# UNIVERSIDAD DE GRANADA

## FACULTAD DE FARMACIA

**DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR II**


# TESIS DOCTORAL

**ESTUDIO DEL ESTADO Y LAS VARIACIONES INTER- E INTRA-SUJETO EN CRECIMIENTO, HEMATOLOGÍA, HIDRATACIÓN, MARCADORES DE OXIDACIÓN, INFECCIÓN E INFLAMACIÓN EN NIÑOS PRESCOLARES CON UNA DIETA PARECIDA. Una investigación utilizando predominantemente, técnicas no-invasivas de recolección de datos biológicos.**

**STUDY ON THE NORMATIVE STATE AND INTER- AND INTRA-INDIVIDUAL VARIATION IN GROWTH, HEMATOLOGY, HYDRATION, AND MARKERS OF OXIDATION, INFECTION, AND INFLAMMATION IN PRESCHOOL CHILDREN WITH A SIMILAR DIETARY INTAKE. An investigation using predominantly, non-invasive collection techniques.**


**MARÍA JOSÉ SOTO MÉNDEZ**


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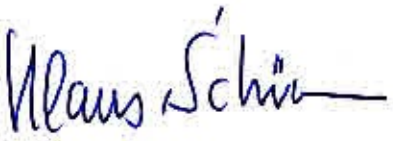

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# LIST OF PUBLICATIONS

- **Soto-Méndez MJ**, Campos-Oliva R, Aguilera CM, Solomons NW, Schümann K, Gil A. **Urinary Osmolality of Preschool Children with a Largely Common Weekday Meal Offering, from the Western Highlands of Guatemala**. *Revista Española de Nutrición Comunitaria* 2014; 20 (Supl. 1):13-19. ISSN 1135-3074.

- **Soto-Méndez MJ**, Aguilera CM, Campaña-Martín L, Martín-Laguna V, Schümann K, Solomons NW,Gil A. **Variation in Hydration Status within the Normative Range is Associated with Urinary Biomarkers of Systemic Oxidative Stress in Guatemalan Preschool Children**. *American Journal of Clinical Nutrition*. 2015 Aug 12. pii: ajcn105429. PMID: 26269363 (in press)

- **Soto-Méndez MJ**, Romero-Abal ME, Aguilera CM, Rico MC, Solomons NW, Schümann K, Gil A. **Associations among Inflammatory Biomarkers in the Circulating, Plasmatic, Salivary and Intraluminal Anatomical Compartments in Apparently Healthy Preschool Children from the Western Highlands of Guatemala**. *PLoS One*. 2015 Jun 15;10(6):e0129158. eCollection 2015.

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- **Soto-Méndez MJ**, Romero-Abal ME, Aguilera CM, Mesa MD, Rico MC, Martín-Laguna V, Campaña-Martín L, Solomons NW, Schümann K, Gil A. **Interactions of Biomarkers of Oxidation and Antioxidation Defense with those of Inflammation Status among Preschool Children of the Western Highlands of Guatemala.** (in preparation).

- **Soto-Méndez MJ**, Schümann K, Gil A, Solomons NW. **Variation of Selected Diagnostic Measurements and Biomarkers in Preschool Children: Implications for Design of Field Surveys, Comparative Studies and Intervention Trials**. (in preparation)

# LIST OF WORKS PRESENTED IN SCIENTIFIC CONVENTIONS

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2. **Soto-Méndez MJ**; Solomons NW, Schümann Klaus, Gil A. **Absence of Geographic-Setting Contrasts in Body Composition across Stunted and Low-stature Guatemalan Preschoolers Offered Common Dietary Fare.** *IUNS 20$^{TH}$ International Congress of Nutrition*; Granada (Spain), September 15-20, 2013.

3. **Soto Méndez MJ**, Aguilera CM, Schümann K, Solomons NW, Gil A. **Association of Urinary Biomarkers of Cellular Oxidation with Urine Volume and Osmolality in Guatemalan Preschoolers**. Oral Competition Award. *I International and III National Hydration Congress*; Madrid (Spain), December 2013.

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5. **Soto-Méndez MJ**, Aguilera CM, Mesa MD, Campaña L, Martín V, Solomons NW, Schümann K, Gil A. **Biomarkers of Oxidative Stress and Antioxidant Defense System among Guatemalan Preschool Children Attending Daycare Centers with a Common Menu Offering.** *Experimental Biology*, San Diego CA (USA). April 2014.

6. **Soto-Méndez MJ**, Aguilera CM, Mesa MD, Campaña L, Martín V, Solomons NW, Schümann K, Gil A. **Relationship between Oxidative Stress Biomarkers in Urine and Erythrocites and to Estimates of Fecal *Giardia intestinalis* Intensity in Guatemalan Preschool Children with a**

**Common Menu Offering**. *Experimental Biology*, San Diego CA (USA). April 2014.

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8. **Soto-Méndez MJ**, Romero-Abal ME, Aguilera CM, Rico MC, Solomons NW, Schümann K, and Gil A. **Biomarkers of Inflammation among Guatemalan Preschool Children Attending Daycare Centers with a Common Menu Offering.** *III World Congress of Public Health Nutrition*; Las Palmas de Gran Canaria (Spain), November 2014.

9. **Soto-Méndez MJ**. **Study on the Normative State, and Inter- and Intra-Individual Variation on Hydration Status Among Guatemalan Preschool Children with Similar Dietary Intake. Hydration Status throughout Different Measurement Methods, Equipment and Storage Systems**. Oral Presentation. *III World Congress of Public Health Nutrition*; Las Palmas de Gran Canaria (Spain), November 2014.

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María José

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| 8-OHdG | = | 8-hydroxydeoxyguanosine |
| Ca | = | calcium |
| CAT | = | catalase |
| F2-Iso | = | 15-isoprostane $F2_t$ |
| GSR | = | glutathione reductase |
| GPX | = | glutathione peroxidase |
| IL-1β | = | interleukin 1-β |
| IL-6 | = | interleukin 6 |
| IL-8 | = | interleukin 8 |
| IL-10 | = | interleukin 10 |
| K | = | potassium |
| Mg | = | magnesium |
| Na | = | sodium |
| O.D. | = | optical density |
| Pi | = | inorganic phosphorus |
| RBC | = | red blood cells |
| SOD | = | superoxide dismutase |
| SOSEP | = | Secretariat of the Beneficial Works of the First Lady (from Spanish) |
| TNF-α | = | tumor necrosis factor alpha |
| Uvol | = | urinary volume |
| Uosm | = | urinary osmolality |
| WBC | = | white blood cells |

# ABSTRACT

**Background**: Despite the characteristics of poverty and undernutrition in Guatemala, there are no specific studies that involve a diversity of measures, including innovative biomarkers that could better elucidate the state of nutrition and health among underprivileged preschoolers in the country.

**Objective:** To describe the distribution, and the inter- and intra- individual variance, and to analyze interactions in a selected series of 61 measures and biomarkers with the aim to characterize six aggregate components of growth, hematology, hydration, infection, oxidation and inflammation of the dissertation, in preschool Guatemalan children, sharing the same institutional-based dietary offering.

**Methods:** In this descriptive, cross-sectional study 87 children (42 girls and 45 boys), from 2-7 years old, attending three daycare centers from the SOSEP system located in semi-urban, marginal-urban and rural settings, from the Western Highlands of Guatemala; enrolled in the 8-week observation / collection period and delivered one sample of saliva, one of spot urine, three of 24-h urine, two of feces and one sample of blood, variously. The samples obtained were processed, aliquoted, stored and subsequently analyzed either in Guatemala or Spain in order to obtain results among the 61 study variables. Data of the values were entered into a SPSS version 20 database and exposed to different statistical procedures in response to the proposed hypotheses and aims outlined in each of the distinct articles, which are part of this dissertation's results section.

**Results:** This exploration generated nine articles that were divided in four sections: Section A, which presents three articles describing the surprisingly adequate and normative hydration status of the children and the interactions with urinary solutes, confirming the determinant roles of urea, sodium and potassium in the variance of hydration status as measured by urinary osmolality. Also of relevance, was the suggestion that a superior hydration status, within the normative range, significantly associates with quantitative

reductions in the urinary excretion of two markers of oxidation (F2-Iso and 8-OHdG). Section B describes the elevation, above the reference levels, of the oxidation and inflammation biomarkers in this population. It also explores their interactions through three articles, finding strong correlations across a Spearman rank-order hemi-matrix among 11 oxidation and antioxidation biomarkers measured in urine and blood (51% of the 55 possible paired-associations). Also of interest, are the 22 significant correlations among 66 paired associations (33%) found in a hemi-matrix, including 12 inflammatory biomarkers measured in the plasmatic, salivary and fecal anatomical compartments. The final article of the trilogy in this section explored correlations between all the measured oxidation and inflammation biomarkers finding 10% of the 132 cells in the full-matrix significantly correlated. Section C, about intestinal health, describes the high prevalence of inflammation in the intestinal lumen as measured by fecal calprotectin. It also confirms the high incidence of *giardiasis* (53%) in this population and the interesting and potential associations between a proxy of intensity of *Giardia intestinalis* infection and some biomarkers related to systemic oxidation. The last segment of results, Section D, presents the distribution and variance for 53 of the 61 field-study variables with a particular focus on refining critical sample-sizes that provides sufficient statistical power to meet criteria for stable assessment of prevalence. Among the 53 variables, relative standard deviation (coefficient of variance) ranged from 0.02-12.42%. Among the 33 categorical variables, the sample size needed to estimate a stable prevalence for a confidence level of 95% ranged from 91-2078. Accordingly, sample-size for comparison with a power (1-ß) of 0.8 ranged from below 10 per group up to 93,000, confirming wide variation in the different biomarkers measured. Finally, for each of the four articles in which Spearman correlation matrices played an important role in the analyses, we examined the probability that a statistical finding could have occurred by chance alone. In the hydration – oxidation, the intra-oxidation, and the intra-inflammation, the chances were severely remote with p values one in a thousand; however, in the oxidation – inflammation analysis a large number of association could have occurred by chance alone.

**Conclusions:** We can confirm that the offering of a common diet, generally adequate in all nutrient requirement, among 87 preschool children from the

Western Highlands, was no assurance of either narrow variance among biomarkers nor of full adequacy of micronutrient status. As indicated in our hypothesis wide variations occurred in diagnostic measures and biomarkers which we must ascribe to non-dietary (genetic, environmental and psychosocial) influences. Additionally, we described interesting associations between and among biomarkers and across geographic sites that are consistent with highly-interactive mechanisms in inflammatory and oxidative biology. Moreover, one does not have to be outside of the normative-range in order for the variable to impact health and nutrition as illustrated by the findings of human hydration status. This opens up in a new world of discovery in both basic human biology and applied public health.

# RESUMEN

**Antecedentes**: A pesar de las condiciones de pobreza y desnutrición en las que se encuentran los niños en Guatemala, no existen estudios específicos que involucren una diversidad de medidas, incluyendo biomarcadores innovadores, que puedan apoyar al conocimiento del estado de nutrición y salud de los prescolares de bajos recursos en el país.

**Objetivos:** Describir la distribución y la varianza inter- and intra- sujeto, además de analizar las interacciones de 61 medidas y biomarcadores, con el objeto de caracterizar seis componentes de esta tesis doctoral (crecimiento, hematología, hidratación, infección, oxidación e inflamación) en niños en edad prescolar, que comparten una oferta de dieta común de la institución a la que asisten.

**Metodología:** En el presente estudio descriptivo y de corte transversal, participaron 87 prescolares (42 niñas y 45 niños), de 2 a 7 años de edad, que asistían a tres guarderías del sistema SOSEP, localizadas en áreas semi-urbana, urbano-marginal y rural del Altiplano Occidental. Durante un periodo de ocho semana se observó la entrega del menú cíclico y los participantes entregaron una muestra de saliva, una de orina al azar, tres de orina de 24 horas, dos de heces y una de sangre, en su totalidad o en diferentes combinaciones. Las muestras que se obtuvieron fueron procesadas, separadas, almacenas y enviadas para ser analizadas en Guatemala o en España con el fin de generar las 61 variables de estudio. Los datos obtenidos fueron ingresados a una base en el programa SPSS versión 20 y sometidas a una serie de análisis estadísticos en respuesta a las hipótesis y metas propuestas en cada uno de los diferentes artículos que son parte de la sección de resultados de esta tesis doctoral.

