and specificity of EH occurrence to detect childhood growth stunting (n=327) (gold standard: early childhood growth stunting, defined by three different HAZ thresholds)

	Sensitivity	Specificity
EH occurrence		
HAZ < -1.0	0.93	0.15
HAZ < -2.0	0.93	0.11
HAZ < -2.5	0.93	0.08

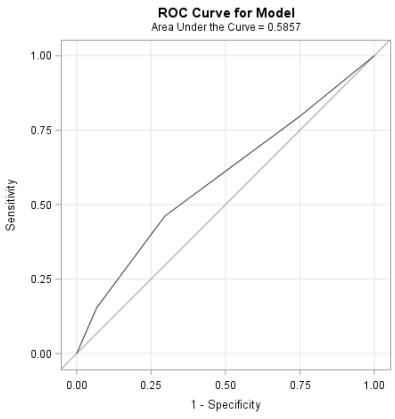
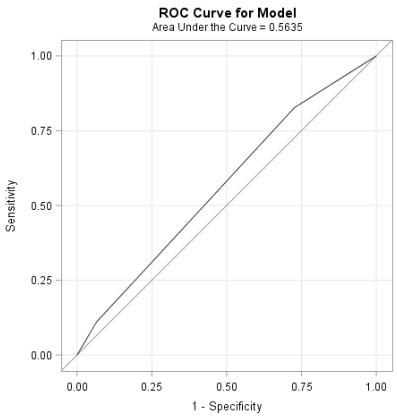
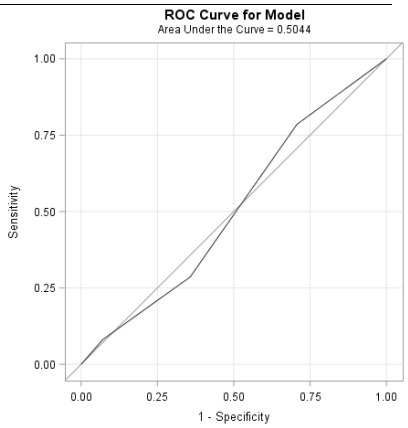
Next, we used ROC analyses to evaluate the use of EH extent to detect stunted growth during early childhood (HAZ < 2.0). In these analyses, sensitivity and specificity were plotted for all possible EH extent cutoff values to further evaluate EH as a retrospective biomarker of early childhood malnutrition (Table 3). EH extent performed better than EH occurrence (AUC 0.52) as a biomarker of stunting. The optimal cutoff threshold by which EH extent discriminated presence/absence of stunted growth during early childhood was between 1/3 and 1/3-2/3 of the tooth surface affected by EH (sensitivity = 0.71, specificity=0.36). The area under the curve was 0.56 (95% CI: 0.50, 0.63), indicating EH extent was not a strong biomarker of early childhood chronic malnutrition, as measured by HAZ < -2.0 (Figure 1). The

performance of EH extent did not differ greatly when -1.0 HAZ or -2.5 HAZ were used as the thresholds by which to define growth stunting during early childhood.

Table 3.
Sensitivity and Specificity of EH Extent (gold standard = early childhood growth stunting) (n=326)

Extent of EH	Growth Stunting (<-2 HAZ) during 1 – 4 years		Cutoff Location	Sensitivity	Specificity
	Present	Absent	←	0.00	1.00
> 2/3	67	14	←	0.27	0.83
1/3 – 2/3	106	38	←	0.71	0.36
< 1/3	56	20	←	0.93	0.11
no EH	16	9	←	1.00	0.00
TOTAL	245	81			

Figure 1.
ROC curve for EH extent, gold standard = early childhood growth stunting by various thresholds

HAZ < 1.0	HAZ < -2.0	HAZ < -2.5
		
AUC = 0.59 (95% CI: 0.49, 0.68)	AUC = 0.56 (95% CI: 0.50, 0.63)	AUC = 0.50 (95% CI: 0.44, 0.57)

Validity of EH as a biomarker for stunted growth during adolescence

Similarly, when we evaluated the accuracy of EH in capturing chronic malnutrition, measured by stunted growth in adolescence (10-17 years of age), we found that EH had a similar sensitivity and specificity to its performance against stunted growth during early childhood. The sensitivity of EH occurrence was

slightly higher for stunted growth in adolescence compared to childhood (96% compared to 93%), and the specificity was lower (10% compared to 11%) (Table 4).

Table 4.
Sensitivity and Specificity of EH Extent (gold standard = growth stunting between 10-17y) (n=336)

	Growth Stunting (<-2.0 HAZ) at 10-17 years of age				
Extent of EH	Present	Absent	Cutoff Location	Sensitivity	Specificity
			←	0.00	1.00
> 2/3	35	48			
			←	0.30	0.78
1/3 – 2/3	46	101			
			←	0.70	0.33
< 1/3	29	51			
			←	0.96	0.10
no EH	5	21			
			←	1.00	0.00
TOTAL	115	221			

We also used EH extent to evaluate a range of thresholds for stunted growth status during adolescence and carried out an ROC analysis to further evaluate EH as a biomarker of chronic malnutrition during adolescence. The area under the curve for EH extent was 0.55 (95% CI: 0.49, 0.61) indicating EH extent was not a strong biomarker of chronic malnutrition measured by HAZ in adolescence. The optimal cutoff threshold by which EH extent discriminates between stunted or not (by HAZ < -2.0) in adolescence was between 1/3 and 1/3-2/3 of the tooth surface affected by EH (sensitivity = 0.70, specificity=0.33).

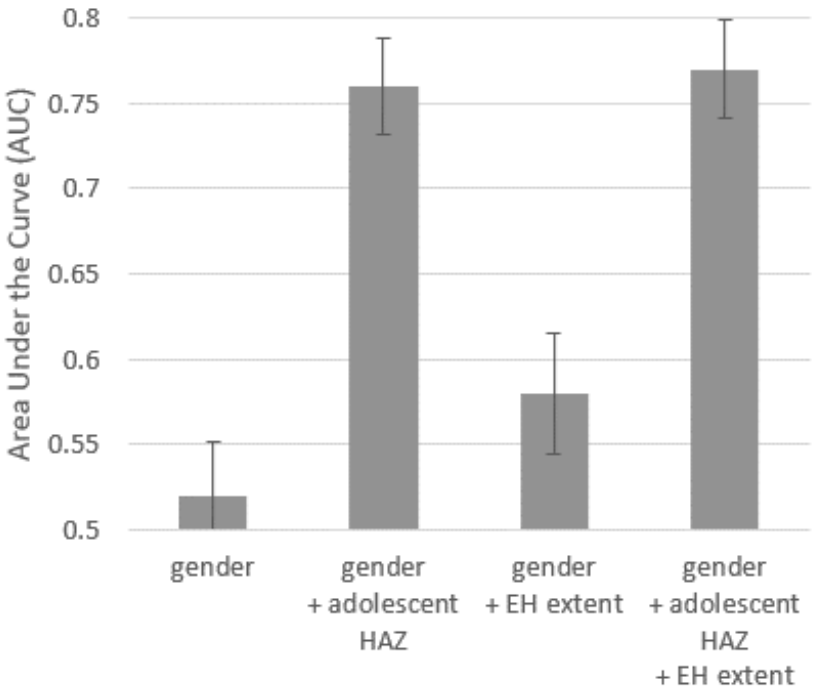
In comparison to childhood growth (HAZ) as a test for adolescent growth, EH extent was less sensitive and less specific. The area under the curve for childhood growth was greater than that for EH extent (AUC=0.77 compared to 0.55 for EH extent). The optimal cutoff threshold by which childhood growth (HAZ) discriminates between adolescent stunting or not was -2.5 HAZ (sensitivity=0.72, specificity=0.74) (Appendix U).

Adolescent stunting vs. EH extent as a proxy for childhood stunting

In many low-resource settings, childhood records of growth and health are not available. We sought to evaluate whether, in such a scenario, adolescent stunting and EH extent may serve as retrospective

proxy measures for early childhood malnutrition. To compare adolescent stunting and EH extent as proxy measures of childhood stunting, we compared the AUC for various combinations of childhood stunting proxy measures. In ROC analyses, addition of adolescent HAZ was a better indicator of childhood stunting than was EH extent when each was added to a set that included gender. However, EH extent slightly improved the AUC for childhood stunting when added to a set that already included gender and adolescent HAZ (Figure 2).

Figure 2.
AUCs of ROC curves corresponding to increasing subsets* of proxy measures of childhood stunting *



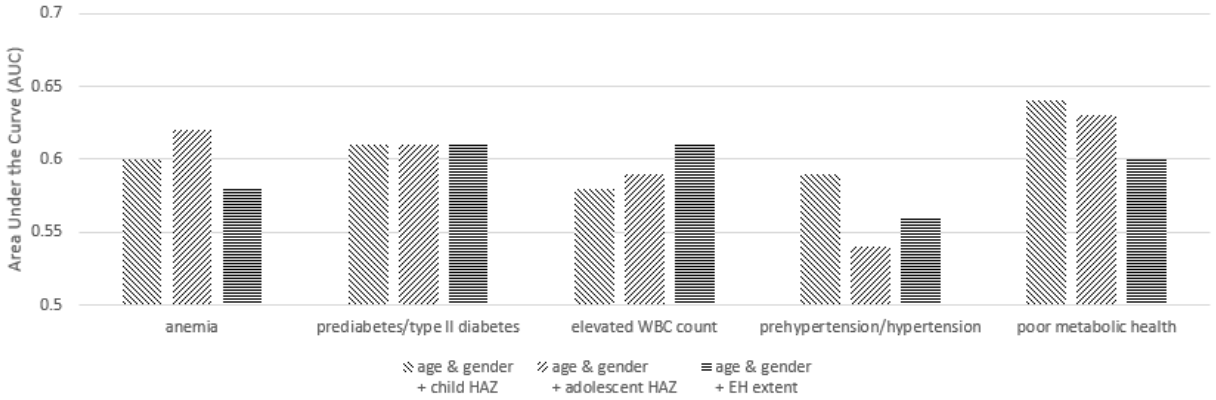
* set 1: gender, set 2: addition of adolescent HAZ to set 1, set 3: addition of EH extent to set 1, set 4: addition of adolescent HAZ and EH extent to set 1

EH extent, childhood stunting, and adolescent stunting as predictors of health outcomes

To comparatively evaluate the performance of three predictors of adolescent biomarker and health outcomes, we compared the AUC for childhood HAZ, adolescent HAZ and EH extent, including age and gender in each AUC. EH extent appeared to be a better predictor of adolescent WBC count in comparison to child and adolescent HAZ, though these differences were not statistically significant

(Figure 3). For predicting adolescent anemia, prediabetes/type II diabetes, prehypertension/hypertension and poor metabolic health, EH extent performance varied compared to adolescent HAZ and child HAZ.

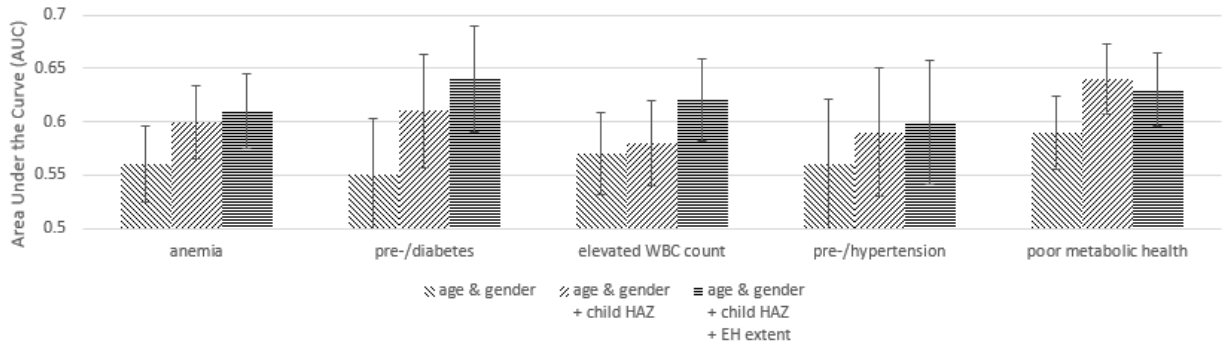
Figure 3.
AUCs of ROC curves corresponding to various subsets* of predictive factors for adolescent health outcomes*



* set 1: age, gender and childhood HAZ, set 2: age, gender and adolescent HAZ, set 3: age, gender and EH extent

Even though EH extent, on its own, performed worse than childhood HAZ for predicting three adolescent outcomes (anemia, prehypertension/hypertension and poor metabolic health), ROC analyses showed that addition of EH extent to a set that already included demographic predictors of health outcomes (age and gender) and childhood stunting improved the AUC for all outcomes except poor metabolic health, although the improvements were not statistically significant (Figure 4).

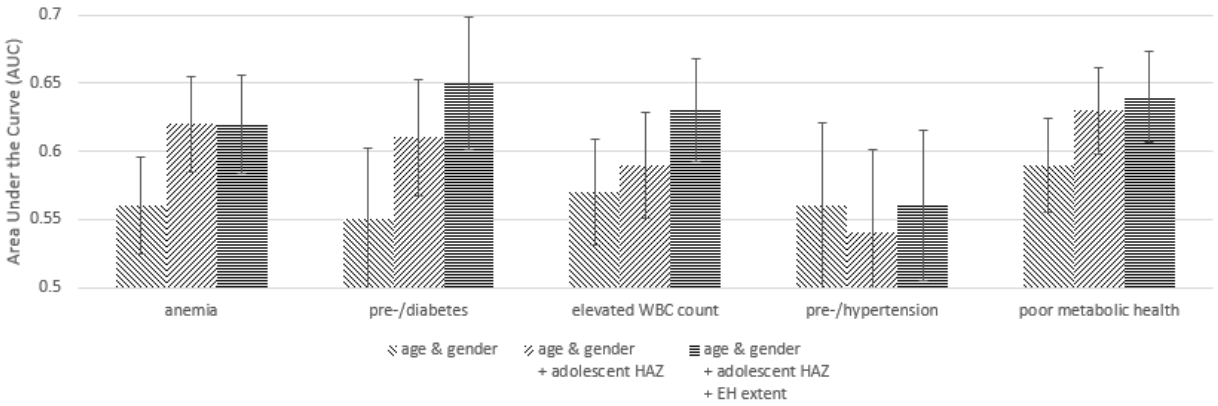
Figure 4.
AUCs of ROC curves corresponding to increasing subsets* of predictive factors for adolescent health outcomes*



* set 1: age and gender, set 2: addition of child HAZ to set 1, set 3: addition of EH extent to set 2

Similarly, addition of EH extent to a set that included demographic predictors of health outcomes (age and gender) and adolescent HAZ improved the AUC for all adolescent health outcomes except anemia. The prediction improvement with the addition of EH extent was especially strong for prediabetes/diabetes and elevated WBC count, although the improvements were not statistically significant (Figure 5).