**Resultados**: Como resultado de la exploración se generó nueve artículos originales que fueron agrupados en 4 secciones: La Sección A, que presenta tres artículos que describen el estado de hidratación, sorprendentemente adecuado, de los niños Guatemaltecos y las interacciones con los solutos urinarios; se confirmó el papel importante que tiene la urea, el sodio y el potasio en la varianza del estado de hidratación, medido a través de la osmolalidad

urinaria. De igual relevancia, fue el hallazgo que un mejor estado de hidratación, dentro de los niveles normales, está significativamente asociado con la disminución cuantitativa de la excreción de dos marcadores de oxidación (F2-Iso y 8-OHdG). La Sección B confirma los niveles elevados en los marcadores de oxidación e inflamación de los niños participantes en este estudio, en comparación con las referencias encontradas. Además, en esta sección, de tres artículos, se exploran interacciones de dichos biomarcadores encontrando, por ejemplo, fuertes correlaciones en 11 variables de oxidación y antioxidación medidas en orina y en sangre (51% de 55 asociaciones posibles fueron significativas). También de interés, fue el hecho de encontrar 22 correlaciones significativas, de las 66 posibles asociaciones de Spearman, en la matriz que incluyó 12 biomarcadores de inflamación medidos en plasma, saliva, y heces. El último capítulo de la trilogía incluida en esta sección exploró las posibles asociaciones entre todos los biomarcadores de oxidación e inflamación, encontrando 10% de 132 celdas en la matriz con correlaciones significativas. La sección C, acerca de salud intestinal, detalla la elevada prevalencia de inflamación, medida por calprotectina en heces. También muestra una alta incidencia de infección por *giardiasis* (53%) en esta población, aunado a las interesantes correlaciones entre un posible marcador de la intensidad de infección por *Giardia intestinalis* y algunos biomarcadores relacionados a la oxidación sistémica. La última parte de resultados, la Sección D, presenta la distribución y varianza de 53 de las 61 variables de estudio con un enfoque especial en el ajuste de tamaños de muestra que proporciona el poder estadístico suficiente para cumplir con los criterios de evaluación de prevalencia. La desviación estándar relativa (coeficiente de variación) varió 0,02 a 12,42% entre las 53 variables. En las 33 variables categóricas, el tamaño de muestra necesario para estimar una prevalencia estable para un nivel de confianza del 95% varió desde 91 hasta 2,078. En consecuencia, el tamaño de muestra para comparación con una poder (1-ß) de 0.8 osciló entre menos de 10 por grupo de hasta 93.000; esto que confirma una amplia variación en los diferentes biomarcadores medidos. Por último, se evaluó la probabilidad de que un hallazgo estadístico podría haber ocurrido por casualidad, para cada uno de los cuatro artículos en los que las matrices de correlación de Spearman jugaron un papel importante. En los capítulos sobre

hidratación - oxidación, intra-oxidación, e intra-inflamación, las posibilidades fueron severamente remotas con p valores de uno de cada mil; sin embargo, en el análisis oxidación – inflamación, un gran número de asociaciones podría haber ocurrido por casualidad.

**Conclusiones:** Se confirma que la oferta de una dieta común, por lo general adecuada en los requerimientos de nutrientes, a 87 niños en edad prescolar del Altiplano Occidental de Guatemala, no fue garantía de menor varianza en los datos de biomarcadores, ni de un adecuado estado de micronutrientes. Como fue propuesto en la hipótesis, se encontró amplia variación en las medidas de diagnóstico y los biomarcadores que es atribuible a influencias no dietéticas (genéticas, ambientales y psicosociales). Además, se describió asociaciones interesantes inter- e intra-biomarcador y a través de los tres sitios de estudio que son consistentes con mecanismos altamente interactivos en la biología inflamatoria y oxidativa. Por otra parte, no es necesario que los marcadores estén fuera del rango adecuado para afecten la salud y la nutrición, como lo confirman los resultados del estado de hidratación humana. Los resultados abren un nuevo mundo de descubrimientos, tanto en la biología humana básica y la salud pública aplicada.

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# I. INTRODUCTION

Sheny Romero, *Descansando en la milpa*, Quetzaltenango, 2012

## Social Setting

The beautiful "*country of eternal springtime*" Guatemala, is located in the center of the Americas, with a total area of 108,889 $km^2$ and an estimated population of 15,806,675 by 2014 (1-3); its name comes from the Náhuatl language expression "Quauhtlemallan" that means "*land in the forest*". Guatemala was recognized as a nation on September 15,1821; but initiated its democratic history as late as 1985 (4). The official language is Spanish, although it is used a second language by the population that communicates in 21 Mayan languages, along with Xinca and Garifuna (Afro-Caribe) (5).

The nation presents outstanding contrasts in cultures, traditions and languages that make the country an interesting place to visit for foreigners. Its economic side, also present contrasts. Being the biggest economy in Central America, Guatemalan levels of inequality ranked amongst the highest in Latin America (6), hosting the highest levels of poverty and the majority of the Mayan population in the rural areas (7).

Guatemalan indexes of maternal and infant mortality are the highest in the Central American region; raking 65 and 77 in the world, respectively (8). Finally, according to the WHO growth standards of 2006 (9), Guatemala has a stunting rate of 54% in children under 5 years of age, which is the highest in America. Nutritional anemia affects 48% of children under five, 21% of non-pregnant in the reproductive age, and 29% of pregnant women in reproductive age (10,11).

A predominant concern in Guatemala is the low coverage of central water supply and sanitation services. UNICEF (12,13) reported 74% coverage of safe water supply (shared or single water tap) in the country. National sanitation coverage was 45% in 2002 (Urban 77% and rural 17%) with no expectation of improvement in the foreseeable future.

## Scientific Setting

Until the sixties, only one institution was in charge of the higher education in the country, the Universidad de San Carlos de Guatemala. Today it is still the only public university in the nation. Founded in 1676, Universidad de San Carlos de Guatemala was the third of its kind instituted in Latin America (14). The Academy of

Medical, Physical and Natural Sciences, established in 1945, gave the initial impetus to national science. However, the scientific advances from the academies and national universities became stalled due to the rise of *de facto* governments with repressive policies, which did not conclude until 1985. New universities were founded in the sixties, all of them being private institutions, either religious or secular.

Activities to organize and promote science and technology in Guatemala started, in a systematic manner as of 1990 with the national law initiative for the promotion of the scientific and technological development. This law was approved by the Guatemalan Congress and published in the official newspaper "*El Diario de Centroamérica*" in 1991. The entity in charge of the promotion and coordination of the scientific activities in Guatemala is The National Council for Science and Technology (CONCYT) (15).

Nutrition and food technology have a history of 66 years in Guatemala (16). In 1949, Dr. Nevin Scrimshaw, came to Guatemala and established the Institute of Nutrition of Central America and Panama (INCAP) with the sponsoring of the Pan-American Health Organization. Since the moment of its inception, INCAP started to train professionals in human nutrition, food science and technology and was recognized for its international collaborations. Currently, INCAP has a series of objectives to be accomplished by 2032, including the Food and Nutritional Security Policy for Central America and the Dominican Republic; along with the nutrition knowledge management in the region (17).

In 1985, one of Dr. Scrimshaw's disciples, Dr. Noel Solomons, finished his education at INCAP and founded the Center for Studies of Sensory Impairment, Aging and Metabolism –CeSSIAM– accompanied by Dr. Fernando Beltranena, Gustavo Hernández Polanco and Dr. Oscar Pineda. The center was first established as the academic stream of the Guatemalan Blind and Deaf Committee located in the Rodolfo Robles Hospital (18). The institution expanded the research scheme of the existent institutions by combining the traditional interest for human nutrition and focused it on the Vitamin A problem and its relation to nutritional blindness (19,20).

Some of the institution's initial studies included preschool children (21-24), and β-carotenes (25-26), along with anthropometry and hematology (27-28). CeSSIAM expanded its interest in nutrition research, which earlier had been focused in rural areas, to include urban areas (20,29,30) and expanded its interest from "undernutrition" to research on "overnutrition" and its implications on non-communicable chronic diseases (31-32). Supported by the Hildegard Grunow Foundation of Munich, Germany, CeSSIAM has been exploring non-invasive methods of analysis in children comparing them with the invasive "gold standards" Examples are non-invasive hemoglobin assessment and detection of vitamin D in saliva and test strips to diagnose giardiasis (33-34).

**The Nature of the SOSEP Community Homes and 40-day Rotating Menu**

Different governments have made huge efforts in order to improve the quality of life in Guatemala. Among them is the effort by the Secretariat of the Social Works of the First Lady (*Secretaria de Obras Sociales de la Esposa del Presidente –SOSEP-*). This government office provides assistance to low-income population, such as the "Community Homes" (*Hogares Comunitarios*), which are community-based daycare centers for preschoolers assisted by mothers from the community (1 per 10 children). The system offers care, education (1 teacher per 20 attendees), and a universal menu to properly feed children in the "Community Homes", that will be called "daycare centers" below (35).

In a previous research (36), CeSSIAM investigators determined the nutritional contribution of foods and beverages provided by SOSEP daycare centers in their 40-day rotating menu with a daily four meal offering from Monday to Friday. The menus supplied most of the nutrients needed in sufficient quantities to cover the preschoolers' daily requirements, even without considering additional consumption of foods and beverages at home. The only exception was vitamin D. The menu offering and its nutritional supply to preschoolers are given in **Annexes 1 and 2**. Dietary and nutrient intake is traditionally considered the variable of interest in human nutritional research. Therefore, a system in which dietary offerings are highly uniform provides the opportunity to explore the variation of other (non-nutrient) variables.

SOSEP gave us the opportunity to analyze these “additional” health and nutritional markers, in a situation of fairly homogenous nutrient and food exposures in their attendees.

## Health and Nutritional Variables in Preschool Children

### *General Concepts of Invasive and Non-invasive Diagnostic Procedures*

The term “invasive” applies to diagnostic procedures that cause pain or expose a subject to risks, stress, radiation, immobilization, prolonged fasting, on suffering, etc. One of its simplest definition is "a procedure that penetrates or breaks the skin", for example by inserting a needle (37). Applied research in children should try to reduce its invasive characteristics to the minimum by testing and validating different non-invasive techniques to diagnose or to describe the normative states of the population.

Non-invasive sampling techniques are increasingly used to monitor a series of biomarkers in research and clinical practice. This type of methodology needs to be further validated. Non-invasive techniques are already in use to obtain data at a population level and to permit easy-to-handle research in humans. It also addresses ethical concerns, mainly in vulnerable populations such as children or elderly (38).

Because it was not possible to obtain all measurements through non-invasive sampling and to compare non-invasive to traditional invasive techniques, a small amount of blood was collected. Both, its plasma fraction and the red-cell sediment were used as obtained by micro-sampling techniques in order to take the maximum advantage of the small sample. The emphasis was on information collected from physical measurement, skin contact and analytes in feces, saliva and urine. As reflected by the title of this thesis the domains of interest were: growth; hematology; hydration; oxidation; inflammation; and infection. Overall, we measured 61 discreet variables (**Table 2**) as arranged in relation to the domains of the dissertation titles.

### *Non-invasive Markers of Hydration Status*

To follow the organization of the result section, we begin with the non-invasive evaluation of the hydration status. Quantitatively, water is the most

abundant and vital of all nutrients (39). Mild dehydration is defined as 1-2% weight loss, secondary to loss of fluids (40). Despite of its significance, there is no worldwide consensus regarding adequate water intake. One of the reasons for this is that water requirements depend on several factors including renal solute load, physical activity, climate, etc. There is no generally accepted laboratory method to assess individual hydration status either (41).

A series of techniques has been used in order to assess hydration status, e.g. isotope dilution, neutron activation analysis, body mass change, bioelectrical impedance analysis, plasma osmolality, salivary flow rate or osmolality, urine color, urine specific gravity and urine osmolality among others (41,42). An advantage of urine over the other biological fluids mentioned is the non-invasive, painless nature of its collection. Urine is hygienically and abundantly available and can be collected either as spot samples or quantitatively over a defined period of time e.g. 24-hour. This argues in favor of urine, especially of its osmolality, as the most promising marker to assess hydration status (42).

To use a normative non-pathological range of the hydration status has turned out as an elusive proposition. As part of a longitudinal study in children from Dortmund, Germany, the DONALD study by Dr. Friedrich Manz and co-workers (39) proposes an operational concept of normative hydration status: “in a subject, minimum and maximum urine osmolality define the range of euhydration”. Acute changes in the hydration status are commonly designated as dehydration or rehydration. Differences in the “continuous” hydration status are called “hypohydration”, “euhydration” or “hyperhydration”.

Renal solute excretion determines the urine volume. Urea excretion has been described to contribute about 40% and sodium chloride about 35–44% of total renal solute excretion (39,43). We attempted to confirm the role of urinary solutes for the hydration status measured in urine, and to determine its influence on urine osmolality in situations where children had similar physical activity routines and common dietary offering.

**Section A** of this dissertation presents our exploration regarding the hydration status markers of urine volume and urinary osmolality (**Chapter 1**). It also

presents the mutual interactions of urine volume and osmolality with two organic compounds (urea and uric acid), four cations (sodium, potassium, calcium and magnesium) and phosphorus as an inorganic anionic compound (**Chapter 2**). As an innovative approach, interactions of hydration status markers with markers of oxidative stress, measured in the same body fluid, (15-isoprostane-$F2_t$ [F2-Iso] and 8-hydroxydeoxyguanosine [8-OHdG] were analyzed (**Chapter 3**).

*Urinary Oxidative Stress Biomarkers and Blood Antioxidant Defense Enzymes and Nutrients*

In aerobic organisms, oxygen is vitally needed, but it also can have toxic effects. Every organism is exposed to oxidative stress disturbing the prooxidant-antioxidant balance (44). As early as 1954, it was proposed that free radicals as well as other reactive oxygen species stealing electrons from healthy cells were responsible for the adverse oxygen effects (45). Oxidation may be caused by several factors, and was related to, among others, cancer, diabetes, cardiovascular disease and neurodegenerative conditions (46).

Free radicals are able to damage cellular and organelle membranes, cell nucleus and protein chains. For example, their action on lipids form a series of prostaglandin-like compounds named isoprostanes (47) that are easily detected and measured in urine or plasma. A biomarker of the oxidative damage to DNA with increasing popularity is 8-OHdG. It was discovered at the end of the 80s by probing its increase in serum and urine in subjects with chronic diseases associated to oxidative stress (48).

**Chapter 3** of this dissertation describes interesting and unexpected findings about the relationship between the steady-state of hydration and the production of these biomarkers of oxidative damage.

Different enzymes protect against oxidative components and decrease their intercellular concentrations (46-49). Thus, superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSR) and glutathione peroxidase (GPX) are the primary intercellular antioxidant enzymes to detoxify the free-radicals produced during normal cellular aerobic respiration (46).

In addition, a series of dietary nutrients have properties to reduce free-radical damage, or work in conjunction with enzymatic antioxidation reaction (50). Retinol, β-carotene, tocopherols and co-enzymes, among others, have antioxidant activity. One of the goals of this dissertation was to explore the mutual interplay and interactions of biomarkers of oxidative stress and antioxidant defense system; corresponding findings are presented in **Chapter 4**.

*Plasmatic, Salivary and Fecal Inflammation Biomarkers*

In present times, acute inflammation can still be recognized with the same characteristics (heat, pain, redness and swelling) that were described by Celsus in the 1$^{st}$ century. However, not all immune responses do present with these classical clinical signs and symptoms. In fact, the immune system is complex, but in its complexity it is a well-organized network that can respond to millions of antigens and protects the body from injurious agents (51). Immune responses are innate or adaptive, and highly integrated by hormonal signaling or cell-to-cell cross-talk (52).

Fernández-Botrán (53), described cytokines as “a group of hormone-like regulatory molecules”, which are secreted by macrophages, T lymphocytes, and other cells. A number of cytokines is produced by white blood cells and constitute a series of “interleukins”, many of them acting as biomarkers of inflammation. Differentiation of helper cells derived from the thymus produce subclasses of cytokines, supporting up-regulating, pro-inflammatory cytokines (Th1) and counter-reacting, down-regulating anti-inflammatory cytokines (Th2). (52). The Th1-directed acute-phase response leads a catabolic intermediary metabolism, tending toward poor tissue growth and wasting of nutrients (54).

Intraluminal intestinal inflammation can be diagnosed and quantified by fecal calprotectin, a protein derived from neutrophils and tissue macrophages in the gut (55). It is involved in antibacterial action and cellular chemotactic activities (56). Fecal calprotectin is recognized as an accurate clinical diagnostic, non-invasive biomarker of the intestinal tract inflammatory status. Different levels of fecal calprotectin in free-living children are documented. Results are comparable between healthy children from developed and developing countries (57-59).

**Chapter 5** presents findings and conclusions of our exploration on white blood cells, calprotectin, and a series of five selected cytokines in plasma and saliva (IL-1β, IL-6, IL-8, IL-10 and TNF-α), and on possible interaction among the biomarkers from different anatomical compartments. **Chapter 6** describes the relationships between inflammation and oxidation biomarkers.

As mentioned above, Guatemala has the highest prevalence of under-five stunting in Latin America (60). In the context of the complexity of immune response, linear growth impairment is associated with immunological alterations such as impaired gut-barrier function, reduced delayed-type hypersensitivity responses (61), and cytokine-induced or infection-induced malnutrition (62). **Chapter 5** also presents the outcomes of the anthropometric measurement of weight and height and their analysis according to the WHO growth standards (9).

Researchers have related poor linear growth to environmental factors. According to FAO, the concept of food and nutritional security was first described in 1970. One of its pillars is the biological utilization, or the "good absorption of the nutrients intake in food or beverages consumption" that depends on health status,, environmental exposure and genetic factors (63). Environmental enteropathy is a subclinical condition found in environments with poor sanitation and hygiene (64). In 1993, Solomons *et al*. (65) proposed an explanation for poor linear growth based on the theory of environmental contamination and poor utilization of nutrient, known as the "dirty chicken" theory.

Because of its predominantly non-invasive collection techniques, this dissertation allowed the collection of fecal samples to determine the level of parasitic infestation, *Giardia intestinalis* infection, and intestinal inflammation as determined by calprotectin. **Section C** presents two articles that refer to intestinal health of SOSEP preschoolers. The exploration of the ratio between asymptomatic *Giardia* infection as measured by optical density and oxidative stress (which are two different approaches) led to the unanticipated and interesting findings described in **Chapter 7**. In addition, **Chapter 8** describes fecal calprotectin levels in our study subjects and compares our findings with studies of children in other countries.

*Variation of Health Measures and Biomarkers.*

Variation can have multiple connotations. One is categorical or classificatory, and relates to a specific status, established against a diagnostic standard. The other is numerical (quantitative and continuous) and related to the width or limits of absolute values across a continuous spectrum.

An example for these two connotations is provided by body mass index. Values can range from as low as 14 $kg/m^2$ to above 50 $kg/m^2$. In terms of classification, a value in an adult below 18.5 $kg/m^2$ signifies "underweight"; a value between 18.5 and 24.9 $kg/m^2$ is considered to be "normal". A value above 25.0 $kg/m^2$ is considered to represent "excess weight" (overweight and obesity). One could have 99 individuals with BMIs ranging from 15 to 30 $kg/m^2$, with 33 in the underweight range, 33 in the normal range, and 33 in the excessive range (i.e. large categorical variance), but the width of the limits is only 15 units (i.e. low numerical variance). On the other hand, one could have another 99 individuals, all of them starting at least at 25.0 $kg/m^2$ and ranging up to 55 units. This is, however, an amplitude of 30 units, twice as wide as the first instance (i.e. wide numerical variance); but in terms of classification, all of the subjects have "excessive weight" (i.e. uniform classification).

In the present study, we were interested in the diversity of the measured variables in both contexts of variation: 1) diversity of classification and 2) amplitude of numerical values. To the extent that variables in our study have established normative limits for preschool children, both connotations of variance can be applied. In other instances, only the numerical limits will be applied. When designing prospective field studies with adequate statistical power to describe prevalence or to test hypotheses; inherent issues in the inter- and intra-individual variation of the variables of interest come into consideration [66, 67]. **Chapter 9** focuses on the variation among the 53 variables measured for this dissertation. Only a few variables, including anthropometrical and hematological ones, have been measured and reported in Guatemalan preschoolers in previous reports.

This dissertation in trying to emphasize on using as many non-invasive collection procedures for diagnostic variables as possible in different anatomical

compartments such as saliva, feces and urine and using only one single 4 ml blood sample as reference to determine biochemical variables.

Having originally been conceived as a 6-domain dissertation (growth, hematology, hydration, oxidation, inflammation and infection); result articles are grouped in the aforementioned four sections to harmonize the major findings of the present Ph.D Thesis.

# II. RATIONALE AND SIGNIFICANCE

Sheny Romero, *Persiguiendo la cámara*, Quetzaltenango, 2012

The history behind the justification for the studies of this dissertation is more than merely scientific in nature. There are contextual and circumstantial factors that led to the directions in this thesis research.

By 2011, I finished my Master thesis. Two of the institutions that supported this project (Hildegard Grunow Foundation in Germany; and CeSSIAM in Guatemala) were trying to promote more research in different fields in Guatemala and planned a longitudinal study in children from the Western Highlands, knowing that research in children requires a larger effort on planning and logistics than studies designed for adults. Related to the focus on young children, both institutions tried to find more convenient ways to test and improve methods of non-invasive sample collection to obtain information about biomarkers that were exclusively analyzed after invasive sampling so far.

Discussion about the distribution of biologic variables in apparently healthy children is ongoing. The sample number in a research project depends on previous findings of inter- and intra-individual variation. This started the interest in a project that would allow the development of other studies in preschool children, living under specific circumstances.

Based on the experience acquired during the year of the Master's in Human Nutrition at the University of Granada, and the support offered by its Department of Biochemistry and Molecular Biology II, it was decided to test and try to establish more variables in children. Some of those variables have never been explored in Guatemala in this age group. Variables regarding the normative state of hydration were explored in the dissertation project of the doctoral candidate, Gabriela Montenegro-Bethancourt, at the Research Institute of Child Nutrition in Dortmund, Germany. The Ibero-American Nutrition Foundation (FINUT) donated the equipment to permit the osmometry measurements in Guatemala.

Based on previous research by Dr. Noel W. Solomons at CeSSIAM regarding the role of poor hygienic conditions –rather than the simple lack of nutrients– on the high prevalence of stunting rates in Guatemala (65), it was decided to take advantage of a group of children in the same age group, spending time in the same

building during the weekdays; who were from the same communities and were on the same adequate diet during five days of the week.

The title of the dissertation project embodied its aims: *"Study on the Normative State and Inter- and Intra-individual Variation in Growth, Hydration, and Markers of Oxidation, Infection and Inflammation in Preschool Children with a Similar Dietary Intake. An investigation using predominantly, non-invasive collection techniques". It* offered access to a preschool population, with the aforementioned similarities, living in the Western Highlands of Guatemala. This population offered the opportunity to expand the knowledge on different health biomarkers in collaboration between four institutions, in three different countries. It may lead to a new cycle of research in low-income populations in developing countries financed by developed countries. Those 4 institutions are: the Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) in Guatemala; the Hildegard Grunow Foundation in Germany, the Ibero-American Nutrition Foundation –FINUT- in Spain, and the Department of Biochemistry and Molecular Biology II, University of Granada.

It is hoped that this dissertation will provide future research with an opportunity to apply biomarkers in children, and to study the interaction of different human biology-domain variables.