Figure 5.
AUCs of ROC curves corresponding to increasing subsets* of predictive factors for adolescent health outcomes*



* set 1: age and gender, set 2: addition of adolescent HAZ to set 1, set 3: addition of EH extent to set 2

Discussion

Although not a strong proxy measure for chronic malnutrition, our findings suggest that EH extent may be an important measure for predicting adolescent health outcomes. EH extent may serve as a useful proxy measure of childhood experience among adolescents in settings where childhood stunting data is not available. Furthermore, EH extent may capture childhood exposures relevant to adolescent health outcomes that are not captured by childhood or adolescent HAZ and thus may be a useful clinical screening tool and/or addition to the "toolkit" of markers of chronic malnutrition for identifying high-risk adolescents.

EH in the permanent dentition and growth stunting share an etiologically-relevant exposure period and common upstream factors (e.g., poverty, diet, infant feeding habits, infection and illness) which is suggested by the high sensitivity of EH. The closer the true causal mechanism of EH is to reflecting the

chosen “gold standard” (chronic malnutrition) and the greater the severity of the exposure, the greater the proportion of true positives and therefore the sensitivity of the measure.[90] However, the particular biological mechanisms by which malnutrition-related EH is thought to occur is related to infectious illness (fever) and/or micronutrient deficiencies during early childhood which disrupt the enamel mineralization process when layers of the enamel matrix are being formed, resulting in a permanent reduction in the enamel thickness.[9,11,36] Growth stunting is a measure of chronic malnutrition and macronutrient deficiencies (protein and energy),[92] which may not be sensitive to acute episodes of infectious illness or micronutrient deficiencies. Although repeated bouts of childhood illnesses and/or persistent micronutrient deficiencies are likely associated with growth stunting, illness episodes and micronutrient deficiencies are more likely to be reflected in acute measures of nutrition status (e.g., wasting or underweight).[92] Therefore, the main limitation of using growth stunting as a gold standard by which to evaluate the validity of EH as a measure of early childhood malnutrition is that growth stunting itself is not a perfect measure of early childhood malnutrition.

Despite the shortcomings of growth stunting – measured in childhood and in adolescence -- as a gold standard measure of early childhood malnutrition, this choice of gold standard measure was strategic because growth stunting is a biomarker commonly used to infer childhood nutrition status. The present study sought to investigate whether EH may be a useful proxy measure of early childhood malnutrition for evaluating long-term health consequences of early childhood malnutrition. Overall, and by the general rule of thumb that the exposure of interest, classification as a true positive must have a greater probability than classification as a false positive (sensitivity $\geq$ (1-specificity)),[90] EH occurrence may be considered a measure of stunted growth and chronic malnutrition.

Our ROC results indicated that EH was not a strong marker for growth stunting. The high sensitivity suggested that few cases of growth stunting were not detected by EH, but the low specificity suggested an underlying etiology of EH that was not related to growth stunting. Active pathways that are unrelated to the gold standard increase the proportion of false positives, thereby decreasing specificity of a marker,[90] and EH has complex and independent causal pathways (e.g., genetics, fluoride over-exposure, primary tooth infection, etc.).

EH extent and growth stunting as predictors of adolescent health

If an EH mechanism is related to subsequent health outcomes, EH may still play a key role in the "toolkit" of biomarkers for studying childhood malnutrition, especially in the absence of childhood growth and health records. Our findings suggest that EH extent may be better predictor of certain adolescent health outcomes, particularly of prediabetes/diabetes and WBC count. For predicting other health outcomes, EH extent performed about the same as adolescent HAZ, yet when added to adolescent HAZ or childhood HAZ, the AUC improved with EH extent for nearly all outcomes, implying that EH extent captures an exposure relevant to adolescent health that is not captured by childhood or adolescent HAZ.

In settings where childhood growth and health records are not available, EH extent and adolescent HAZ combined may be useful proxies for childhood stunting and for general health screening. When childhood health records are available, EH extent may contribute additional information to childhood HAZ that is useful for predicting long-term health.

Limitations

A limitation to these analyses is the potential measurement error of EH relating to the participants not having brushed their teeth prior to the photographs (due to feasibility in field conditions) and the unique pattern of EH observed in this study sample may have prevented us from detecting stronger associations. Study findings should not be generalized to populations outside Amerindian populations living in the Amazonian Basin and in the midst of a dietary and lifestyle transition like the Tsimane'. Measurement of EH and the varying patterns it presents in, its etiology and a better understanding of the mechanisms underlying the relationship between childhood malnutrition and subsequent outcomes in these populations will be necessary before EH can be reliably used as an indicator of or screening tool for disease risk.

Chapter 7. Conclusions and future directions

This dissertation has (1) identified the need for and opportunities to improve enamel hypoplasia (EH) measurement, (2) contributed to our understanding of EH etiology through employment of a prospectively-collected dataset including multiple malnutrition-related childhood exposures in a population with high prevalence of EH, and (3) demonstrated the potential for EH to be a useful screening tool for general health risk in adolescence. Subsequent work that builds on this project will be directed toward improving measurement of EH, including further characterization of the spectrum of enamel defects observed in the human dentition, systematically investigating EH etiology across populations and further developing EH as a useful predictor of long-term health by evaluating additional health outcomes, associations in more populations and employing advanced methodology.

Enamel defect measurement

The discrepancy in our intra- and inter-examiner results were likely influenced more strongly by use of the Modified Developmental Defects of the Enamel (DDE) Index than by the digital photography method itself. The strong intra-examiner reliability may support the reproducibility of EH measurement using digital photography, and the weak inter-examiner reliability may be attributable to a vague index by which to identifying and classify EH. We draw this conclusion partially based on anecdotal evidence and observations made by the examiners involved in the study. The Modified DDE Index, last updated more than 25 years ago, in 1989, offers little description to classify types of DDEs and subtypes of EH.[34] For example, we obtained clinical consensus that the EH pattern in the Tsimane' study sample was indeed EH, yet it did not fit any of the subtype descriptions/examples included in the Modified DDE Index. The only other record of enamel defects in an Amerindian Amazonian population, like in the Tsimane', reported an extremely high prevalence of EH in the permanent in the central maxillary incisors, the lowest frequency of EH occurrence at age 4 years (compared to 1-3 years), and little difference in EH between sexes. Like the Tsimane', this other group also have a high protein-energy malnutrition and anemia prevalence, are burdened with endemic infectious and parasitic disease, have been in contact with

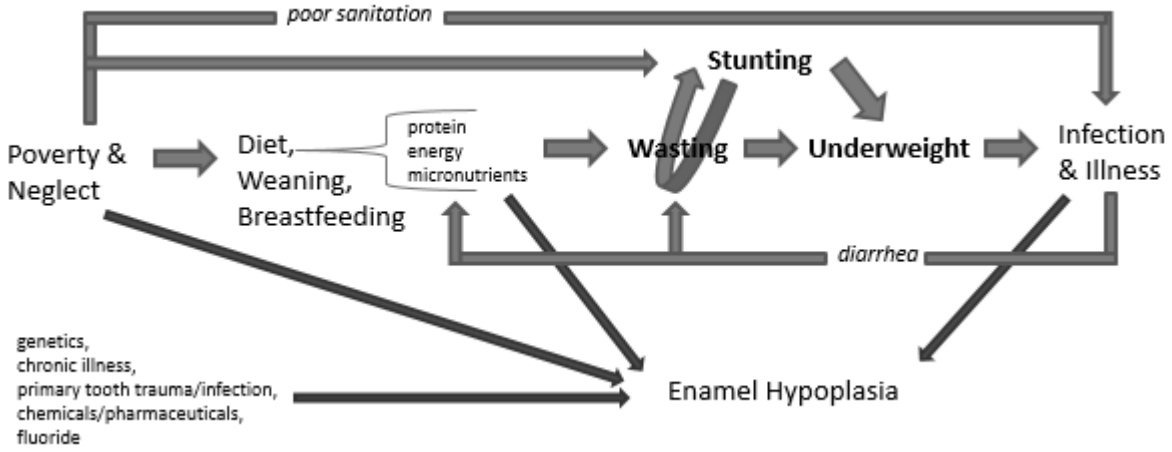
national society for nearly 100 years, and their overall health status reflects an inadequate diet, poor sanitation and a shortage of health care services.[49] It would be beneficial to know if the prevalent EH pattern in this group fits the description of the EH observed in the Tsimane', but – in accordance with the Modified DDE Index – the hypoplasia was described simply as "linear or missing enamel", a very vague description. No photos or further description was offered, making comparisons and etiological inference extremely uncertain.[49]

Digital photography and photography software developed since the last modification of the DDE Index may provide the opportunity to better characterize enamel defect patterns through high quality example photos and photo editing software abilities to help detect and differentiate between minute variations in the enamel surface. Consistency of enamel defect classification across studies could help to hone in on pathogeneses that may be unique to each particular pattern. Photo software may also be able to more precisely measure EH extent, one measure on the existing Modified DDE Index, as a proportion of a defined tooth surface, which would presumably include less error than examiners' subjective and extremely error-prone classification as <1/3, 1/3-2/3, >2/3 of the tooth surface (the current Modified DDE Index standard).

Enamel hypoplasia etiology

Despite errors in the measurement of EH, the present study provided evidence in support of a relationship between chronic malnutrition and infection in early childhood and a rough, cobblestone-like hypoplastic pattern of EH on the surface of the permanent central maxillary incisors. Given the high prevalence of EH and limited sample size, analyses may have lacked the statistical power to detect significant relationships between acute malnutrition (weight-for-age) or hemoglobin and EH. These findings are in line with the prevailing hypotheses that EH is caused by fever, diarrheal episodes, micronutrient deficiencies and other measures related to childhood malnutrition.[9,11,93] However, despite the high prevalence of EH, the particular EH pattern observed and small effect sizes detected make us suspicious that the primary cause of EH in this sample may not have been evaluated in this study (Figure 1).

Figure 1.
Conceptual framework, potential mechanisms and alternative explanations for malnutrition-EH



Additional factors that could influence EH include ingested fluoride, diet/micronutrient deficiency, genetics or epigenetic factors. Fluoride seems unlikely to be the cause, assuming the Tsimane' have essentially no exposure to topical fluoride and given what we know about natural fluoride levels in the region. According to the Pan American Health Organization (PAHO, 2000), Trinidad, a town in the Beni Department of Bolivia within which the Tsimane' Territory is also located and which is located approximately 125 miles from the heart of the Territory, has recorded natural fluoride concentrations in the local water of 1.0 ppm.[94] Severe fluorosis (an enamel defect attributable to ingestion of fluoride) like the EH observed in the permanent Tsimane' dentition at this fluoride concentration is highly improbable. On other hand, dietary causes such as a micronutrient deficiency, is a plausible causal factor of EH among the Tsimane'. One of the strongest EH studies in the human population literature evaluated an intervention in which a group of Mexican children that had received nutritional supplementation (milk/protein & vitamin A, thiamin, riboflavin, nicotinamide, ascorbic acid, vitamin D - given to mother at first missed menstrual period, during lactation, given directly to child at 4 months; sandwiches and milk until adolescence) from time *in utero* until adolescence was compared to a control group that did not receive the supplementation. They found the risk of enamel defects in the non-supplemented adolescents' teeth was nearly double that of the nutrition supplementation group, and that those who had shorter vertical stature and a greater frequency of gastrointestinal and upper-respiratory infections were

also more likely to have enamel defects.[82] The traditional Tsimane' diet consists of plantains, *yuca* (manioc/cassava), wild game, fish and rice. Although an estimated 75% of the study households' diets are still sourced from traditional hunting-horticultural activities, with increasing exposure to mainstream Bolivian society, the Tsimane' dietary transition to a diet of purchased market foods (refined sugar, candies, soft drinks, salt, oil, noodles, canned foods) is well underway.[62] A diet-related cause of EH would fall in line with the observed pattern of EH (for 69% of the study sample, > 1/3 of the tooth surface is affected), which is suggestive of an underlying and continuous exposure opposed to an acute exposure – such as a fever – that would presumably leave a more defined and limited hypoplastic defect in the tooth surface. Investigation of genetic and epigenetic influences on EH is not possible with the existing data, and so would require further data collection to study. Perhaps the most viable alternative explanation is primary tooth infection due to rampant caries, which occur on top of the forming permanent tooth enamel. This explanation would also be in line with the lack of evidence throughout the Tsimane' dentition for a systemic cause, but could still explain the high concordance of EH on both central maxillary incisors. If children with rampant primary tooth decay due to sugars/carbohydrates in their diet had lost their primary central incisors to caries by age 4 years, this may also explain why those with no EH at age 4 years had a higher average HbA_{1c} than those who had EH at age 4 years. Pilot data collected in 2015 that included a few children with primary teeth suggests rampant tooth decay in the primary dentition is common. Although systematic reviews of molar-incisor hypoplasia (MIH) and EH in the primary dentition have been published, a review of the literature that provides evidence for EH etiology in the permanent dentition has not and is a logical next step to better understanding the various causes of EH in the permanent dentition. Results of our ROC analyses revealed that EH extent may detect an underlying childhood exposure that is not detected by stunted growth, which further emphasizes the importance of identifying causal factors of EH so as to better be able to employ EH as a biomarker.