# III. HYPOTHESES AND OBJECTIVES

Jeniece Alvey, *Los niños*, Quetzaltenango, 2012

## HYPOTHESES

For this dissertation, we explored a wide variety of topics, some of which were never explored in children before. Primary hypotheses referred to the variance among 61 analyzed variables. As we entered the project with very little understanding of the inter-individual variance of most of the collected biomarkers, we expected a wide range of variability, extending from those with virtually no variation to those with an extraordinarily wide variance.

Based on available knowledge and on environmental differences between the three participating daycare centers, the hypotheses also refer to differences by sex and by location in some variables, especially with regards to growth attainment, hemoglobin and parasitic and protozoan infections.

Moreover, we expect to find associations, especially between biomarkers from the same domain and in the same anatomical compartment, e.g. among oxidation biomarkers measured in urine and those of inflammation in saliva.

## GENERAL OBJECTIVE

To estimate the inter- and intra-individual variances in a selected series of biological markers; e.g., of growth, hematology, hydration, infection, oxidation and inflammation, in preschool children sharing the same institutional-based diet offered in SOSEP daycare centers.

## SPECIFIC OBJECTIVES

- To determine the inter-individual variation of growth and of non-invasively- and invasively-determined hemoglobin in preschool children, with a common dietary offering who attend SOSEP daycare centers and to compare between settings.
- To determine the inter-individual variation of markers for oxidative stress, antioxidation sytems and antioxidant nutrients in preschool children with a common dietary offering.
- To describe the inter-individual variation of systemic and intra-intestinal inflammation in preschool children.

- To determine the inter-individual variation of gastrointestinal parasite infestation in preschool children.
- To describe the inter- and intra-individual variation of the hydration status in preschool children, and to compare between semi-urban and rural areas.
- To explore intra-individual associations in variables of the same biological domain variables or variables across different domains.

# IV. SUBJECTS AND METHODS


María José Soto, *Día de la Independencia*, Quetzaltenango 2012

## Study Design

This is an exploratory, cross-sectional and descriptive study, focused on the variation among children, having a similar diet but living in different households. Thus, they were exposed to distinct environments and showed genetic diversity.

## Geographical Setting and Sites

The study was conducted in the province of Quetzaltenango, located at about 2600 meters above sea-level in a verdant canyon valley. The terrain is traversed by multiple rivers and streams. Annual rainfall is ~380 mm, and the average daytime high temperature is 22$^{o}$C. The major economic pursuits of the region include finance and banking, textile manufacture, subsistence farming of maize and wheat, and commercial cropping of coffee, seasonal fruits, and truck-garden vegetables, as well as livestock production.

The provincial population is primarily of Mayan ascendancy, with a minority of mixed European-indigenous ancestry (mestizo or ladino), the latter concentrated in urban areas. Homes of low-income families are often of rudimentary constructions, poorly ventilated with indoor cooking stoves. Household piped water and electricity have become universal, but sewage disposal remains unsatisfactory.

The sites of the study were designated by the SOSEP director in the Quetzaltenango region. Center A is in a semi-urban setting in a suburb, La Esperanza, of the main city. Center B is situated in a marginal-urban area of the city itself, in the barrio of La Puerta del Llano. Finally, Center C is located in a truly rural, agricultural zone in the hamlet of San Martín Chile Verde.

## Study Subjects

Preschool children, who were between 24 and 72 months and were attending semi-urban, marginal-urban and rural SOSEP daycare centers in Quetzaltenango, Guatemala. The initial project target was to enroll 100 subjects. Eighty-seven children had signed consent forms and delivered samples that let us perform some or all measurements. Depending on the combination of variables explored, characteristics of the subjects, along with the inclusion and exclusion criteria, are

detailed on each of the articles in the result sections. **Figure 1** provides the description of the 87 schoolchildren enrolled in the dissertation project.

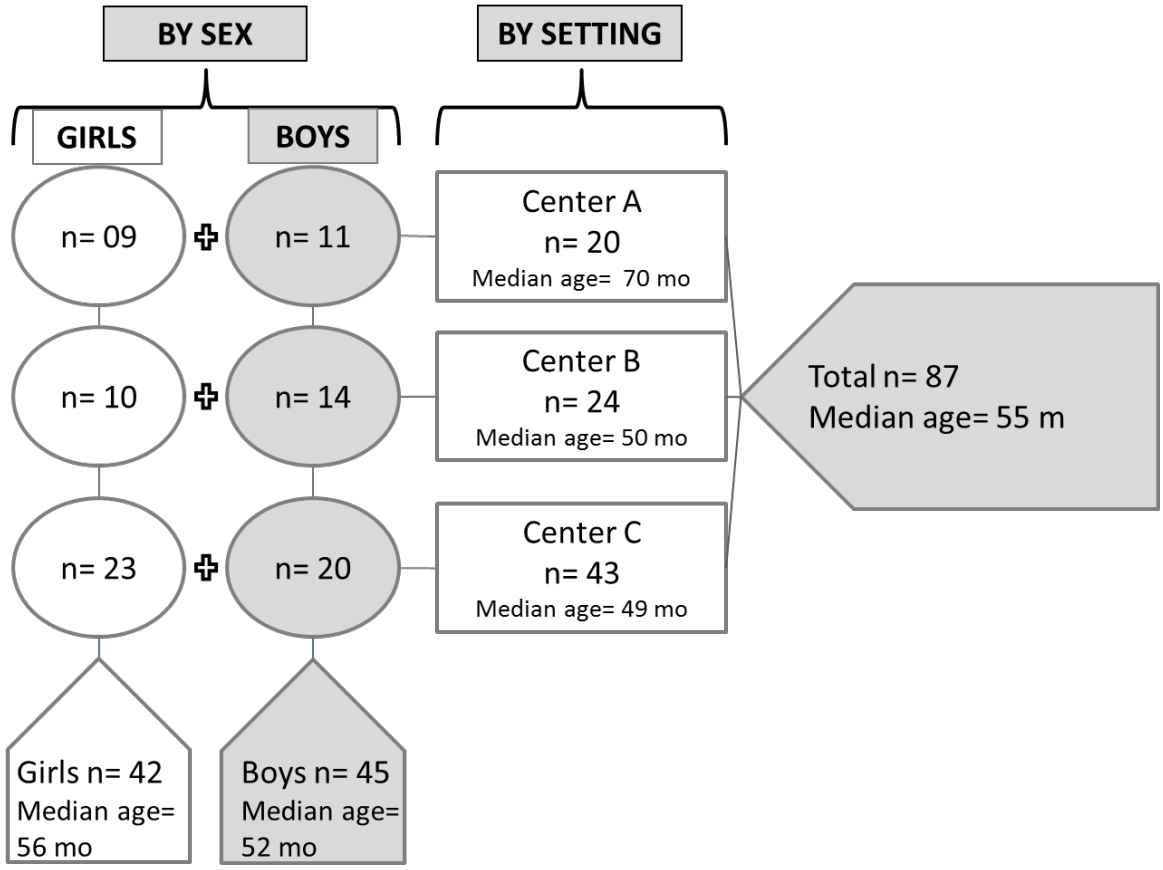


**Figure 1.** Characteristics of the study population.

## Ethical Considerations

The study protocol was presented and approved by the University of Granada. Ethical approval was granted by the Human Subjects Committee from the Center for the Studies of Sensory Impairment, Aging, and Metabolism in Guatemala (**Annex 3**). A parent or legal guardian signed a written consent form after the nature, purpose, risks and benefits were explained (**Annex 4**). As the findings of this dissertation needed to be published for the knowledge of the scientific community, the study was registered at clinicaltrials.gov as NCT02203890 (**Annex 5**).

A physician responded to the findings of the diagnostic tests (e.g. hemogram, stool microscopy and urinalyses tests), and delivered deworming treatment along with medical prescriptions, e.g. for oral iron supplementation, or medical care, e.g. antibiotics for urinary tract infection. When required, the diet offered to the children was complemented by study funds in order to provide all food items prescribed by the menu.

**Observation / Collection Period**

For methodological reasons, we decided to spend the eight weeks that would complete a cycle of the 40-day SOSEP rotating menu at each of the three daycare centers that were assigned for the study. The first five weeks were exclusively observational: one of the collaborators of this study spent the whole day with the children sharing activities and making notes about the items included in the daily food offering; for example, the menu prescribed a fruit portion as midmorning snack, we registered the fruit selected each day by the daycare center caregivers. We were also able to buy any missing item that was included in the prescribed menu in order to complete the food offering. From the $6^{th}$ to the $8^{th}$ week, we collected the samples and made all measurements that generated all the data included in this dissertation. In **Table 1**, we compiled all samples collected, according to the observation / collection week of the cycle and arranged by dissertation domain.

**Study Variables**

**Table 2** classifies the 61 output variables measured in this dissertation in according to the anatomical compartment, dissertation domain and the study location where laboratory analyses were performed. Finally, for a better understanding of this dissertation organization, we are including a column with the chapter number(s) where results for each variable have been presented and related to other findings.

**Statistical Analyses**

Several analyses were performed in order to determine variance, compare magnitudes or correlatively cross-associate the aforementioned study variables. **Chapters 1** to **9** of the Results section in this dissertation include the statistical

details and the tests used to confirm our findings. The software SPSS version 20 (IBM, Chicago, IL, USA) was used to perform statistical analyses.

**Table 1**: Samples collected on the last three weeks of the 8-week cycle at three daycare centers in the Western Highlands of Guatemala.

| WEEK (OBSERVATION /COLLECTION) | SAMPLE | DISSERTATION COMPONENT |
|---|---|---|
| 1st to 5th (Observation) | --- | --- |
| 6th (Observation /Collection) | 24-hour urine | Hydration |
| 7th (Observation / Collection) | 24-hour urine | Hydration |
| | Feces | Infection / Inflammation |
| | Weight | Growth |
| | Height | Growth |
| 8th (Observation / Collection) | 24-hour urine | Hydration / Oxidation |
| | Spot urine | Infection |
| | Feces | Infection |
| | Skin transmission | Hematology |
| | Blood | Hematology / Inflammation / Oxidation |
| | Saliva | Inflammation |

**Table 2:** Study variables classified by dissertation domain, anatomical compartment, location of findings in result section, and institution that performed laboratory analyses.