Enamel hypoplasia as a biomarker for predicting adolescent health

Our results indicated that EH extent was consistently associated with several adverse adolescent outcomes, including shorter height, lower weight, lower hemoglobin, and higher white blood cell (WBC) count. Interestingly, the occurrence of EH at an estimated 4 years of age compared to those with EH, but

who were only affected by it at 1, 2, and/or 3 years of age (but not 4 years) seemed to be associated with better adolescent outcomes overall, including taller height, higher hemoglobin, and lower WBC count. It is plausible that height, hemoglobin and WBC count are related because high WBC count indicates immune system activation and increased investment in immune function is associated with reduced growth.[95,96] Although low hemoglobin (anemia) is often attributed to dietary deficiencies in iron, B12 or folate, infection can also lower hemoglobin through iron deficiency, particularly helminth infection.[92] Given the high prevalence of helminth infection, WBC count and c-reactive protein levels among the Tsimane', it seems plausible that latter of the two causes of anemia in this population is the more important.

The EH-adolescent outcome associations we observed could be indicative of early life influences on subsequent health or of persistent exposures (common causes) or susceptibility throughout the life course. If indicative of early life influences, given the existing knowledge regarding the developmental origins of health and disease (DOHaD),[97,98] our findings suggest that mechanisms relating to the developmental origins of immune function in particular may be at work in this study sample. Early life origins of immune function are not well understood, but exposure to infectious agents in early childhood likely has long-term effects on immune development and function.[95,96] New hypotheses related to life history challenge the prior assumption that developmental impairment explains the long-term immune system consequences (upregulation) of postnatal physiological stress due to high-pathogen environments.[95] Furthermore, the immune system is often one of the first systems to fail, so WBC count may also be an early sign of subsequent health problems to come. The other explanation for the observed associations is that factors upstream to EH play a role as time-varying confounders that afflict the study sample throughout the life course and thus account for the EH-adolescent outcome relationships observed. Examples of such factors include socioeconomic status and poverty, sanitation and hygiene practices, water contamination and source, repeated and/or chronic infection and diet (particularly with regard to micronutrients). Employment of advanced methods that are able to adjust for the influence of these factors after early childhood through the time of the outcome measurement would help to draw conclusions regarding whether DOHaD mechanisms are at work or whether the continuous influence of these factors across the life course accounts for the study findings. Critical evaluation of these multiple, inter-connected factors will require advanced epidemiologic design and statistical

modeling, such as consideration of life course frameworks (accumulation of risk, chain of risk and critical periods) as well as multilevel and marginal structural models.

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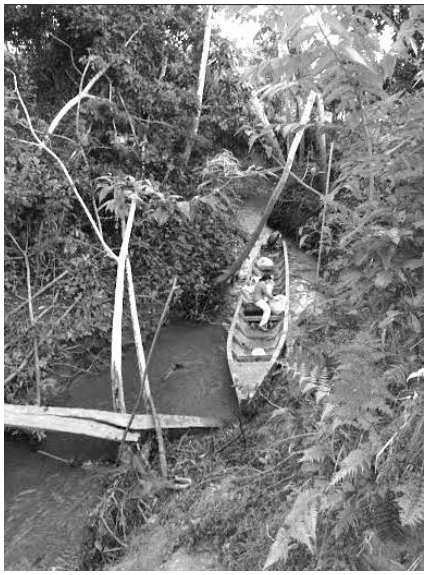
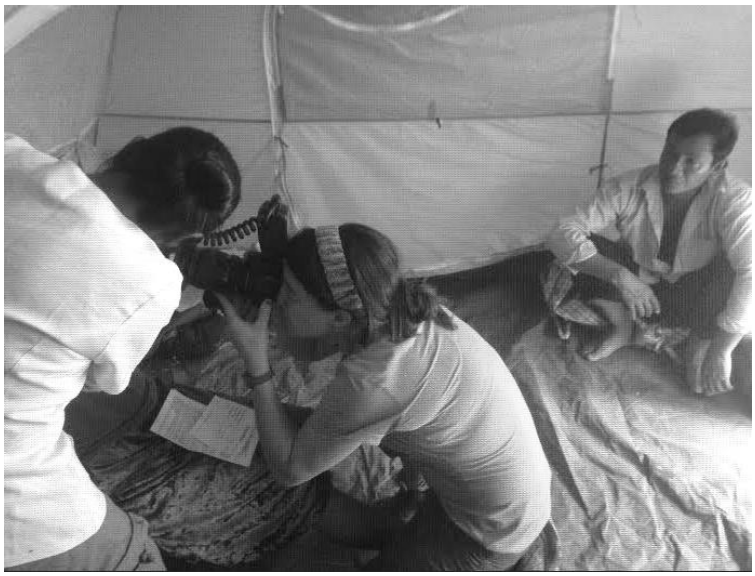
Appendices

Appendix A. Photos from the field: Tsimane' Territory, Beni Department, Bolivia





Appendix B1. Demographic characteristics of study sample and eligible individuals

Variable	Study Sample (n=349 individuals, n=1,476 0-5y child-years)		Eligible Individuals (n=655 individuals, n=2,410 0-5y child-years)	
	mean ± SD	**n (%)**	**mean ± SD**	**n (%)**
Per Child				
Gender, male	-	180 (51.6)	-	326 (49.8)
Age at first year of TAPS enrollment, in years	1.4 ± 1.4	-	1.6 ± 1.5	-
<1	-	105 (30.1)	-	190 (29.0)
1	-	114 (32.7)	-	182 (27.8)
2	-	52 (14.9)	-	95 (14.5)
3	-	35 (10.0)	-	85 (13.0)
4	-	37 (10.6)	-	86 (13.1)
5	-	6 (1.7)	-	17 (2.6)
Child-years of TAPS enrollment	4.2 ± 1.5	-	3.7 ± 1.7	-
1	-	15 (4.3)	-	78 (11.9)
2	-	44 (12.6)	-	121 (18.5)
3	-	44 (12.6)	-	94 (14.4)
4	-	66 (18.9)	-	109 (16.6)
5	-	102 (29.2)	-	144 (22.0)
6	-	78 (22.4)	-	109 (16.6)
Age in 2015, in years	13.1 ± 2.2	-	13.3 ± 2.3	-
10	-	53 (15.2)	-	94 (14.4)
11	-	47 (13.5)	-	86 (13.1)
12	-	61 (17.5)	-	91 (13.9)
13	-	44 (12.6)	-	77 (11.7)
14	-	42 (12.0)	-	85 (13.0)
15	-	45 (12.9)	-	85 (13.0)
16	-	28 (8.0)	-	69 (10.5)
17	-	29 (8.3)	-	68 (10.4)
Per Child-Year				
Gender, male	-	780 (52.8)	-	1,218 (50.5)
Age in 2015, in years	3.0 ± 1.6	-	2.9 ± 1.6	-
<1	-	105 (7.1)	-	190 (7.9)
1	-	212 (14.4)	-	348 (14.4)
2	-	256 (17.3)	-	413 (17.1)
3	-	286 (19.4)	-	452 (18.8)
4	-	309 (20.9)	-	513 (21.3)
5	-	308 (20.9)	-	494 (20.5)

Appendix B2. Childhood experiences of study sample and eligible individuals

Variable	Study Sample (n=1,476 child-years)	Eligible Individuals (n=2,410 child-years)
	mean ± SD or n (%)	mean ± SD or n (%)
Anthropometrics		
height-for-age z-score		
< 1 year	0.5 ± 4.2	0.8 ± 4.4
1	-2.2 ± 1.9	-2.2 ± 1.9
2	-2.3 ± 1.3	-2.2 ± 1.6
3	-2.2 ± 1.2	-2.2 ± 1.4
4	-2.2 ± 1.1	-2.1 ± 1.4
5	-2.0 ± 1.1	-1.9 ± 1.4
weight-for-age z-score		
1	-1.4 ± 0.8	-1.4 ± 0.9
2	-1.1 ± 0.8	-1.0 ± 1.0
3	-0.9 ± 0.7	-0.8 ± 0.8
4	-0.9 ± 0.7	-0.8 ± 0.9
5	0.8 ± 0.7	-0.7 ±1.3
Hemoglobin (Hb)	N=114	N=267
mean ± SD	10.8 ± 1.4	10.8 ± 1.4
By age (mean, SD):		
2 (n=53)	10.8 (1.0)	10.9 (1.0)
3 (n=83)	10.7 (1.5)	10.7 (1.4)
4 (n=85)	10.7 (1.6)	10.7 (1.6)
5 (n=43)	11.1 (1.2)	11.2 (1.0)
Immune activation (CRP)	N=111	N=247
mean ± SD	2.8 ± 5.0	3.1 ± 5.3
By age (mean, SD):		
2 (n=35)	2.7 (5.7)	3.1 (5.4)
3 (n=78)	2.6 (4.6)	2.9 (4.9)
4 (n=91)	2.8 (3.1)	3.4 (5.4)
5 (n=42)	3.5 (7.7)	3.0 (6.0)
Hookworm infection	N=115	N=211
yes	57 (49.6)	115 (54.5)

Appendix C. Instructions for digital dental photograph data abstraction

Procedures:

Assessments will be made on a tooth-by-tooth basis. For each of the six teeth assessed (four maxillary incisors and two mandibular cuspids), examiners will:

(1) evaluate the tooth to detect (Photoshop 'gradient map' tool) the occurrence of DDEs
(2) (a) complete the data abstraction form (instructions below)
(b) mark (using the Photoshop 'brush' tool) DDEs recorded in data abstraction form on saved photo
(3) save edited photos to OneDrive account in person subfolder
(4) estimate the timing of the DDE development (using enamel formation measurement grid)

Instructions:

- Access subsample of photos on OneDrive, link will be emailed
    - List of ID number of photos to evaluate will be sent separately
    - Use .CR2 (RAW) file formats for all evaluations

- Download Photoshop: https://creative.adobe.com/products/download/photoshop?promoid=IICUB
    - Open/load photos by selecting: File > Open …
    - "Gradient Map" can be accessed by the top dropdown menu: Image > Adjustments > Gradient Map
        - Try selecting "Copper" and "reverse, Spectrum"
    - "Brush" tool appears as a paintbrush icon midway down the left toolbar
        - Select a blue color to mark hypoplasias
        - Select a red color to mark opacities
        - Select a yellow color to mark discolorations and other types of DDEs
    - Save photos by selecting: File > Save As…
        - Save file as: "ID#.IMGxxxx.your initials" for the file name [ex: 1234.IMG_8972.WS for ID 1234, RAW Image #8972]
        - Save in a new personal folder that can be easily uploaded to OneDrive
        - Let file format stay as default "Photoshop" (.PSD, .PDD)
    - To estimate timing of defect occurrence:
        - Load brush-marked photos slide-by-slide into a MS Powerpoint file
        - Copy tooth-specific timing grids (based on Reid and Dean 2006) onto each photo and size grids to each tooth

- Electronic data collection/abstraction form may be accessed at www.kobotoolbox.org
    - Click the three stacked lines in the upper left corner
        - Select projects
        - Select "Bolivia 2015_DDE_10.2.15" or "Bolivia 2015_DDEtiming_2.2.16"
        - Click "enter data in browser" under "Add Data" on the right hand side of the screen
    - Be sure to click "Submit" at the end of each tooth evaluation/submission
    - Please follow "Definitions of Terms" below closely

Bolivia 2015_DDE_10.2.15 --

VARIABLE NAME	DESCRIPTION
Study ID	study ID (careful not to enter the image, IMG_xxxx, number)
Tooth enamel can be classified for DDEs?	The tooth must be present and the enamel visible to evaluate it for DDEs
Why can't it be classified?	Reason tooth cannot be evaluated and classified
Tooth surface	Evaluations for DDEs are made on a tooth by tooth surface, select which tooth is being

	evaluated (there will be 6 entries for each Study ID)
Any occurrence of DDEs?	Yes/no – any type of DDE detected on tooth
Type of DDE	See definitions below, be sure to study example photos
Subtypes of DDEs	See definitions below, be sure to study example photos
Number of DDEs	See definitions below
Location of DDEs	See definitions below
Extent of DDEs	See definitions below

Bolivia 2015_DDEtiming_2.2.16 --

VARIABLE NAME	**DESCRIPTION**
Study ID	study ID
1y	Yes/No for presence of defect during ~12-24m
2y	Yes/No for presence of defect during ~24-48m
3y	Yes/No for presence of defect during ~48-60m
4y	Yes/No for presence of defect during ~60-72m
5y	Yes/No for presence of defect during ~72-84m **n/a to maxillary teeth of interest*
None	No defects at any location on the tooth
Not fully erupted or severely worn	(cannot see full tooth surface to estimate timing)
Cannot evaluate (missing or decayed)	(cannot see full tooth surface to estimate timing)

Definitions of Terms *(from: DDE Index 1982 & Modified DDE Index 1989)*

1. Types of Defects (select all that apply, see training photos starting on the next page)
    a. Hypoplasia – quantitative defect of enamel visually and morphologically identified as involving the surface of the enamel (an external defect) and associated with a reduced thickness of enamel
        i. Pits – shallow or deep pits or rows of pits arranged horizontally in a linear fashion across the tooth surface or generally distributed over the whole or part of the enamel surface
        ii. Grooves – small or large, wide or narrow grooves
        iii. Missing enamel – partial or complete absence of enamel over small or considerable areas of dentine (includes severe erosion or 'chipping' of the enamel – see ex. IMG1956 below)
        iv. "cobblestone" – see ex. IMG0803 below, mild and severe forms should be included
    b. Opacity – a qualitative defect of enamel identified visually as an abnormality in the translucency of enamel; in all cases the enamel surface is smooth and the thickness of enamel is normal (except in some cases when associated with hypoplasia)
        i. Diffuse – lines, patchy or lacks well-defined margins
            1. Diffuse, fine white lines – distinct lines of opacity which follow the pattern of the perikymata, confluence of adjacent lines may be observed
            2. Diffuse, patchy – irregular, cloudy areas of opacity
        ii. Demarcated – well demarcated from the adjacent normal enamel
    c. Discolored Enamel – an obvious abnormal appearance of the enamel which because of its color and distribution cannot be considered within the normal range of variation in color and shade of tooth enamel (excluding opacities)

2. Number of DDE appearances on tooth (by type and subtype)
    a. Single – only one lesion is visible on the tooth surface
    b. Multiple – more than one defect

3. Location of DDE(s) on tooth (by type and subtype, select all that apply)
    a. Gingival half – adjacent to the gingival margin
    b. Incisal half – adjacent to the incisal tip
    c. Cuspal – tip of a cusp, specifically; should not be confused with incisal and should be used only when a defect is distinctly confined to the cusp

4. Extent of DDE on tooth (by type and subtype, select one)
    a. <1/3 – less than one third of the tooth surface area is affected by the DDE
    b. ≥ 1/3 and < 2/3 – at least 1/3 and less than 2/3 of the tooth surface area is affected by the DDE
    c. ≥ 2/3 – at least 2/3 of the tooth surface area is affected by the DDE

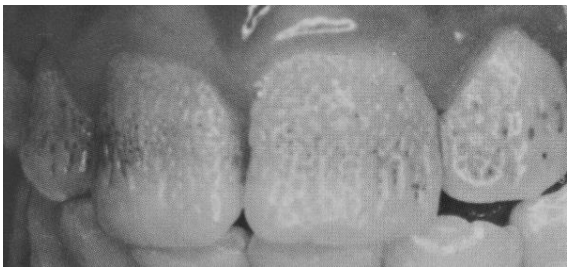
5. Estimated timing of physiological stressor, based on histologically-determined age estimates
    a. Any defect or none at: 1, 2, 3, 4, 5 years of age

Notes on Recording and Coding of Data:

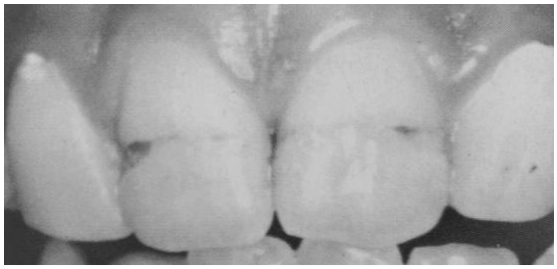
- Examiners may evaluate frontal photos only (do not use the lateral or occlusal shots for this data abstraction)
- Examiners may use Photoshop "gradient map" tool and zoom to evaluate the photo(s) and help detect DDEs
- Examiner must be familiar with the defects as visually defined in the reference color prints (see below)
    - Classification must be strictly based on DDE Index definitions and criteria above and photos below
- If the defect does not resemble any of the listed specific defects then it is coded as "other defect"
- If more than one type of defect occurs on the same surface, each type should be coded

Training photos (from 1982 DDE Index, modified to fit Bolivia 2015 study):

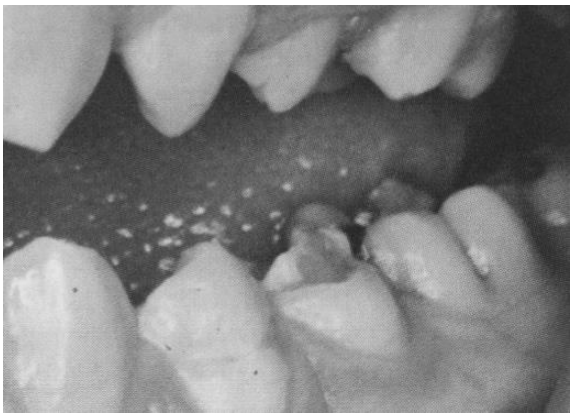
Hypoplasia, pitting –



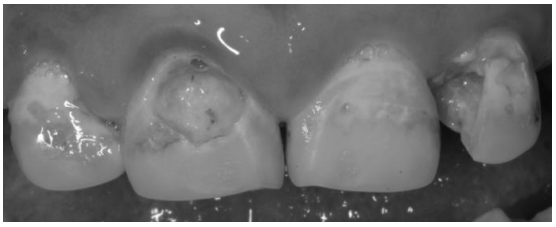
Hypoplasia, horizontal grooves –



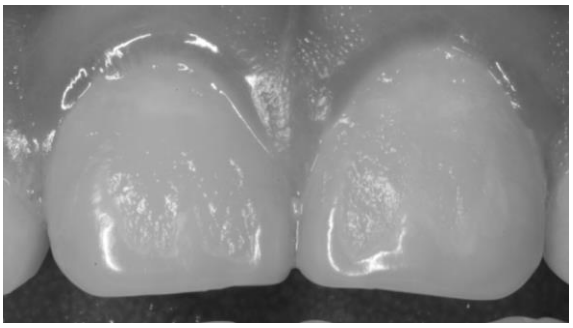
Hypoplasia, missing enamel –



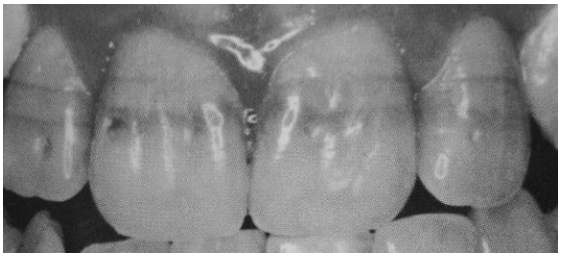
Hypoplasia, missing enamel –



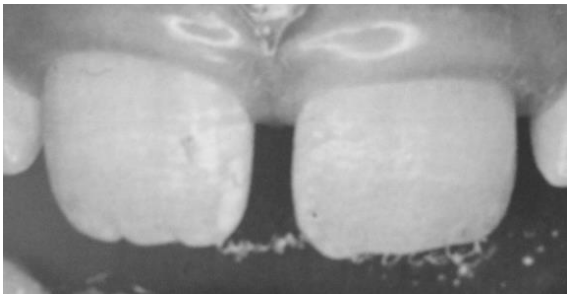
Hypoplasia, “cobblestone” pattern –



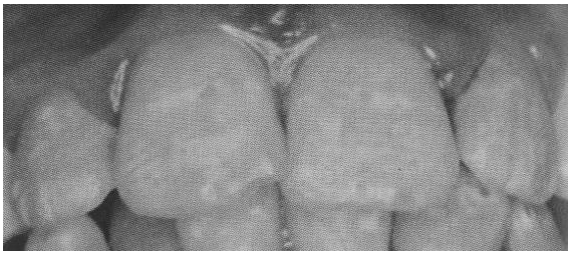
Discolored enamel –



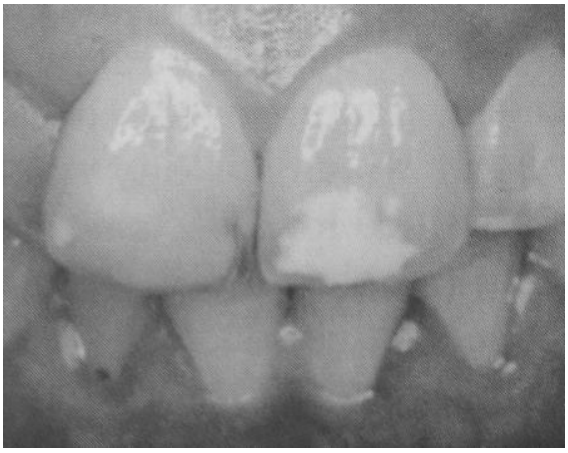
Diffuse opacities, white, lines –



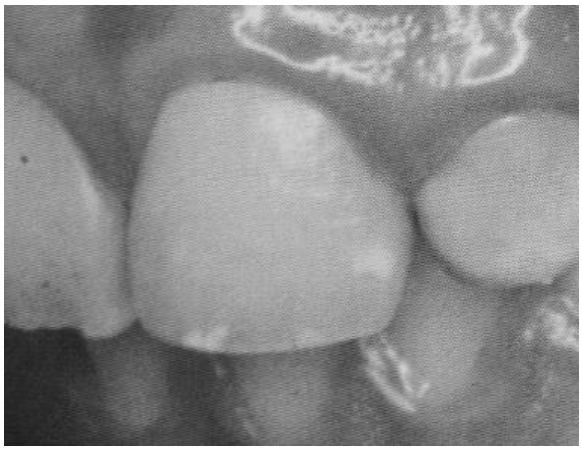
Diffuse opacity, white, patchy –



Demarcated opacity, white, single/tooth –



Demarcated opacity, white, multiple/tooth –

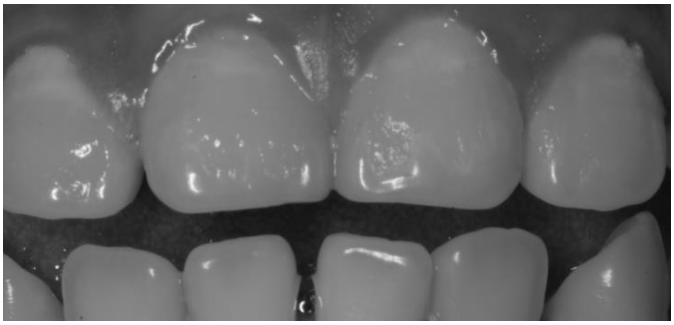


Photoshop Tools & Examples

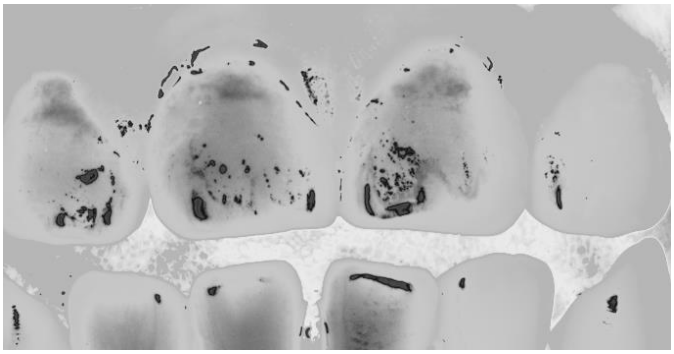
Gradient Map Tool

- note that colors in the maps correspond uniquely to the flash, opacities and the hypoplastic defects
    - For example, in the “reverse Spectrum” gradient map –
        - the cobblestone pattern is detectible in this photo on all 4 max incisors (dark blue specks)
        - patch diffuse opacities in the gingival half of the max incisors is also visible (med blue shading)

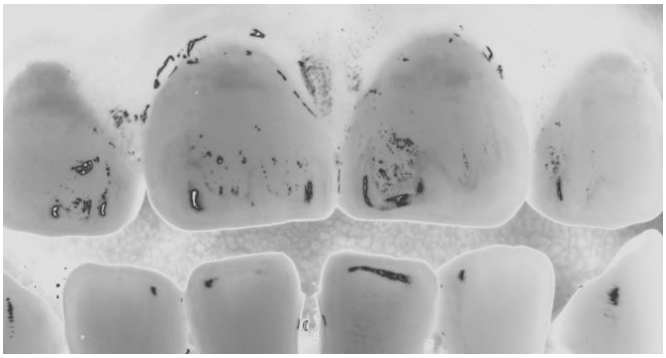
Original Photo:



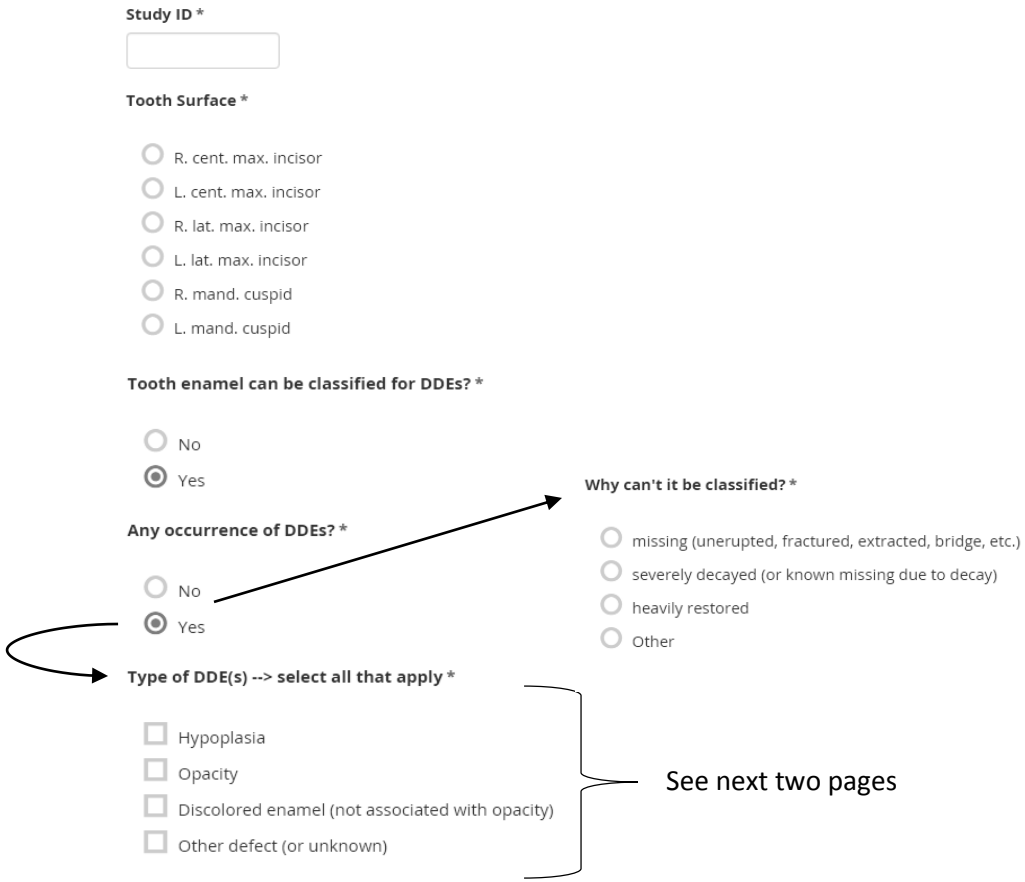
“Reverse Spectrum” Gradient Map:



“Copper” Gradient Map:



Appendix D. Data abstraction form for digital dental photographs



See next two pages

Subtype of hypoplasia --> select all that apply *

- [ ] Pits
- [ ] Grooves, horizontal
- [ ] Grooves, vertical
- [ ] Missing enamel
- [ ] "cobblestone" pattern

Pits - number of clusters of pitting on tooth *

- ( ) Single
- ( ) Many

Pits - location of pitting on tooth --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Pits - proportion of tooth affected by pitting (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Horiz. Grooves - number of grooves on tooth *