| Dissertation Domain | Anatomical Compartment | Variable (measurement unit) | Section in Results | Chapter in Results | Location of lab. Analyses |
|---|---|---|---|---|---|
| Growth | Observational / Anthropometric | Weight | D | 9 | Study setting (each daycare center) CeSSIAM, Quetzaltenango, Guatemala |
| | | Height (cm) | C, D | 7, 9 | |
| | | Weight for age -WAZ- (z-score) | B, C, D | 5, 7, 9 | |
| | | Height for age -HAZ- (z-score) | B, C, D | 5, 7, 9 | |
| | | Weight for height -WHZ- (z-score) | B, C, D | 5, 7, 9 | |
| | | Body mass index -BMI- ($kg/m^2$) | D | 9 | |
| | | BMI for age -BAZ- (z-score) | D | 9 | |
| Hematology | Skin Transmission | Hemoglobin, non-invasive -Hb- (mg/dL) | D | 9 | |
| | Blood | Hematocrit -Ht- (%) | D | 9 | La Democracia Hospital Laboratory, Quetzaltenango Guatemala |
| | | Hemoglobin, whole blood -Hb- (mg/dL) | D | 9 | |
| | | Red blood cell count -RBC- ($10^6/mm^3$) | D | 9 | |
| | | Mean corpuscular volume -MCV- (fL) | D | 9 | |
| | | Mean corpuscular hemoglobin -MCH- (pg) | D | 9 | |
| | | Mean corpuscular hemoglobin concentration -MCHC- (g/dL) | D | 9 | |
| | | Red cells distribution width -RDW- (%) | D | 9 | |
| | | Platelet count ($10^3/mm^3$) | D | 9 | |
| | | Platelet distribution width -PDW- (%) | D | 9 | |
| Hydration | Urine | Urine volume (mL/24hour) 6th cycle week | A | 1 | CeSSIAM Quetzaltenango, Guatemala |
| | | Urine volume (mL/24hour) 7th cycle week | A | 1 | |
| | | Urine volume (mL/24hour) 8th cycle week | A, D | 1, 2, 3, 9 | |
| | | Urinary osmolality (mOsm/kg) 6th cycle week | A | 1 | |
| | | Urinary osmolality (mOsm/kg) 7th cycle week | A | 1 | |
| | | Urinary osmolality (mOsm/kg) 8th cycle week (Guatemala) | A | 1 | |
| | | Urinary osmolality (mOsm/kg) 8th cycle week (Granada) | A, D | 1, 2, 3, 9 | Department of Biochemistry and Molecular Biology II, Granada University, Spain |
| | | Urinary uric acid (mg/dL) | A, D | 2, 9 | Scientific Instrumentation Center, Granada University, Spain |
| | | Urinary urea (mg/dL) | A, D | 2, 9 | Scientific Instrumentation Center, Granada University, Spain |
| | | Urinary creatinine (mg/dL) 8th cycle week | A, D | 1, 3, 9 | |
| | | Plasma creatinine (mg/dL) | D | 9 | |
| | | Sodium (mg/dL) | A, D | 2, 9 | |
| | | Potassium (mg/dL) | A, D | 2, 9 | |

| | | | | | |
|---|---|---|---|---|---|
| Hydration | Urine | Calcium (mg/dL) | A, D | 2, 9 | Scientific Instrumentation Center, Granada University, Spain |
| | | Magnesium (mg/dL) | A, D | 2, 9 | |
| | | Inorganic phosphorus (mg/dL) | A, D | 2, 9 | Department of Biochemistry and Molecular Biology II, Granada University, Spain |
| Oxidative Stress and Antioxidant Defense | Urine | 8-hydroxydeoxyguanosine -8-OHdG- (ng/mL) | A, B, C, D | 3, 4, 6, 8, 9 | |
| | | 15-Isoprostanes $F2_t$ -F2-Iso- (ng/mL) | A, B, C, D | 3, 4, 6, 8, 9 | |
| | Blood | Catalase activity -CAT- (nmol/seg/g Hb) | B, C, D | 4, 6, 8, 9 | |
| | | Superoxide dismutase activity -SOD- (U/g Hb) | B, C, D | 4, 6, 8, 9 | |
| | | Glutathione reductase activity -GSR- (umol/min/g Hb) | B, C, D | 4, 6, 8, 9 | |
| | | Glutathione peroxidase activity -GPx- (µU/g Hb) | B, C, D | 4, 6, 8, 9 | |
| | | Retinol (mg/L) | B, D | 4, 6, 9 | Scientific Instrumentation Center, Granada University, Spain |
| | | α-tocopherol (mg/L) | B, D | 4, 6, 9 | |
| | | β-carotene (mg/L) | B, D | 4, 6, 9 | |
| | | Coenzyme $Q_9$ -CO-$Q_9$- (mg/L) | B, D | 4, 6, 9 | |
| | | Coenzyme $Q_{10}$ -CO-$Q_{10}$- (mg/L) | B, D | 4, 6, 9 | |
| Inflammation | Blood | White blood cell count -WBC- ($10^3$/$mm^3$) | B, D | 5, 6, 9 | La Democracia Hospital Laboratory, Quetzaltenango Guatemala |
| | | Plasma IL-1β (pg/mL) | B, D | 5, 6, 9 | Department of Biochemistry and Molecular Biology II, Granada University, Spain |
| | | Plasma IL-6 (pg/mL) | B, D | 5, 6, 9 | |
| | | Plasma IL-8 (pg/mL) | B, D | 5, 6, 9 | |
| | | Plasma IL-10 (pg/mL) | B, D | 5, 6, 9 | |
| | | Plasma TNF-α (pg/mL) | B, D | 5, 6, 9 | |
| | Saliva | Salivary IL-1β (pg/mL) | B, D | 5, 6, 9 | |
| | | Salivary IL-6 (pg/mL) | B, D | 5, 6, 9 | |
| | | Salivary IL-8 (pg/mL) | B, D | 5, 6, 9 | |
| | | Salivary IL-10 (pg/mL) | B, D | 5, 6, 9 | |
| | | Salivary TNF-α (pg/mL) | B, D | 5, 6, 9 | |
| | Feces | Fecal calprotectin (mg/Kg) | B, C, D | 5, 6, 7, 9 | CeSSIAM Quetzaltenango, Guatemala |
| Infection | Urine | Urinary white blood cells per field | D | 9 | La Democracia Hospital Laboratory, Quetzaltenango Guatemala |
| | Feces | *Giaridia intestinalis* intensity (OD) | C, D | 7, 8, 9 | |
| | | Fecal parasites (helminthes and protozoa) by Microscopy | C | 7 | |
| | | Quantitative fecal helminthes' eggs by Kato-Katz | C | 7 | CeSSIAM Quetzaltenango, Guatemala |

# V. RESULTS

Jeniece Alvey, *Las niñas*, Quetzaltenango 2012

# SECTION A

# HYDRATION STATUS AND ITS INTERACTIONS

Sheny Romero, *Las instrucciones*, Quetzaltenango 2012

# Chapter 1

## Urinary Osmolality of Preschool Children with a Largely Common Weekday Meal Offering, from the Western Highlands of Guatemala

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# PUBLISHED

## URINARY OSMOLALITY OF PRESCHOOL CHILDREN WITH A LARGELY COMMON WEEKDAY MEAL OFFERING, FROM THE WESTERN HIGHLANDS OF GUATEMALA.

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**Abstract**

*Introduction:* Urine volume and osmolality are within the most practical methods for measuring human hydration status.

*Objectives:* To describe the distribution and central tendency of urinary osmolality (Uosm) for preschool children, and to assess the reproducibility of Uosm measurements after frozen storage and when determined in two different osmometers.

*Methods:* We collected three samples of 24-hour urine over three consecutive weeks among children attending three day-care centres in different settings in Quetzaltenango, Guatemala.
Volume was determined and samples were stored at different temperatures for different periods of time. Finally, Uosm was measured on two different osmometers in different countries. Data were analyzed using SPSS version 20.

*Results:* Twenty-four hour urine volumes ranged from 65 to 1,670 ml, with a median value of 485 ml; urine osmolality ranged from 158 to 1,088 mOsm/kg, with a median of 475 mOsm/kg (n = 234 urine collections), without differences by sex, centre, or collection order. Seventy-six subjects completed 3 collections; Coefficients of Variation ranged from 1 to 68%. When refrigerated urine samples were compared to split-sample aliquots frozen at -80º, the correlation was r = 0.89 and the difference in medians was 0.2%. Values from frozen samples be-tween a Vogel-Löser 815 and Gonotec Osmomat 030 had a correlation of r = 0.83, with an 11% difference in the median.

*Conclusions:* Guatemalan children show some of the lowest median Uosm values so far reported. A good reproducibility was found when measuring after different storage times and temperatures, but on the same equipment. However, reproducibility across different osmometer brand, was not within acceptable limits.

Keywords: *Hydration, Urine Osmolality, preschool children, reproducibility, Guatemala.*

Abbreviations: *Uosm = Urinary Osmolality, SOSEP = Secretaría de Obras Sociales de la Esposa del Presidente, mOsm = milliosmoles, FINUT = Fundación Ibe-roamericana de Nutrición, FUNDANIER = Fundación del Niño Enfermo Renal, CeS-SIAM = Centre for the Studies of Sensory Impairment, Aging, and Metabolism.*

**OSMOLIDAD URINARIA DE NIÑOS PRE-ESCOLARES CON UN MISMO MENU OFRECIDO DURANTE DIAS HABILES, EN EL ALTIPLANO OCCIDENTAL DE GUATEMALA.**


## Resumen

*Introducción:* El volumen y la osmolalidad urinarios son dos de los métodos más prácticos para medir hidratación en humanos.

*Objetivos:* Describir la distribución y tendencia de osmolalidad urinaria (Uosm) en preescolares y determinar los efectos del almacenamiento en congelación y la medición en diferentes osmómetros sobre la reproducibilidad de resultados.

*Métodos:* Se recolectaron tres muestras de orina de 24 horas durante tres semanas, en niños que asistían a tres guarderías de diferentes lugares en Quetzaltenango, Guatemala; se determinó volumen y las muestras fueron conservadas durante diferentes tiempos y temperaturas. Finalmente, la Uosm fue medida en dos diferentes osmómetros y países. Los datos se analizaron usando SPSS versión 20.

*Resultados:* El volumen de orina varió entre 65 y 1.670 ml, con una mediana de 485 mlL; la Uosm entre 158 y 1.088 mOsm/kg con 475 mOsm/kg de mediana, (234 muestras de orina), sin diferencias por sexo, centro ni semana de recolección. 76 sujetos completaron las 3 recolecciones y los CV oscilaron entre 1 y 68%. Al analizar las alícuotas refrigeradas y compararlas con las congeladas (-80ºC), la correlación fue r = 0,89 y la diferencia de medianas fue de 0,2%. Las mediciones de las muestras congeladas entre el Vogel, Löser, y el Gonotec, Osmomat, tuvieron una correlación de r = 0,83 con un 11% de diferencia de medianas.

*Conclusión:* Guatemala tiene uno de los valores más bajos de Uosm observados hasta el momento. Se encontró una buena reproducibilidad en las mediciones en diferentes tiempos y temperaturas, pero en el mismo equipo. La reproducibilidad no fue aceptable entre diferentes marcas de osmómetros.



*Palabras clave:* Hidratación, Osmolalidad urinaria, niños prescholares, reproducibilidad, Guatemala.


*Abreviaturas:* Uosm = Osmolalidad urinaria, SOSEP = Secretaría de Obras Sociales de la Esposa del Presidente, mOsm = milliosmoles, FINUT = Fundación Iberoamericana de Nutrición, FUNDANIER = Fundación del Niño Enfermo Renal, CeSSIAM = Centre for the Studies of Sensory Impairment, Aging, and Metabolism.

## Introduction

Water has been called the primary and most important nutrient[1]. Mild dehydration is defined as 1-2% loss of body weight secondary to fluid loss; this impairs exercise performance and increases the risk of urinary stone disease[2]. Among the different techniques that can help in the assessment of hydration status are: isotope dilution, neutron activation analysis, bioelectrical impedance, body mass change, plasma osmolality, per cent plasma volume change, 24-hour urine volume, salivary flow rate or osmolality, urine color, urine specific gravity, urine conductivity, and urine osmolality. However, estimating the state of hydration within the normal (non-pathological) range is an elusive proposition[3]. Manz[4] provides guidance toward an operational concept of normative hydration: “in a subject, minimum and maximum urine osmolality define the range of euhydration.” Urine has the advantage over the other biological fluids mentioned in the non-invasive nature of collection, its sanitation and the availability of ample volumes[5]. Urinary osmolality (Uosm) alone has been reported widely in studies to approximate hydration state, including in children[6-12].

However, obtaining quantitative 24-hour urine collections in young children is a notoriously significant challeng [13].

Twenty-four-hour urine collections and osmolality measurements were two of the

activities programmed in a field project called: “Study on the normative state and inter and intra-individual variation in growth, hematology, hydration, and markers of oxidation, infection and inflammation in pre-school children with a similar dietary intake,” conducted among day-care centre attendees in the Western Highlands of Guatemala. The centres were in different geographical locations, and of ethnic make-up and social-economic status. A common feature was the 4-meal menu offered in each daycare centre. In the present study, we describe Uosm distributions and central tendencies in the children of the sample. We also compare our findings with other pediatric studies. On the methodological side, we examine the effects of different storage periods and temperatures and compared osmometers of two different manufacturers.

## Methods

### *Setting*

Guatemala is the country of 108,889 $Km^2$ located in the center of the American continent. Its name comes from the Náhuatl language expression “Quauhtlemallan” that means “land of trees”. Guatemala has great variety of microclimates and different cultures[14]. Guatemala also has the highest under-5 stunting index in Latin America, with nutritional anemia that affects 26% of children between 1-5 years and 26% of the women between 18 to 45 years. Infant mortality rate is the third highest in the world[15].