- ( ) Single
- ( ) Many

Horiz. Grooves - location of grooves on tooth --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Horiz. Grooves - proportion of tooth affected by grooves (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Vert. Grooves - number of grooves on tooth *

- ( ) Single
- ( ) Many

Vert. Grooves - location of grooves on tooth --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Vert. Grooves - proportion of tooth affected by grooves (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Missing Enamel - number of sections of missing enamel on tooth *

- ( ) Single
- ( ) Many

Missing Enamel - location of missing enamel on tooth --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Missing Enamel - proportion of tooth affected by missing enamel (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Cobblestone Pattern - number of cobblestone pattern clusters on tooth *

- ( ) Single
- ( ) Many

Cobblestone Pattern - location of cobblestone pattern on tooth --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Cobblestone Pattern - proportion of tooth affected by cobblestone pattern (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Subtype of opacity(/ies) --> select all that apply *

- [ ] Diffuse
- [ ] Demarcated

Subtype of DIFFUSE opacity(/ies) --> select all that apply *

- [ ] fine white lines
- [ ] patchy

Lines, diffuse - color of opacity(/ies) *

- ( ) white/cream
- ( ) yellow/brown

Lines, diffuse - location of opacity(ies) on the tooth --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Lines, diffuse - proportion of tooth affected by]opacity(/ies) (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Patchy, diffuse - color of opacity(/ies) *

- ( ) white/cream
- ( ) yellow/brown

Patchy, diffuse - location of opacity(ies) on tooth --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Patchy, diffuse - proportion of tooth affected by opacity(/ies) (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Demarcated opacity - color of opacity(/ies) *

- ( ) white/cream
- ( ) yellow/brown

Demarcated opacity - number of opacity(/ies) *

- ( ) single
- ( ) multiple

Demarcated opacity - location of opacity(/ies) --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Demarcated opacity - proportion of tooth affected by opacity(/ies) (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Discolored enamel - description *

Discolored enamel - location of discoloration --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Discolored enamel - proportion of tooth affected by discoloration (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Other or unknown DDE - description *

Other or unknown DDE - location of defects --> select all that apply *

- [ ] gingival half of the tooth
- [ ] cervical half of the tooth
- [ ] cuspal

Other or unknown DDE - proportion of tooth affected by defect (extent) *

- ( ) < 1/3 of the tooth
- ( ) at least 1/3, <2/3 of the tooth
- ( ) at least 2/3 of the tooth

Appendix E. DDE and Modified DDE Indices

DDE Index (1982)

Classification of Enamel Defects

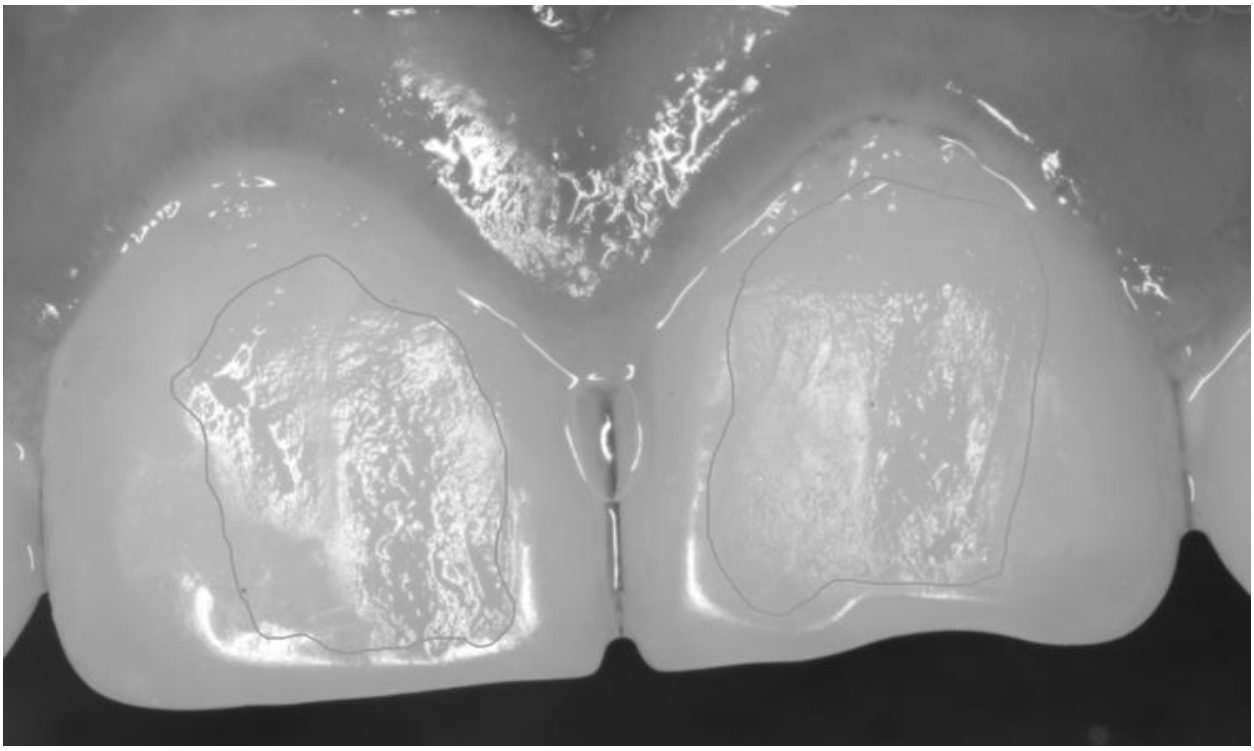
1. *Types of Defect*
   - Normal
   - Opacity (white/cream)
   - Opacity (yellow/brown)
   - Hypoplasia (pits)
   - Hypoplasia (grooves: horizontal)
   - Hypoplasia (grooves: vertical)
   - Hypoplasia (missing enamel)
   - Discoloured enamel (not associated with opacity)
   - Other defects
   - Combination of defects
2. *Number and Demarcation of Defects*
   - Single
   - Multiple
   - Diffuse, fine white lines
   - Diffuse, patchy
3. *Location of Defects*
   - Gingival one-half
   - Incisal one-half
   - Occlusal
   - Cuspal

Modified DDE Index (1989)

MODIFIED DDE INDEX FOR USE IN GENERAL PURPOSE EPIDEMIOLOGICAL STUDIES

	Code
Normal	0
Demarcated opacities:	
white/cream	1
yellow/brown	2
Diffuse opacities:	
Diffuse - Lines	3
Diffuse - Patchy	4
Diffuse - Confluent	5
Confluent/patchy + staining + loss of enamel	6
Hypoplasia:	
Pits	7
Missing Enamel	8
Any other defects	9
Extent of Defect	
Normal	0
< 1/3	1
at least 1/3 < 2/3	2
at least 2/3	3

Appendix F. Rough, cobblestone-like EH pattern in the study sample



Appendix G. Distribution of EH by gender and age (in 2015)

EH by gender:

	Male (n=175)	Female (n=162)	Overall (n=337)
Any EH	94.3%	90.1%	92.3%
Extent ǂ			
none	5.7%	9.9%	7.7%
<1/3	22.2%	25.9%	24.0%
1/3 – 2/3	47.7%	39.5%	43.8%
> 2/3	24.4%	24.7%	24.6%
Timing of EH ǂ			
EH at 4y	62.9%	61.1%	62.0%

ǂ n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)

EH by age in 2015:

	10y (n=53)	11y (n=47)	12y (n=61)	13y (n=44)	14y (n=40)	15y (n=40)	16y (n=26)	17y (n=26)	Overall (n=337)
Any EH	90.6%	93.6%	96.7%	93.2%	87.5%	87.5%	96.3%	92.3%	92.3%
EH Extent ǂ									
none	9.4%	6.4%	3.3%	6.8%	12.5%	12.5%	3.7%	7.7%	7.7%
<1/3	15.1%	31.9%	27.9%	22.7%	25.0%	20.0%	14.8%	34.6%	24.0%
1/3 – 2/3	45.3%	38.3%	54.1%	47.7%	45.0%	40.0%	48.2%	19.2%	43.8%
> 2/3	30.2%	23.4%	14.8%	22.7%	17.5%	27.5%	33.3%	38.5%	24.6%
Timing of EH ǂ									
EH at 4y	50.9%	63.8%	54.1%	63.6%	72.5%	60.0%	69.2%	76.9%	62.0%

ǂ n=336 due to 1 participant with partially erupt central maxillary incisors (only teeth with all enamel zones present are included)

Appendix H. Present vs. missing central maxillary incisors, by demographic and growth

	central maxillary incisors present (n=337)	missing central maxillary incisors (n=12)	chi square or ttest
	n (%) or mean ± SD	n (%) or mean ± SD	p-value
Gender, male	175 (52%)	4 (33%)	0.21
Age	13.0 ± 2.2	15.5 ± 1.1	**<0.0001**
Average childhood HAZ	-2.3 ± 1.3	-1.7 ± 0.7	**0.03**
Average childhood WAZ	-1.0 ± 0.7	-0.5 ± 0.7	**0.01**
Adolescent HAZ	-1.7 ± 0.9	-1.3 ± 0.5	0.15
Adolescent BAZ	0.2 ± 0.8	0.5 ± 0.7	0.20

bolded results indicate statistically significant estimates at p< 0.05

Appendix I. Early childhood exposures & EH, adjusted for household SES and diet

Early childhood experience and EH regression results (1 record/child) (N=336) (prevalence or odds ratios and 95% CIs reported)*

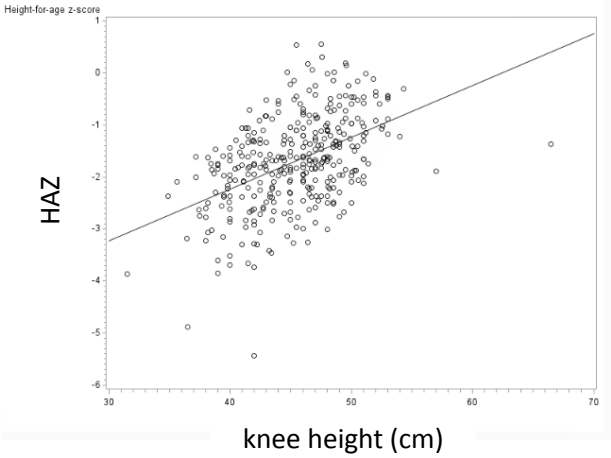
	Any EH occurrence	EH extent			
	prevalence ratio	none odds ratio	< 1/3 odds ratio	1/3-2/3 odds ratio	> 2/3 (ref.)
avg. HAZ		**1.55**	1.07	1.12	--
(adj. for SES)		**(1.05, 2.28)**	(0.84, 1.37)	(0.90, 1.40)	
(adj. for sugar)		**1.54**	1.03	1.11	--
		(1.04, 2.27)	(0.81, 1.32)	(0.89, 1.38)	
(adj. for plantain)		**1.55**	1.06	1.12	--
		(1.05, 2.30)	(0.83, 1.36)	(0.90, 1.40)	
(adj. for manioc)		**1.52**	1.05	1.12	--
		(1.03, 2.24)	(0.82, 1.34)	(0.90, 1.39)	
avg. CRP (mg/L)	1.01				--
(adj. for SES)	(1.00, 1.02)				
(adj. for sugar)	**1.01**				--
	(1.00, 1.03)				
(adj. for plantain)	**1.01**				--
	(1.00, 1.03)				
(adj. for manioc)	**1.01**				--
	(1.00, 1.03)				
hookworm		0.40	**0.27**	**0.31**	--
infection (v. not)		(0.06, 2.57)	**(0.08, 0.94)**	**(0.11, 0.86)**	
(adj. for SES)					
(adj. for sugar)		0.42	**0.28**	**0.31**	--
		(0.07, 2.78)	**(0.08, 0.95)**	**(0.11, 0.87)**	
(adj. for plantain)		0.36	**0.27**	**0.31**	--
		(0.06, 2.19)	**(0.08, 0.93)**	**(0.11, 0.87)**	
(adj. for manioc)		0.40	**0.27**	**0.31**	--
		(0.06, 2.66)	**(0.08, 0.94)**	**(0.11, 0.87)**	

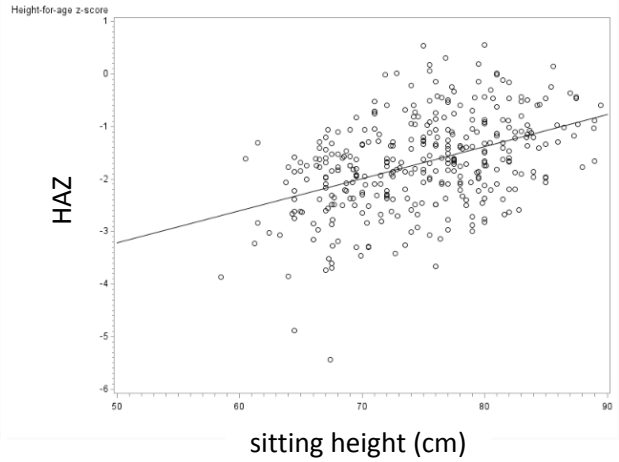
Early childhood experience and EH regression results (1 record/ child-year) (odds ratios and 95% CIs reported)*

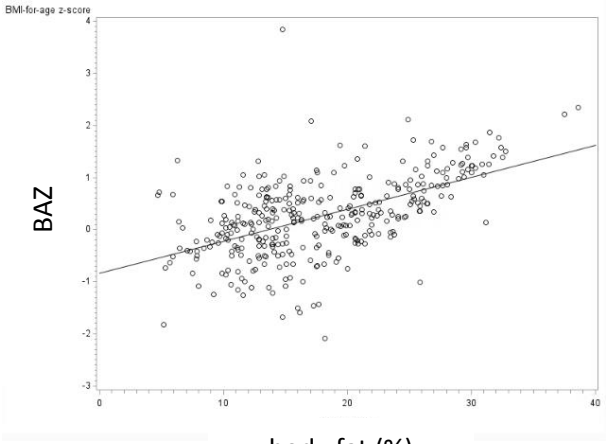
		Any EH occurrence, by year of age
	child-years (n)	prevalence ratio
HAZ		
(adj. for SES)	982	**0.98 (0.96, 1.00)**
(adj. for sugar)	982	**0.98 (0.96, 1.00)**
(adj. for plantain)	982	**0.98 (0.96, 1.00)**
(adj. for manioc)	982	**0.98 (0.96, 1.00)**
CRP (mg/L)		
(adj. for SES)	81	1.01 (0.99, 1.02)
(adj. for sugar)	81	**1.02 (1.00, 1.03)**
(adj. for plantain)	81	1.01 (1.00, 1.02)
(adj. for manioc)	81	**1.02 (1.00, 1.03)**

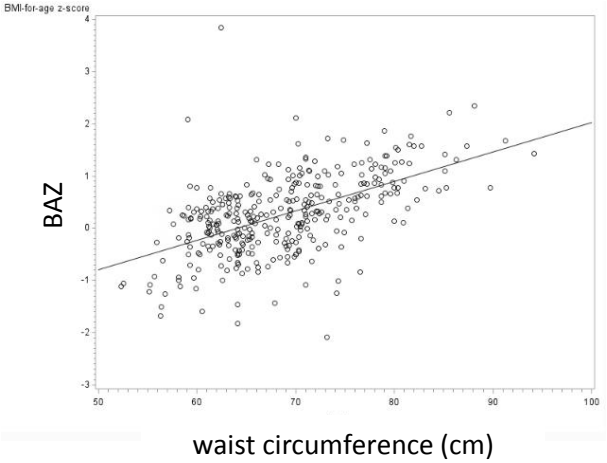
** adjusted for age at childhood measure and sex*

Appendix J. Adolescent anthropometric correlations

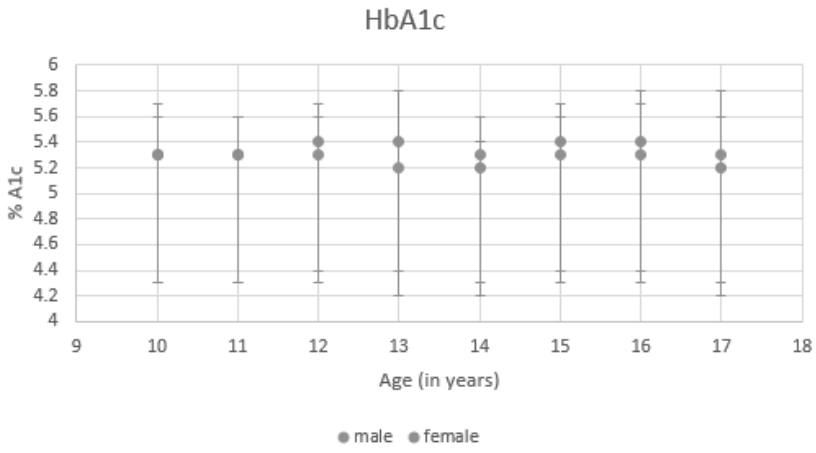


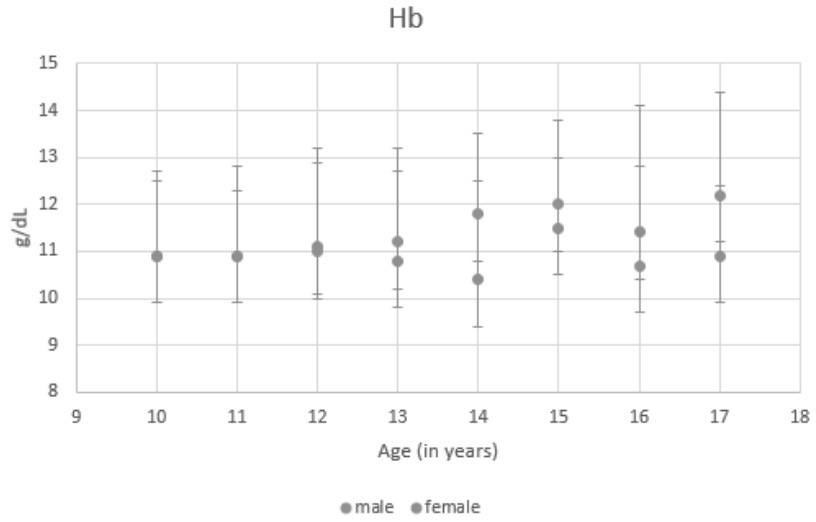


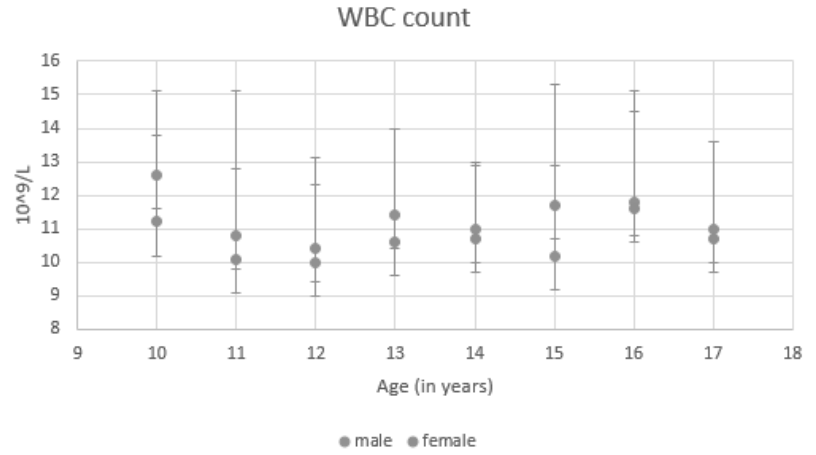


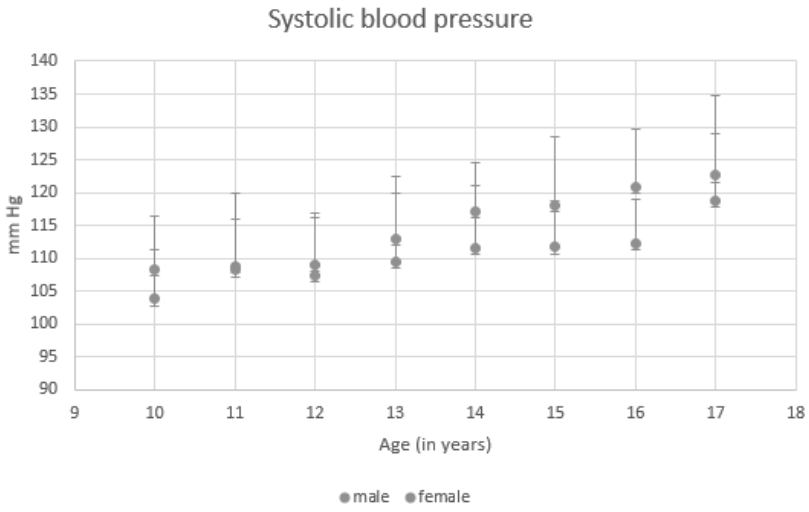


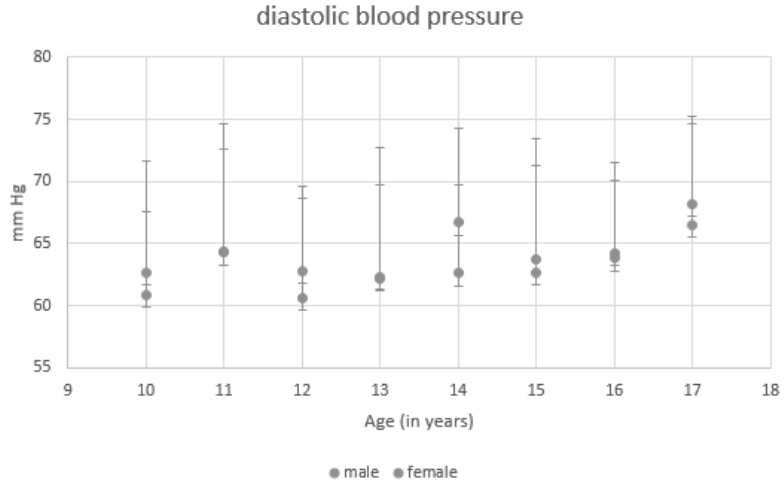
Appendix K. Adolescent outcomes by age and gender



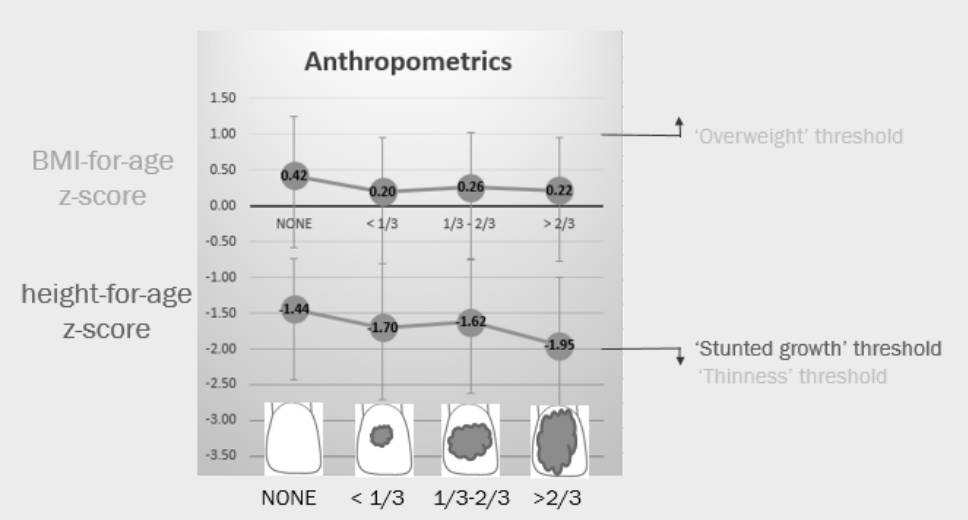


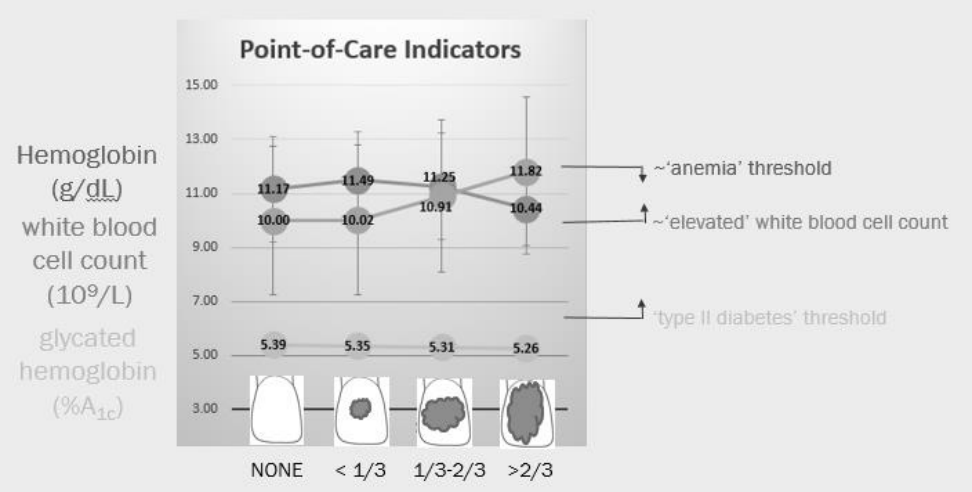


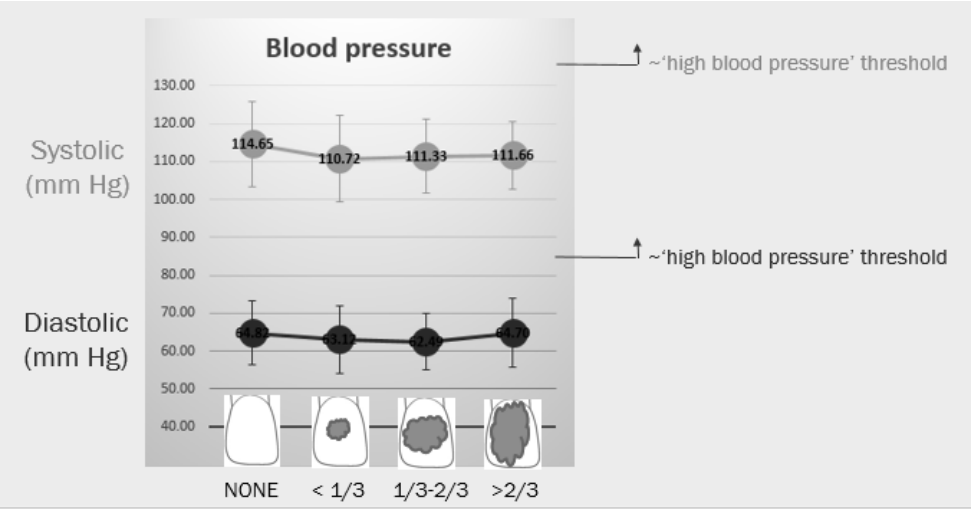

Systolic blood pressure
140
135
130
125
120
115
110
105
100
95
90
mm Hg
9
10
11
12
13
14
15
16
17
18
Age (in years)
male
female


diastolic blood pressure
80
75
70
65
60
55
mm Hg
9
10
11
12
13
14
15
16
17
18
Age (in years)
male
female

Appendix L. Adolescent outcome means by EH extent







Appendix M. Adolescent (2015) measures by estimated age at EH occurrence

Description of adolescent demographics, anthropometrics and health indicators, by EH at estimated age 4 years or not (N=337)