The study was conducted in the Western Highlands of Guatemala in the province of Quetzaltenango, which is located 220 Km from the capital and 2357 m above the sea level, with a land area of 1,943 $km^2$ and 24 municipalities[16]; SOSEP designated three centres to our study, one located in La Esperanza (Centre A), another in La Puerta del Llano (Centre B) and the last one -and most rural- in San Martín Sacatepéquez (Centre C).

### *Study Design and Subjects*

The study is a descriptive, cross-sectional field survey and was conducted in three day-care centres (Hogares Comunitarios) of the SOSEP system. One site, Centre A, was located in La Esperanza, a semi-urban area 2 miles away from downtown Quetzaltenango, Guatemala. The second, Centre B, was located in the outskirts of the city of Quetzaltenango close to rural areas in La Puerta del Llano; for that reason it was classified as marginally urban.

The rural area or Centre C was located 15 miles away from Quetzaltenango in La Estancia, San Martín Sacatepéquez. Almost all children attending the centres were indigenous; however, some habits, pastimes and physical characteristics varied between centres.

### *Exclusion Criteria*

Eligible children were required to attend the selected SOSEP centre and to be 2 to 7 years old. Also, subjects needed to attend at least 80% of the centre working days during the study. Eligible subjects were apparently healthy and with no restrictions to consume the foods offered by the SOSEP menu. Children who refused to adhere to the urine collection routine or to participate in the study, or whose parents refused to sign the consent form were excluded.

### *Ethical considerations*

The Centre for the Studies of Sensory Impairment, Aging, and Metabolism’s Human Subjects Committee in Guatemala City granted ethical approval of the study protocol. A consent form was to be signed by a parent or guardian. Previous authorization was obtained from the Director of SOSEP for the Quetzaltenango area. Whenever the situation required, we complemented the

diet offered to the children in order to provide all items prescribed on the menu. A local physician took care of the results of the hemogram, stool and urine tests and delivered deworming treatment along with medical prescription or medical care (e.g. of urinary infection), if required.

*Sample collection and Storage*

*Urine collection:* Three samples of 24-hour urine were collected, one each in the 3 collection weeks in order to measure urinary osmolality (Uosm). BD Vacutainer® No.364999 plastic 24-hour collection container (Becton, Dickinson and Company, New Jersey, United States) were used. Urine collection was started at the time when each child arrived in the day-care centre and was supervised by investigators and SOSEP personnel. Collection was continued at home with the parents' assistance, and was finished after 24 h

*Aliquoting and Storage:* All collected 24-hour urine samples were well mixed. Aliquots of each sample were stored at 0°C in Quetzaltenango for 16 to 25 wee-ks variously between August, 2012 and February, 2013. During the third round of urine collection, 2 additional 4-ml aliquots were stored at -80°C in an ultra-cold freezer in Guatemala City. One of tho-se specimens was shipped to Granada, Spain, on dry ice and stored at -80ºC un-til analyses (total storage time: 43 to 52 weeks). The final specimen remained in storage in Quetzaltenango until analysis after a total storage time of 50 to 59 weeks.

*Laboratory Procedures*

Analyses in Quetzaltenango, Guatemala, were performed using the Vogel-Löser 815 osmometer (Vogel, Giessen, Germany).
For the determinations performed in Granada, Spain, we used the Gonotec-Osmomat 030 (Gonotec, Berlin, Germany), each according to manufacturers' specifications. Both instruments work on the principle of freezing-point depression of Peltier. Immediately before measurement, the aliquots were mixed for approximately 3 minutes and 100 µl or 50 µl were transferred to the measurement vessels for the Löser osmometer and the Osmomat model, respectively. Results are expressed in milliosmoles per kilogram of urine (mOms/kg).

Urinary creatinine concentrations were measured in Guatemala City using the colorimetric Jaffe reaction method. This value was applied in a predictive formula developed in Dortmund Germany by Remer[17] to screen for completeness of 24-hour urine collections in pediatric studies.

*Statistical Analyses*

Data were entered into a SPSS Version 20 database. Descriptive statistics were expressed as median, 95% Confidence interval (CI) and minimum and maximum. Central tendencies were compared between two or among three sub-groups using a non-parametric statistical test (e.g. Independent Median, Mann-Whitney U, Wilcoxon, Friedman and Kruskal-Wallis tests), as the Uosm values were not normally distributed. Association of values collected at different points in time was tested using Pearson product-moment correlation coefficient or Spearman rank-order coefficient, as appropriate. A probability level of $< 0.05$ was accepted as statistically significant.

## Results

*Distribution and Variation in Urinary volume and Osmolality*

We collected a total of 234 urine

specimens of supposedly 24-hour duration. The volumes ranged from 65 to 1670 ml with a median of 485 ml. Table I shows descriptive statistics (median, 95% CI, minimum and maximum). No significant differences were found, neither between the volumes collected in all patients of a subgroups as compared to those meeting the Remer criterion[17] (p = 0.120), nor between sexes (p = 0.807), nor among sequences of collection (p = 0.670), nor among sites (p = 0.194).

*Within Individual Stability of Urinary Osmolality*

To test the intra-individual reproducibility of Uosm across the three collection days we performed Pearson correlations. Figure 1 shows the r values of the comparison between first collection vs second (r = 0.222 p = 0.0.025), second vs third (r = 0.368 p < 0.001), and first vs third (r = 0.155 p = 0.088). Coefficients of variance (CV) of Uosm for all subjects who provided urine samples on the three occasions varied from 1% to a high extreme of 68%. The median CV for repeat collections was 22%.

*Correspondence of Measurements after Cold Storage vs Frozen Storage*

When samples were measured by two different operators in the Vogel Löser 815 osmometer in Quetzaltenango, Guatemala, the median value of the 0ºC storage specimens was 484 mOsm/kg (464-538 mOsm/kg, 95% CI). After -80°C corresponding values were 486 mOsm/kg (456-529 mOsm/kg, 95% CI) (p = 0.275 by Wilcoxon test). A significan correlation, r = 0.893 (p < 0.0001), was found using Spearman test (fig. 2) when samples stored at either temperature.

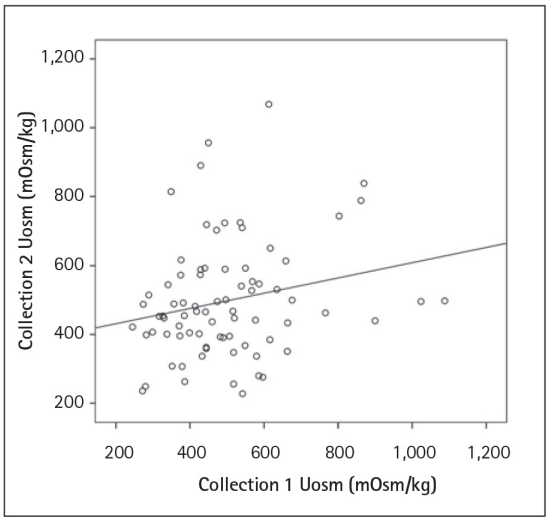
*Correspondence of Measurements with Frozen Storage in Two Different Osmometers*

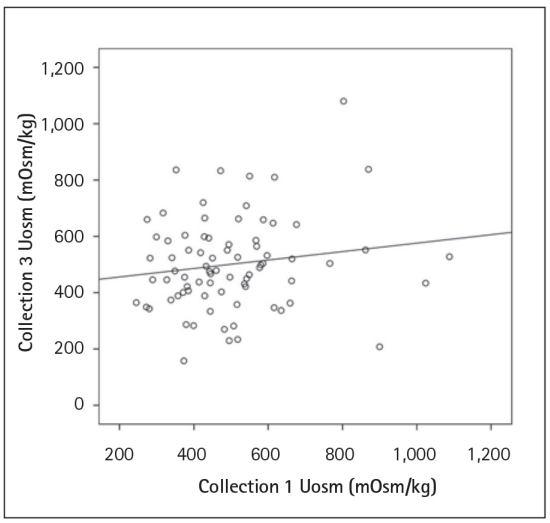
When samples were measured by two different observers, and by use of two different osmometers in Quetzaltenango (Löser 815) and Granada (Osmomat 030), respectively, the median value of A significant correlation, r = 0.828 (p <

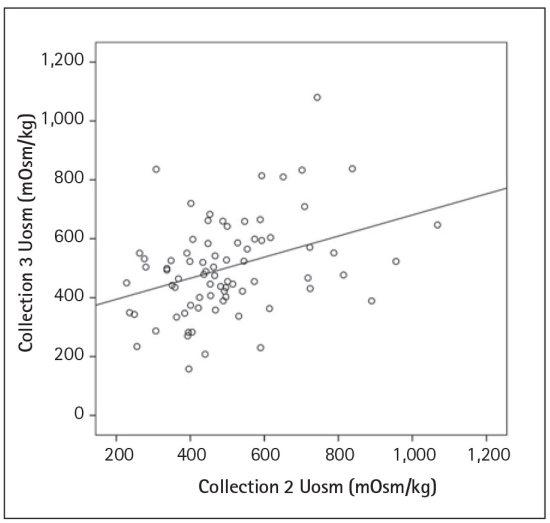
Table 1. Median and Variance of Urinary Osmolality by Whole-Group and Sub-Group Tabulations

| Subgroup | N | Uosm in mOsm/kg | | |
|---|---|---|---|---|
| | | Median | 95% CI | Min-Max |
| All collections | 234 | 475 | 478 / 521 | 158 – 1088 |
| All Remer complete* | 134 | 495 | 490 / 541 | 263 – 1088 |
| *p value* | | *p = 0.120 (Independent median test)* | | |
| All Girls' collections | 116 | 475 | 466 / 531 | 208 – 1088 |
| All Boys' collections | 113 | 475 | 470 / 527 | 158 – 1068 |
| *p value* | | *p = 0.807 (Mann-Whitney U-test)* | | |
| First collection | 77 | 474 | 462 / 539 | 245 – 1088 |
| Second collection | 74 | 467 | 455 / 532 | 228 – 1068 |
| Third collection | 78 | 484 | 464 / 538 | 158 – 1080 |
| *p value* | | *p = 0.670 (Friedman test)* | | |
| Centre A | 57 | 489 | 469 / 545 | 274 – 1088 |
| Centre B | 71 | 504 | 470 / 543 | 208 – 900 |
| Centre C | 101 | 446 | 451 / 524 | 158 – 1080 |
| *p value* | | *p = 0.194 (Kruskal-Wallis test)* | | |

**Figure 1:** Association or Urinary Osmolality Measurement Between Collections

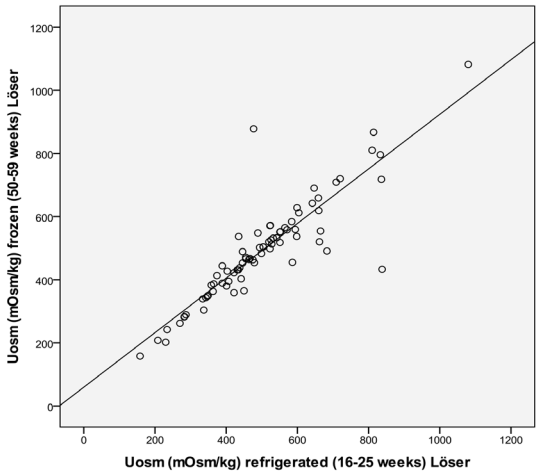


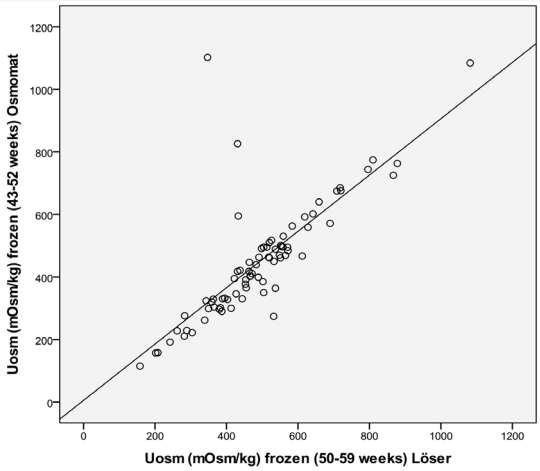

To determine association between the values of Uosm among different week of collection Pearson correlations were run, obtaining r values first collection vs second (r=0.222 p=0.025); second vs third (r=0.368 p<0.001); and first vs third (r=0.155 p=0.088 .