MEASURE	No EH (n=26)	EH, but not at age 4y (n=102)	EH at age 4y (n=209)
	% or mean ± SD	% or mean ± SD	% or mean ± SD
Gender, percent male	38.5%	53.9%	52.6%
Age, in years	13.1 ± 2.2	12.5 ± 2.0	13.2 ± 2.2
Anthropometrics			
Height (cm)	145 ± 13	141.1 ± 12.0	142.3 ± 11.6
Height-for-age z-score (HAZ)	-1.4 ± 0.7	-1.6 ± 0.9	-1.8 ± 0.9
short stature (< -2 HAZ)	19.2%	31.4%	37.3%
Weight (kg)	43 ± 12	38.2 ± 10.2	41.3 ± 10.9
BMI-for-age z-score (BAZ)	0.42 ± 0.8	0.19 ± 0.7	0.25 ± 0.8
overweight (> +1 BAZ)	30.8%	10.8%	15.8%
thin (< -2 BAZ)	3.9%	0%	0%
Health Outcomes±			
Hemoglobin (Hb), g/dL	11.2 ± 2.0	11.4 ± 1.8	11.0 ± 1.9
anemia	72.0%	74.5%	76.0%
Glycated hemoglobin (HbA_{1c}), $\%A_{1c}$	5.39 ± 0.3	5.4 ± 0.3	5.3 ± 0.3
prediabetes	21.7%	13.4%	6.5%
type II diabetes	0%	2.1%	0.5%
White blood cell (WBC) count, 10^9/L	10.0 ± 2.8	10.5 ± 3.2	11.2 ± 2.7
"elevated"	52.9%	60.5%	69.3%
Systolic blood pressure, mmHg	115 ± 11	110 ± 10	112 ± 10
Diastolic blood pressure, mmHg	65 ± 8	61 ± 8	64 ± 8
prehypertension	8.0%	5.9%	5.3%
stage 1 hypertension	8.0%	2.0%	4.3%
poor metabolic health**			
0	46.2%	72.6%	70.8%
1	42.3%	23.5%	26.3%
2	11.5%	2.9%	2.9%
3	0%	1.0%	0%

± reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

Appendix N. EH extent & adolescent outcomes

EH and adolescent outcomes, linear regression results (n=336) ǂ
(difference in means and 95% CIs reported)

	height (cm)**	HAZ *	weight (kg) **	BAZ *
EH extent				
> 2/3 (ref.)	**--**	**--**	--	--
1/3-2/3	**3.17**	**0.33**	**1.94**	0.04
	(1.29, 5.05)	**(0.09, 0.57)**	**(0.28, 3.60)**	(-0.17, 0.25)
< 1/3	**2.69**	0.26	1.54	-0.02
	(0.55, 4.83)	(-0.02, 0.53)	(-0.35, 3.42)	(-0.26, 0.21)
none	**4.87**	**0.51**	**3.97**	0.21
	(1.79, 7.94)	**(0.12, 0.90)**	**(1.26, 6.68)**	(-0.13, 0.54)
type III sum of squares	p < 0.01	p = 0.02	p = 0.02	p = 0.60

	hemoglobin (g/dL) **	glycated hemoglobin ($\%A_{1c}$) **	white blood cell count (10^9/L) **	systolic BP (mmHg) **	diastolic BP (mmHg) **
EH extent					
> 2/3 (ref.)	--	--	--	--	--
1/3-2/3	**0.84**	0.05	**-0.96**	0.38	-2.06
	(0.33, 1.34)	(-0.04, 0.14)	**(-1.81, -0.12)**	(-2.09, 2.85)	(-4.29, 0.17)
< 1/3	**1.07**	0.09	**-1.79**	-0.56	-1.48
	(0.50, 1.64)	(-0.02, 0.19)	**(-2.77, -0.80)**	(-3.38, 2.25)	(-4.01, 1.06)
none	0.76	0.12	**-1.80**	3.28	0.24
	(-0.07, 1.60)	(-0.03, 0.27)	**(-3.29, -0.31)**	(-0.82, 7.38)	(-3.46, 3.94)
type III sum of squares	p < 0.01	p = 0.28	p < 0.01	p = 0.33	p = 0.24

EH and adolescent outcomes, log-binomial regression results (n=336)ǂ (prevalence ratios and 95% CIs reported)**

	short stature (HAZ <-2)	**overweight (BAZ >+1)**	**anemic*****	**prediabetes or diabetes (%A_{1c} > 5.7)**	**elevated WBC count*****	**prehyper-tension or stage 1 hyper-tension*****	**poor metabolic health**
EH extent							
> 2/3 (ref.)	--	--	**--**	--	**--**	--	--
1/3-2/3	0.75	0.93	**0.80**	1.70	0.87	0.83	1.01
	(0.53, 1.07)	(0.49, 1.77)	**(0.70, 0.92)**	(0.64, 4.50)	(0.72, 1.05)	(0.35, 1.94)	(0.66, 1.54)
<1/3	0.86	1.00	**0.86**	1.81	0.78	0.88	1.08
	(0.58, 1.26)	(0.49, 2.05)	**(0.73, 1.00)**	(0.63, 5.14)	(0.61, 1.01)	(0.34, 2.32)	(0.67, 1.75)
none	0.46	1.99	0.83	**3.18**	0.74	1.63	**1.80**
	(0.20, 1.04)	(0.85, 4.19)	(0.64, 1.08)	**(1.01, 10.06)**	(0.46, 1.19)	(0.53, 5.02)	**(1.11, 2.92)**
type III likelihood ratio	p = 0.13	p = 0.27	p = 0.03	p = 0.29	p = 0.19	p = 0.70	p=0.13

ǂ reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

* age- and gender- standardized, by WHO reference data

** adjusted for covariates (gender and age) in adolescence

*** gender and age specific thresholds for disease status

Appendix O. EH & adolescent biomarkers, adjusted for adolescent anthropometrics

EH and adolescent health indicators linear regression results, adjusted for adolescent anthropometrics (n=336)[#] [±]
(difference in means and 95% CIs reported)

	Hemoglobin (g/dL)	Glycated Hemoglobin (%A_{1c})	White Blood Cell count (10^9/L)	Diastolic BP (mm Hg)
Adolescent anthropometrics				
Height (cm)	**0.05**	**0.01**	**-0.09**	-0.11
	(0.02, 0.08)	**(0.00, 0.01)**	**(-0.14, -0.04)**	(-0.24, 0.02)
HAZ	**0.36**	**0.05**	**-0.68**	-0.61
	(0.14, 0.59)	**(0.01, 0.09)**	**(-1.06, -0.30)**	(-1.60, 0.39)
Weight (kg)	**0.04**	**0.01**	**-0.08**	0.04
	(0.00, 0.07)	**(0.00, 0.01)**	**(-0.14, -0.02)**	(-0.11, 0.18)
BAZ	0.04	0.03	-0.23	1.10
	(-0.23, 0.31)	(-0.02, 0.07)	(-0.72, 0.27)	(-0.08, 2.27)
EH extent	**-0.36**	**-0.04**	**0.74**	0.19
(from Tables 2 & 3)	**(-0.59, -0.13)**	**(-0.08, 0.00)**	**(0.35, 1.14)**	(-0.82, 1.21)
EH extent, adj. for:				
Height (cm)	**-0.30**	-0.03	**0.63**	0.03
	(-0.53, -0.07)	(-0.07, 0.01)	**(0.24, 1.03)**	(-0.99, 1.05)
HAZ	**-0.31**	-0.03	**0.67**	0.11
	(-0.55, -0.09)	(-0.07, 0.01)	**(0.27, 1.06)**	(-0.91 1.14)
Weight (kg)	**-0.33**	-0.03	**0.69**	0.07
	(-0.56, -0.10)	(-0.07, 0.01)	**(0.29, 1.08)**	(-0.96, 1.10)
EH Timing				
(from Table 3)				
none	0.25	0.10	-1.17	0.75
	(-0.54, 1.04)	(-0.04, 0.24)	(-2.60, 0.26)	(-2.65, 4.16)
EH, but NOT at age 4	**0.50**	**0.09**	**-0.79**	**-2.66**
years	**(0.05, 0.95)**	**(0.01, 0.17)**	**(-1.58, -0.00)**	**(-4.62, -0.70)**
EH at age 4 years (ref.)	**--**	--	**--**	**--**
type III sum of squares	p = 0.09	p = 0.05	p = 0.06	p = 0.02
EH Timing, adj. for:				
Height (cm)				
none	0.10	0.08	-0.78	1.10
	(-0.69, 0.88)	(-0.06, 0.22)	(-2.20, 0.63)	(-2.33, 4.52)
EH, but NOT at age 4	0.42	0.08	-0.65	**-2.48**
years	(-0.03, 0.87)	(-0.00, 0.16)	(-1.42, 0.13)	**(-4.45, -0.51)**
EH at age 4 years (ref.)	**--**	--	**--**	**--**
type III sum of squares	p = 0.18	p = 0.12	p = 0.19	p = 0.03
HAZ				
none	0.13	0.08	-0.88	0.94
	(-0.65 0.91)	(-0.06, 0.22)	(-2.29, 0.53)	(-2.48, 4.37)
EH, but NOT at age 4	0.44	**0.08**	-0.66	**-2.56**
years	(-0.01, 0.89)	**(0.00, 0.15)**	(-1.44, 0.11)	**(-4.53, -0.60)**
EH at age 4 years (ref.)	**--**	--	**--**	**--**
type III sum of squares	p = 0.16	p = 0.10	p = 0.16	p = 0.02
Weight (kg)				
none	0.15	0.08	-0.97	0.63
	(-0.64, 0.93)	(-0.06, 0.22)	(-2.40, 0.45)	(-2.80, 4.07)

EH, but NOT at age 4 years	**0.48 (0.03, 0.93)**	**0.08 (0.00, 0.16)**	-0.74 (-1.52, 0,04)	**-2.69 (-4.66, -0.72)**
EH at age 4 years (ref.)	**--**	--	**--**	**--**
type III sum of squares	p = 0.12	p = 0.09	p = 0.10	p = 0.02

ǂ adjusted for covariates (gender and age) in adolescence

± reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

Appendix P. EH & adolescent outcomes, adjusted for early childhood exposures

EH and health indicators linear regression results, adjusted for childhood experiences (n=336) ł (difference in means and 95% CIs reported)

	Height-for-age z-score	BMI-for-age z-score	Hemoglobin (g/dL) ±	Glycated Hemoglobin (% A_{1c}) ±	White Blood Cell count (10^9/L) ±
Childhood experiences					
avg. HAZ	**0.42**	**0.18**	0.10	0.02	**-0.41**
	(0.35, 0.48)	**(0.11, 0.24)**	(-0.07, 0.26)	(-0.01, 0.05)	**(-0.71, -0.11)**
avg. WAZ	**0.54**	**0.40**	0.24	0.03	-0.35
	(0.42, 0.67)	**(0.29, 0.51)**	(-0.05, 0.54)	(-0.02, 0.09)	(-0.89, 0.18)
avg. CRP (mg/L)	0.03	0.01	-0.03	0.01	-0.02
	(-0.02, 0.08)	(-0.05, 0.06)	(-0.17, 0.11)	(-0.01, 0.04)	(-0.26, 0.21)
avg. hemoglobin (g/dL)	-0.04	0.08	0.30	0.00	-0.05
	(-0.15, 0.08)	(-0.04, 0.19)	(-0.01, 0.61)	(-0.05, 0.06)	(-0.60, 0.50)
hookworm infection	0.16	0.23	-0.15	0.06	0.44
(v. not)	(-0.16, 0.48)	(-0.07, 0.52)	(-0.85, 0.56)	(-0.04, 0.16)	(-0.86, 1.74)
EH extent	**-0.14**	-0.03	**-0.36**	**-0.04**	**0.74**
(from Tables 2 & 3)	**(-0.24, -0.03)**	(-0.12, 0.06)	**(-0.59, -0.13)**	**(-0.08, 0.00)**	**(0.35, 1.14)**
EH extent, adj. for:					
avg. HAZ	-0.07	-0.01	**-0.30**	-0.03	**0.73**
	(-0.16, -0.02)	(-0.10, 0.09)	**(-0.53, -0.06)**	(-0.07, 0.01)	**(0.32, 1.13)**
avg. WAZ	**-0.10**	-0.01	**-0.30**	-0.03	**0.72**
	(-0.20, 0.00)	(-0.10, 0.08)	**(-0.53, -0.07)**	(-0.07, 0.01)	**(0.32, 1.13)**
EH timing					
(from Tables 2 & 3)					
EH at age 4 years (ref.)	--	--	--	---	--
EH, but NOT at age 4	0.19	-0.06	**0.50**	**0.09**	**-0.79**
years	(-0.02, 0.40)	(-0.24, 0.12)	**(0.05, 0.95)**	**(0.01, 0.17)**	**(-1.58, -0.00)**
No EH	0.35	0.17	0.25	0.10	-1.17
	(-0.01, 0.72)	(-0.14, 0.48)	(-0.54, 1.04)	(-0.04, 0.24)	(-2.60, 0.26)
type III sum of squares	p = 0.06	p = 0.37	p = 0.09	p = 0.05	p = 0.06
EH timing, adj. for:					
avg. HAZ					
EH at age 4 years (ref.)	--	--	--	---	--
EH, but NOT at age 4	0.09	-0.11	**0.50**	0.08	-0.69
years	(-0.09, 0.26)	(-0.28, 0.07)	**(0.04, 0.96)**	(-0.00, 0.16)	(-1.48, 0.11)
No EH	0.06	0.03	0.16	0.07	-0.84
	(-0.25, 0.36)	(-0.27, 0.34)	(-0.64, 0.96)	(-0.08, 0.21)	(-2.29, 0.62)
type III sum of squares	p = 0.61	p = 0.45	p = 0.09	p = 0.13	p = 0.17
avg. WAZ					
EH at age 4 years (ref.)	--	--	--	---	--
EH, but NOT at age 4	0.14	-0.10	**0.49**	**0.08**	-0.73
years	(-0.05, 0.34)	(-0.27, 0.07)	**(0.03, 0.94)**	**(0.00, 0.16)**	(-1.54, 0.07)
No EH	0.26	0.11	0.20	0.08	-0.97
	(-0.08, 0.60)	(-0.19, 0.40)	(-0.60, 0.99)	(-0.07, 0.22)	(-2.44, 0.50)
type III sum of squares	p = 0.16	p = 0.35	p = 0.11	p = 0.11	p = 0.12

ł adjusted for covariates (gender and age) in adolescence

± reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

Appendix Q. 2x2 tables – childhood stunting and adolescent outcomes

		Adolescent stunting			
		+	-		
Childhood HAZ < -2.0	+	96	109	prev.| childhood stunting: 46.8%	
	-	17	116	prev.| no childhood stunting: 12.8%	PR = 3.65

		Adolescent overweight			
		+	-		
Childhood HAZ < -2.0	+	22	183	prev.| childhood stunting: 10.7%	
	-	30	103	prev.| no childhood stunting: 22.6%	PR = 0.47

		Adolescent anemia			
		+	-		
Childhood HAZ < -2.0	+	161	43	prev.| childhood stunting: 78.9%	
	-	90	42	prev.| no childhood stunting: 68.2%	PR =1.16

		Adolescent pre-/diabetes			
		+	-		
Childhood HAZ < -2.0	+	14	180	prev.| childhood stunting: 7.2%	
	-	20	107	prev.| no childhood stunting: 15.7%	PR =0.46

		Adolescent elevated WBC			
		+	-		
Childhood HAZ < -2.0	+	109	47	prev.| childhood stunting: 69.9%	
	-	60	40	prev.| no childhood stunting: 60.0%	PR =1.17

		Adolescent prehyper-/hypertension			
		+	-		
Childhood HAZ < -2.0	+	17	188	prev.| childhood stunting: 8.2%	
	-	13	119	prev.| no childhood stunting: 9.8%	PR =0.84

		Adolescent poor metabolic health			
		+	-		
Childhood HAZ < -2.0	+	47	158	prev.| childhood stunting: 22.9%	
	-	57	76	prev.| no childhood stunting: 42.9%	PR =0.53

Appendix R. Household SES and diet, EH and adolescent outcomes

Description of SES and diet (measured in childhood), by EH (n=336)

MEASURE	No EH (n=26)	Any EH (n=311)	EH Extent			Overall (n=349)
			< 1/3 (n=80)	1/3 – 2/3 (n=147)	> 2/3 (n=83)	
	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
Total wealth (SES), bolivianos (Bs)	3,957 ± 2,852	3,388 ± 2,308	3,634 ± 2,899	3,280 ± 2,144	3,327 ± 1,948	3,425 ± 2,344
Sugar (kg consumed in hh in past week)	1.5 ± 1.0	1.4 ±1.0	1.7 ± 1.0	1.4 ± 0.9	1.2 ± 1.0	1.5 ± 1.0
Plantains (kg in past week)	43.7 ± 16.4	49.8 ± 21.1	47.4 ±18.3	49.9 ± 21.1	51.9 ± 23.3	49.7 ± 22.4
Yuca (kg in past week)	8.0 ± 6.5	10.7 ± 9.1	10.1 ± 9.8	10.8 ± 9.0	11.1 ± 8.7	10.5 ± 9.1

SES and diet (measured in childhood) and adolescent anthropometrics, linear regression results (n=349)
(difference in means and 95% CIs reported)

	Height (cm)	HAZ	Weight (kg)	BAZ
Total wealth (SES), unit=100 Bs	-0.02 (-0.06, 0.01)	-0.00 (-0.01, 0.00)	-0.01 (-0.04, 0.02)	-0.00 (-0.00, 0.00)
Sugar	**0.98 (0.18, 1.78)**	0.10 (-0.00, 0.20)	**0.86 (0.16, 1.56)**	0.03 (-0.06, 0.12)
Plantains	-0.02 (-0.06, 0.01)	-0.00 (-0.01, 0.00)	-0.02 (-0.06, 0.01)	-0.00 (-0.00,0.00)
Yuca	0.00 (-0.08, 0.09)	-0.00 (-0.01, 0.01)	-0.03 (-0.11, 0.04)	-0.01 (-0.01, 0.00)

SES and diet (measured in childhood) and adolescent health indicator linear regression results (n=336)[±]
(difference in means and 95% CIs reported)

	Hemoglobin (g/dL)	Glycated Hemoglobin (%A_{1c})	White Blood Cell count (10^9/L)	Systolic BP (mmHg)	Diastolic BP (mmHg)
Total wealth (SES), unit=100 Bs	0.00 (-0.00, 0.01)	0.00 (-0.00, 0.00)	-0.01 (-0.03, 0.00)	0.03 (-0.01, 0.07)	0.03 (-0.01, 0.07)
Sugar	**0.24 (0.02, 0.45)**	**0.06 (0.02, 0.09)**	**-0.37 (-0.74, -0.00)**	0.23 (-0.80, 1.27)	-0.74 (-1.68, 0.20)
Plantains	0.00 (-0.01, 0.01)	0.00 (-0.00, 0.00)	0.01 (-0.01, 0.02)	0.00 (-0.05, 0.05)	-0.01 (-0.05, 0.04)
Yuca	-0.01 (-0.03, 0.02)	0.00 (-0.00,0.00)	0.00 (-0.04, 0.04)	0.05 (-0.06, 0.16)	**0.11 (0.01, 0.21)**

± reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

Appendix S. EH & adolescent outcomes, adjusted for household SES and diet

EH and adolescent health indicator linear regression results, adjusted for household SES and sugar access (n=336) [ł]
(difference in means and 95% CIs reported)

	Height (cm)	HAZ	Weight (kg)	BAZ	Hemoglobin (g/dL)*	Glycated Hemoglobin (%A_{1c})*	White Blood Cell count (10^9/L)*	Diastolic BP (mmHg)*
EH occurrence	-2.69	-0.29	**-2.66**	-0.19	-0.09	-0.07	0.92	-1.65
(Tables 2 & 3)	(-5.52, 0.14)	(-0.65, 0.07)	**(-5.13, -0.19)**	(-0.50, 0.11)	(-0.87, 0.69)	(-0.21, 0.07)	(-0.50, 2.33)	(-4.98, 1.78)
adjusted for	-2.43	-0.26	-2.29	-0.16	-0.03	-0.05	0.56	-1.58
SES	(-5.31, 0.46)	(-0.62, 0.11)	(-4.84, 0.25)	(-0.48, 0.15)	(-0.81, 0.76)	(-0.19, 0.09)	(-0.89, 2.02)	(-5.05, 1.88)
adjusted for	-2.18	-0.24	-2.16	-0.16	-0.02	-0.05	0.58	-1.83
sugar access	(-5.05, 0.69)	(-0.61, 0.12)	(-4.69, 0.37)	(-0.47, 0.15)	(-0.81, 0.76)	(-0.19, 0.09)	(-0.87, 2.04)	(-5.30, 1.63)
EH extent	**-1.35**	**-0.14**	**-0.98**	-0.03	**-0.36**	**-0.04**	**0.74**	0.19
(Tables 2 & 3)	**(-2.21, -0.50)**	**(-0.24, -0.03)**	**(-1.73, -0.23)**	(-0.12, 0.06)	**(-0.59, -0.13)**	**(-0.08, 0.00)**	**(0.35, 1.14)**	(-0.82, 1.21)
adjusted for	**-1.27**	**-0.12**	**-0.92**	-0.03	**-0.31**	-0.03	**0.69**	0.37
SES	**(-2.14, -0.41)**	**(-0.23, -0.01)**	**(-1.69, -0.15)**	(-0.12, 0.07)	**(-0.54, -0.07)**	(-0.07, 0.01)	**(0.28, 1.10)**	(-0.66, 1.41)
adjusted for	**-1.09**	-0.11	**-0.79**	-0.02	**-0.27**	-0.03	**0.68**	0.20
sugar access	**(-1.97, -0.22)**	(-0.22, 0.00)	**(-1.57, -0.02)**	(-0.12, 0.07)	**(-0.51, -0.04)**	(-0.07, 0.01)	**(0.26, 1.09)**	(-0.85, 1.25)
Estimated age at EH occurrence *(Tables 2 & 3)*								
EH at age 4 years (ref.)	--	--	--	--	--	--	--	--
EH, but NOT at age 4 years	**1.83** **(0.14, 3.49)**	0.19 (-0.02, 0.40)	0.74 (-0.73, 2.22)	-0.06 (-0.24, 0.12)	**0.50** **(0.05, 0.95)**	**0.09** **(0.01, 0.17)**	**-0.79** **(-1.58, -0.00)**	**-2.66** **(-4.62, -0.70)**
No EH	**3.28** **(0.41, 6.14)**	0.35 (-0.01, 0.72)	**2.90** **(0.38, 5.42)**	0.17 (-0.14, 0.48)	0.25 (-0.54, 1.04)	0.10 (-0.04, 0.24)	-1.17 (-2.60, 0.26)	0.75 (-2.65, 4.16)
type III overall stat. sig	p =0.02	p = 0.06	p = 0.07	p = 0.37	p = 0.09	p = 0.05	p = 0.06	p = 0.02
adjusted for SES								
EH, but NOT at age 4 years	**1.85** **(0.14, 3.56)**	0.21 (-0.01, 0.42)	0.71 (-0.81, 2.22)	-0.06 (-0.25, 0.13)	**0.50** **(0.04, 0.96)**	**0.09** **(0.00, 0.17)**	-0.72 (-1.52, 0.09)	**-2.77** **(-4.78, -0.77)**
No EH	**3.04** **(0.12, 5.96)**	0.33 (-0.05, 0.70)	2.53 (-0.07, 5.12)	0.14 (-0.18, 0.46)	0.19 (-0.61, 0.99)	0.08 (-0.06, 0.22)	-0.81 (-2.28, 0.67)	0.68 (-2.81, 4.17)
type III overall stat. sig	p=0.03	p=0.07	p=0.14	p=0.48	p=0.10	p=0.09	p=0.16	p=0.02

adjusted for sugar access								
EH, but NOT at age 4 years	1.34 (-0.40, 3.08)	0.16 (-0.06, 0.38)	0.29 (-1.25, 1.82)	-0.08 (-0.27, 0.11)	0.43 (-0.03, 0.90)	0.06 (-0.02, 0.14)	-0.64 (-1.46, 0.19)	**-2.38 (-4.43, -0.33)**
No EH	2.63 (-0.30, 5.55)	0.29 (-0.08, 0.66)	2.26 (-0.32, 4.83)	0.14 (-0.18, 0.45)	0.17 (-0.63, 0.96)	0.07 (-0.07, 0.21)	-0.80 (-2.28, 0.67)	1.06 (-2.44, 4.56)
type III overall stat. sig	p=0.11	p=0.16	p=0.23	p=0.43	p=0.19	p=0.28	p=0.23	p=0.04

ł adjusted for covariates (gender and age) in adolescence

* reduced sample due to point-of-care device errors: hemoglobin n=335, glycated hemoglobin n=319, white blood cell count n=256 and blood pressure n=336

Appendix T. EH occurrence and stunted childhood growth, 2x2 tables

Sensitivity and specificity at various mean HAZ thresholds during childhood

Mean childhood HAZ (1-4 years)	Growth Stunting (<-2.0 HAZ) between 10 – 17 years: Present	Absent	Cutoff Location	Sensitivity	Specificity
			←	0.00	1.00
< -3.0	52	28			
			←	0.45	0.88
-3.0	31	32			
			←	0.72	0.74
-2.5	16	57			
			←	0.85	0.50
-2.0	8	49			
			←	0.92	0.29
-1.5	7	29			
			←	0.98	0.16
-1.0	0	17			
			←	0.98	0.09
-0.5	1	9			
			←	0.99	0.05
> 0.0	1	12			
			←	1.00	0.00
TOTAL	116	233			

Childhood stunting, defined by various HAZ thresholds and EH occurrence:

		< -1.0 mean HAZ between 1-4 years	
		+	-
Any EH	+	278	33
	-	20	6

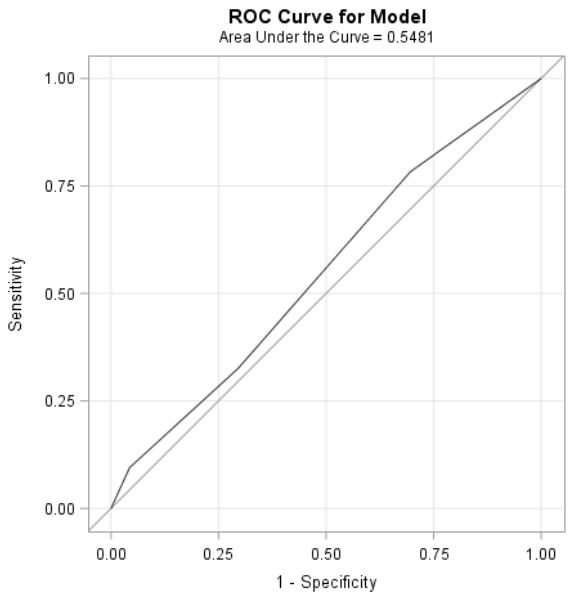
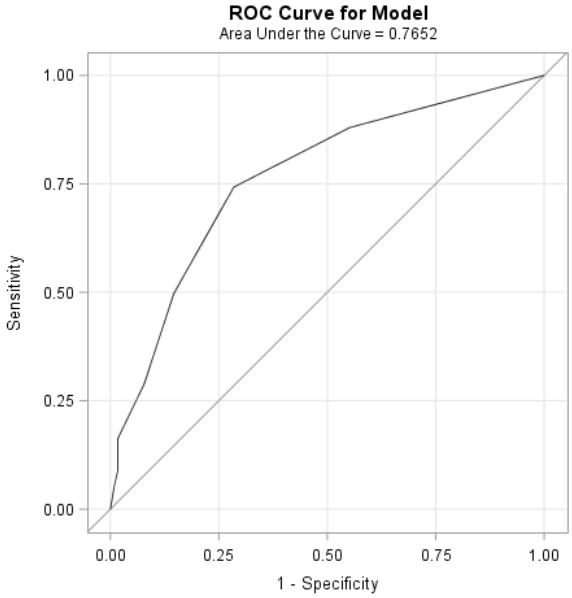
		< -2.0 mean HAZ between 1-4 years	
		+	-
Any EH	+	198	113
	-	12	14

		< -2.5 mean HAZ between 1-4 years	
		+	-
Any EH	+	131	180
	-	10	16

Appendix U. Adolescent stunting, classified by EH occurrence and by childhood growth (HAZ) thresholds

		< -2.0 HAZ at 10-17 years	
		+	-
Any EH	+	110	201
	-	5	21

ROC curve, gold standard = HAZ < -2.0 at 10-17 years of age

Test = EH Extent	Test = mean HAZ < -2.0 during childhood
	
AUC = 0.55 (0.49, 0.61)	AUC = 0.77 (0.71, 0.82)