**Figure 2:** Comparison of Urinary Osmolality Measurements between Zero- and minus Eighty-degree Storage Temperatures, Third Collection Specimens




Spearman test was run to determine correlation between Uosm values obtained from refrigerated and frozen aliquots on the same osmometer; r=0.893 (p<0.0001).

**Figure 3:** Comparison of Urinary Osmolality Measurements between Osmometers in Deep-Frozen, Third Collection Specimens




Spearman test was run to determine correlation between Uosm values obtained from two frozen aliquots on different osmometers; r=0.828 (p<0.0001).

0.0001), was found between groups using Spearman test (fig. 3).

## Discussion

The non-invasive nature of urine collection is a major advantage of urinary biomarkers although urinary biomarkers cannot provide a full description of human hydration status. Many reports on Uosm in adults[18-21] and children[8,9] used spontaneous ambient urine sample for analyses. Other studies, even in children[6,7,10-12], aspired to make a complete 24-hour collection, which provides the advantage of leveling out changes in concentration during the course of a day. Completeness of urinary collection over 24 hours was called the "Remer criterion", developed in the DONALD study[17] in Dortmund, Germany. The younger the child, the more challenging is the complete collection for the full 24-hour urines. Complete collection of urine over 24 hours was our goal in the community and institutional setting investigated here. However, in spite of intense efforts, only 57% of the samples collected in our preschoolers met the "Remer criterion" for completeness.

In our experience the adherence to that criterion did not influence the overall Uosm estimate. Still, all but two of our collected urine volumes were in excess of 100 ml in one setting. In our study staff and parents were trained how to collect urine and supervised intensely. As a result, correspondence of Uosm in the second and third collection period improved considerably, showing the impact of experience with the urinary collection procedure. Table II shows arithmetic mean values for 8 reports from 6 countries, with the present study being the only one of non-European or Middle-Eastern origin. Comparing the mean of the distribution may not be ideal since, at least in our experience, the data are not normally distributed. With that caveat, Guatemala has the lowest median value. Belgium and Guatemala are the only two sites with mean Uosm below 600 mOsm/kg, and the only two presenting results obtained with instrumentation of the 21st century. The German values were highest and those from France and Denmark were in-between. The German study[8] may have provided the highest values, possibly because the samples may have been collected early in the day when the urine is most concentrated. One may also speculate that the lower values could be due to the improved and possibly more valid technology of modern osmometers, or to differences in hydration, or both.

The first author was present at each study site at which the common menu was served. It offered 3 beverages of 200 mL volume per day plus a 240 g of a usually succulent fruit which offer ~800 mL of water. Children could request additional servings of the beverages. Additional unknown amounts of liquids were probably consumed at home in the evenings. Between liquids and fruit, without considering the moisture in other plant and animal items of the diet, we have the basis of abundant water consumption.

Preschoolers are, by nature, physically active. However, in the rainy season at an altitude of 2,357 m, the ambient mean temperature of 15ºC reduces perspiration and water losses by transpiration. Preschoolers in our study were in the narrow age range from 2-6 years. Other studies specified the ages ranges up to 12 to 18 years [6,7,10]. This goes along with the relatively larger body surface and lung capacity and leads to greater absolute water losses in older children. We do not offer the values with the older children omitted in the Results Section, nor do we interpret these values although the Guatemalan values are the lowest in the international comparison, we feel that the values of 531 mOsm/kg (average) or 475 mOsm/kg (median) are plausible and consistent with the described conditions of collection, measurement, and the children's succulent diet, body-surface area and temperate environment.

Table 2: Mean Urinary Osmolality Values across Pediatric Studies of Combined Sexes in the Literature Review

| Authors | Location | n | Age (y) | Mean Uosm (mOsm/kg) |
|---|---|---|---|---|
| Robers & Manz (1996) (6) | Germany | 231 | 3 – 18 | 801 |
| Remer et al. (2006) (7) | Germany | 358 | 6 – 12 | 730 |
| Stolley & Schlage (1977) (8) | Germany | 566 | 5 | 860* |
| Phillip et al. (1993) (9) | Israel | 200 | 2-6 | 791* |
| Chaptal et al. (1963) (10) | France | 46 | 3 – 14 | 755 |
| Rittig et al. (1989) (11) | Denmark | 22 | Unspecified | 676 |
| Vande Walle et al. (2000) (12) | Belgium | 24 | Unspecified | 560 |
| Soto-Méndez et al. (present) | Guatemala | 78 | 2 – 6 | 531 |

*Data from spontaneous non-24-hour collection

The measurement reproducibility studies have important implications for the design and conduct of coordinated, multicentre urinary osmolality research.

Here, we performed 2 comparisons, one related to the temperature of urine storage and another on the correspondence between two osmometers of different brands. In theory, on the one hand, the deep freezing of urine could cause precipitation of some of the less soluble constituents. These constituents might not be fully re-dissolved when the urine specimen is thawed and brought to room temperature. This would predict a decrease in Uosm value after freezing. On the other hand, any sublimation of water from the frozen urine would tend to reduce liquid volume and concentrate the constituents in solution.

The results of our comparison of 0ºC and -80ºC storage showed no statistical significance ($p = 0.275$), the difference of the medians was as low as 0.4%, and the degree of association was almost 0.89 (fig. 2). For practical purposes, therefore, urine can be deep frozen for storage and later thawed and analyzed months after wards without major distortion of Uosm results.

A multi centre study, therefore, with urines shipped in frozen from the various participating centres to a common reference laboratory would run no risk of error.

Secondly, if Uosm in a multicentre trial were to be measured independently by use of osmometers in place at different study site, the degree of correspondence between the readings by different instruments become of interest. There was an 11% difference ($p < 0.001$) between the Löser and Osmomat outputs, and the association was 0.828 (fig. 3). This is obvious-ly unacceptable, if data were to be pooled. Perhaps, with a set of common test urines or standard solutions, the instruments in different laboratories could be calibrated to an acceptable range. However, our re-sults caution that confiding in the identity of outputs of different instruments would be unwise.

## Conclusions

Although they differ from Uosm/kg values in other pediatric series we believe our values to be plausible and valid for preschoolers of the Guatemalan Western Highlands. As in other series no differences between sexes were seen, and circumstantially, no Uosm differences were found from centre to centre. Most importantly, since the group data from each of the three collection periods was identical, only one round of collections will be needed to achieve the consensus estimate on a group basis. However, experience with collection process seems important to improve the

quality and consistency of the data for the individual.

Our studies on reproducibility reveal that frozen storage has no effect on the consistency of measurement in a single osmometer. Samples collected at different moments and places can be combined in comparative analyses. However, it will not be prudent to measure Uosm on different equipment without having a rigorous crossed-standardization and common calibration of the instruments involved.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# Chapter 2

# The Contribution of Selected Urinary Solutes to the Determination of Urinary Osmolality in Guatemalan Preschool Children consuming a Common Menu Offering

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Submitted

# SUBMITTED

## THE CONTRIBUTION OF SELECTED URINARY SOLUTES TO THE DETERMINATION OF URINARY OSMOLATY IN GUATEMALAN PRESCHOOL CHILDREN CONSUMING A COMMON MENU OFFERING

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## Abstract

*Background:* Although water is the most abundant and most vital of all human nutrients, hydration is among the most ignored aspects of human nutrition. Many different solutes are eliminated by the kidneys in the urine flow, potentially contributing to the osmotic charge of this body fluid and acting as determinants of the urinary osmolality [Uosm].

*Objectives:* To measure urine osmolality concurrently with urea, uric acid, calcium, magnesium, potassium, sodium and inorganic phosphorus in a 24-h sample and to determine the patterns of their mutual interactions towards assessing the primary determinants of Uosm.

*Methods:* Seventy-eight children from 2 to 7 years old, 40 girls and 38 boys, with median age of 57 mo underwent 24-h urine collections, with an aliquot separated for measuring urine osmolality by

freezing-point-depression osmometry and solute concentrations by various analytical chemistry techniques.
Spearman correlations and multiple regression models were run to assess interactions.

*Results:* Backward-elimination multiple-regression models found that the urinary concentrations of inorganic phosphorus, urea, sodium, potassium and magnesium explained 95.1% of the variance in Uosm among the seven analytes quantified; calcium and uric acid made negligible contribution.
*Conclusions:* The analyses allowed us to confirm the determinant roles of urea and the principal electrolytes, sodium and potassium, for urine osmolality and to appreciate coordination in the collateral collinear associations with other excreted solutes.

Keywords: *Urinary osmolality; Hydration; Electrolytes; Preschool children; Guatemala.*

Short Title: Urine solutes and osmolality in children.

## Introduction

Although water is the most abundant and most vital of all human nutrients,[1,2] hydration is among the most ignored aspects of human nutrition. This is especially true for juvenile populations, for which there are limited data based on quantitative urine connections.[3]
Interestingly, in a sample of preschool-aged children attending government-sponsored day-care centers in the western highlands of Guatemala, the urinary osmolality [Uosm] was among the lowest of those reported across the pediatric literature,[3] signifying a relatively superior hydration status.
The volume of water from all sources, including beverages, water in recipes, intrinsic food moisture and metabolic water from energy-substrate oxidation, is one determinant of hydration status.[4] The minute-by-minute retention or excretion of this water is governed, in part, by renal regulatory mechanisms that filter the circulating stream of plasma containing a host of small, electrically-charged and neutral uncharged constituents to be excreted in the urine. These atomic and molecular species, themselves, initially originate in the diet. In some instances, they can be turned over and excreted on the same day of ingestion, as in the case of sodium [Na], potassium [K] and chloride. Alternatively, they may have been incorporated for various time intervals in bone or muscle, as with calcium [Ca], magnesium [Mg] and phosphorus [Pi], or in any soft tissue such as muscle or visceral organs, as with the amino acid-derived nitrogen, excreted as urea. Furthermore, the nucleic acids in the plant and animal cells in the diet and from endogenous sources of human tissues yield uric acid, eliminated by the kidneys in the urine flow. As ions and minute molecules in the urine, these -and many other solutes - contribute to the os-motic potential of this body fluid and cons-titute the basis for the Uosm. For young children then, the daily water balance plus the dietary food selection and the turnover of growing tissues would interact, in combination, to determine the osmolality as measured in urine.
What the resultant variation in the excretion of these solutes and the influence on Uosm would be in situations where children had similar physical activity routines and common dietary offering is not known. In the present study, the 24-h urine specimens were collected from the aforementioned convenience sample of Guatemalan preschool children and a select group of two organic compounds (urea and uric acid) and four cationic electrolytes (Ca, Mg, K and Na) and one inorganic anionic compound (Pi) were measured. Uosm was determined by freezing-point depression osmometry, a putative indicator of human hydration status.[5] We present here the patterns of mutual interactions, and multiple regressions to assess the primary determinants of osmolality.

## Subjects and Methods

*Settings*

The geographical setting, day-care center sites and the routines and menu offerings at the centers have been presented previously.[3] The study was conducted in the Western Highlands of Guatemala in the province of Quetzaltenango, located 220 km from Guatemala City and 2357 m above the sea level, with a land area of 1,943 km$^2$ and 24 municipalities;[6] the Secreta-riat for Beneficial Works of the First Lady [SOSEP] named three different daycare centers to our study, one located in La Es-peranza, a semi-urban location (Center A); another located really close to the city, La Puerta del Llano, but classified as marginal-urban, (Center B); and the last one, and in the most rural setting, San Martín Sacatepéquez, (Center C). The centers share an 8-wk menu cycle in common, with a 2-meal plus 2-snack daily offering.

*Subjects*

Seventy-eight children from 2 to 7 years old attending daycare centers from the SOSEP system in three areas of Quetzaltenango, Guatemala were enrolled in the study. The majority of them of them were of Mayan indigenous ascent; however, certain cultural behaviors, pastimes and physical characteristics varied among centers.

*Exclusion Criteria*

Children were excluded if they did not attend one of the 3 selected daycare centers at least 80% of the center`s working-days during the 8-wk within the study period. Also excluded were children whose parents refused to adhere to the urine collection routine, to participate in the study, or whose parents refused to sign the con-sent form were excluded. Eligible subjects were apparently healthy and with no res-trictions in consuming the foods and beverages offered within the SOSEP menu.

*Ethical considerations*

Study protocol obtained ethical approval from The Center for the Studies of Sensory Impairment, Aging, and Metabolism's Human Subjects Committee in Guatemala City. Participants were required to have a consent form signed by a parent or legal guardian. Previous authorization was obtained from the director of SOSEP for the Quetzaltenango area. When the situation required, the investigation purchase the prescribed items to assure the complete offering of the assigned SOSEP menu. A physician responded to the findings of the diagnostic tests (e.g. hemogram, stool and urine tests), and delivered deworming treatment along with medical prescription, e.g. for oral iron supplementation, or medical care, e.g. antibiotics for urinary tract infection.

*Urine collection, aliquoting and storage*

After two previous collections, third sample of 24-h urine was obtained in plastic 24-h collection container (BD Vacutainer® No.364999 Becton-Dickinson Co. NJ, USA). Urine collection was started in the daycare center at the time each child arrived. Collection in daycare centers was supervised by investigators and SOSEP personnel and continued at home with the parents' assistance until it was finished, 24 h thereafter. When collection became incomplete either at daycare center or home, it was restarted the following day.

Three rounds of 24-h urine collection were conducted in Quetzaltenango, Guatemala between August and November 2012. During the third one, samples were well mixed, urine volume was determined, and two additional 4 mL aliquots were stored at -80ºC in Guatemala City. One of those was shipped to Granada, Spain on dry ice and stored at -80ºC until analyses, total storage time from 43 to 52 wks.

*Measurement of urinary osmolality*

After volume measurement with volumetric cylinders and 4 mL aliquots storage at -80ºC in ultra-cold freezers, the samples were mixed for about 3 minutes to assure homogeneity. A 50 µL aliquot of urine was pipetted into the sampling chamber of an Osmomat 030 osmometer (Gonotec, Berlin, Germany) and the Uosm was read out in mOsm/kg of urine. The technique had a measurement precision with a coefficient of variation of 1%.

*Measurement of urinary analytes*

The series of seven constituents were measured across different analytical laboratories of Scientific Instrumentation Center (CIC) of the Center of Biomedi-cal Research, University of Granada). The CIC bioanalysis unit ran the tests to determine urea, and uric acid using the automatic biochemical analyzer BS200 (Mindray, Shenzhen, China). Analysis of Na, K, Ca and Mg by atomic emission spectroscopy with inductive-coupled plasma (ICP-AES) using the model Op-tima 8300 (PerkinElmer, Waltham, MA, USA) in the structures analysis and de-termination unit. Pi was measured using the Fiske-Subbarow, direct method, kit (catalog #997609, QCA, S.A., Spain).

*Data handling and statistical analysis*

All descriptive and analytical statistics were calculated using SPSS version 20 (IBM, Chicago, IL, USA). Normality in the data distribution was determined using the Kolmogorov-Smirnov test. Descriptive statis-tics are presented as mean ± SD, median, 95% confidence interval and range for 24-h total production and concentration for each solute and for urine volume and osmolality. Spearman correlation coefficient was determined for all variables, and a multiple regression backwards step analysis was run in order to predict values of urine osmolality from the urine solutes measured for this study. A probability level of <0.05 was accepted as the criterion for statistical significance.

## Results

*Characteristic of the subjects*

There were a total of 78 subjects, 40 girls and 38 boys, whose urinary data were complete for the at least one of variables. Median age was 57 mo with mean of 57 +/- 15 and a range from 23 to 81 mo. Disaggregated characteristics by sex and site are shown in Figure 1.

*Descriptive statistics of urine volume, Uosm and selected solutes*

The descriptive statistics of median, 95% confident intervals and maximal and minimal values for urine volume and Uosm along with the solutes of interest are presented in Table 1. The solutes are described both as concentration (units/dL) in the middle panel and as amount per full collection (units/d) in the lower panel. With the exception of Na concentrations, all con-centration and daily-output values for the solutes did not have a normal, Gaussian distribution.

*Associations among the variables*

As seen in the cross-variable Spearman hemi-matrix (Figure 2), a total of 22 correlation coefficients (61%) met the 5% probability criterion when the total number of subjects having both paired variables is included. Of these 13 were direct and 9 were inverse. When only the 64 children with all variable reported are analyzed, this declines to 19 (53%, 12 direct and 7 inverse) (data not shown). Of the statistical significant associations, r values ranged from -0.260 (urinary volume with Na, $P = 0.022$) to 0.836 (Uosm with Na, $P < 10^{-3}$).

**Figure 1:** Demographic characteristics of the sample.

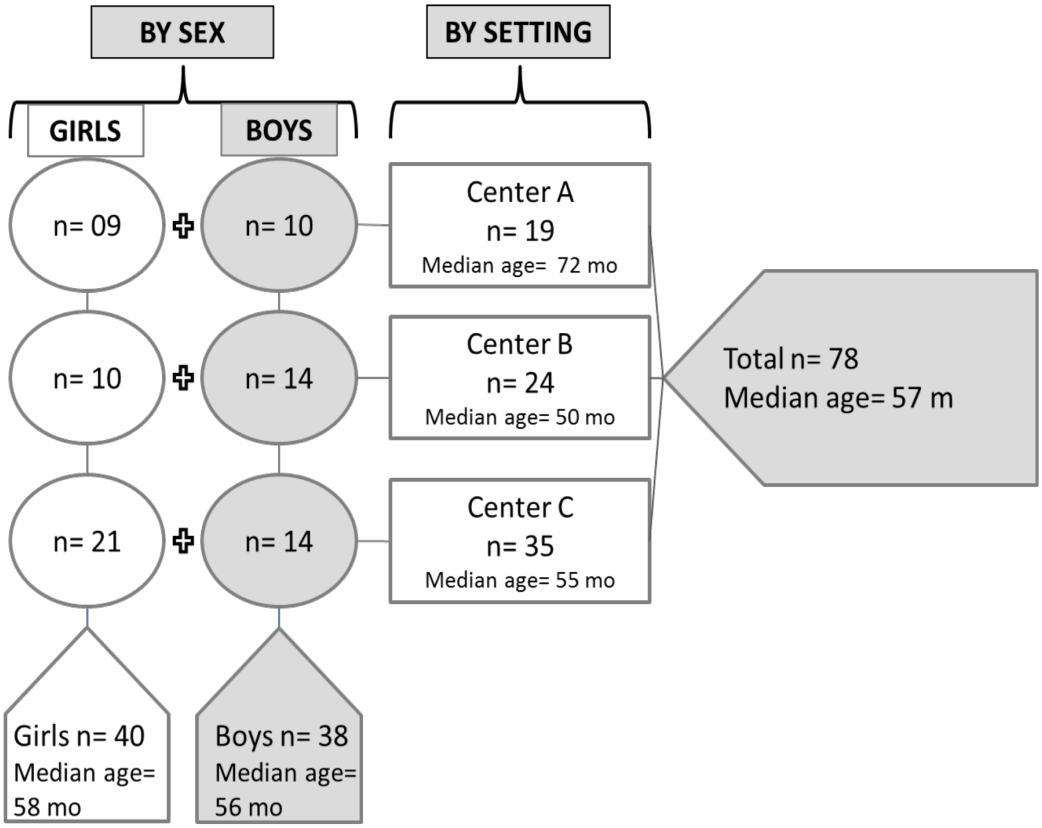


**Figure 2:** Spearman correlation hemi-matrix of Uvol, Uosm and 7 selected urinary analytes con-centrations for 78 preschool children.

| Variable | | Uvol (mL/24hour) | Uosm (mOsm/kg) | Urinary Uric Acid (mg/dL) | Urinary Urea (mg/dL) | Na (mg/L) | K (mg/L) | Ca (mg/L) | Mg (mg/L) | Pi (mg/dL) |
|---|---|---|---|---|---|---|---|---|---|---|
| Uvol (ml/24hour) | r-value | 1.000 | | | | | | | | |
| | P-value | | | | | | | | | |
| | n | 78 | | | | | | | | |
| Uosm (mOsm/kg) | r-value | -0.363** | 1.000 | | | | | | | |
| | P-value | 0.001 | | | | | | | | |
| | n | 78 | 78 | | | | | | | |
| Urinary Uric Acid (mg/dl) | r-value | 0.090 | 0.024 | 1.000 | | | | | | |
| | P-value | 0.436 | 0.833 | | | | | | | |
| | n | 78 | 78 | 78 | | | | | | |
| Urinary Urea (mg/dl) | r-value | -0.079 | -0.742** | 0.131 | 1.000 | | | | | |
| | P-value | 0.532 | <0.001 | 0.299 | | | | | | |
| | n | 65 | 65 | 65 | 66 | | | | | |
| Na (mg/L) | r-value | -0.260* | 0.836** | 0.138 | -0.516** | 1.000 | | | | |
| | P-value | 0.022 | <0.001 | 0.230 | <0.001 | | | | | |
| | n | 77 | 77 | 77 | 64 | 77 | | | | |
| K (mg/L) | r-value | -0.312** | 0.580** | 0.051 | -0.297* | 0.544** | 1.000 | | | |
| | P-value | 0.006 | <0.001 | 0.658 | 0.017 | <0.001 | | | | |
| | n | 77 | 77 | 77 | 64 | 77 | 77 | | | |
| Ca (mg/L) | r-value | 0.003 | 0.289* | 0.207 | -0.330** | 0.334** | -0.063 | 1.000 | | |
| | P-value | 0.981 | 0.011 | 0.070 | 0.008 | 0.003 | 0.584 | | | |
| | n | 77 | 77 | 77 | 64 | 77 | 77 | 77 | | |
| Mg (mg/L) | r-value | -0.178 | 0.771** | 0.178 | -0.577** | 0.629** | 0.321** | 0.407** | 1.000 | |
| | P-value | 0.122 | <0.001 | 0.121 | <0.001 | <0.001 | 0.004 | <0.001 | | |
| | n | 77 | 77 | 77 | 64 | 77 | 77 | 77 | 77 | |
| Pi (mg/dL) | r-value | -0.173 | 0.785** | -0.038 | -0.651** | 0.524** | 0.459** | 0.138 | .695** | 1.000 |
| | P-value | 0.130 | <0.001 | 0.743 | <0.001 | <0.001 | <0.001 | 0.232 | .000 | |
| | n | 78 | 78 | 78 | 65 | 77 | 77 | 77 | 77 | 78 |

Uvol=urine volume; Uosm=urine osmolality; Na=sodium; K=potassium; Ca=calcium; Mg=magnesium; Pi= inorganic phosphorous *Significant at a 0.05 level
**Significant at a 0.01 level

*Multiple regression model of prediction of Uosm*

In order to determine the relative contribution of the various urinary variables to the resultant Uosm measured across these preschool children, we performed backward-elimination multiple regression in conjunction with ANOVA (Table 2). With all 9 variables as the standard of explaining 100% of the measurable balance, the $r^2$ variables remained stable, virtually no loss in explained variance for predicting Uosm down to the 5 analytes. The variables eliminated in penultimate and ultimate steps of backward elimination were Ca and uric acid, respectively. The 5/five analytes remaining in the final round Pi, K, Na and Mg, all expressed as concentration in mg/dL; these explained 95.1% of the variance in Uosm.

## Discussion

In the assessment of human hydration, we cannot rely on the osmolality of the plasma in the circulation, itself,[7] as it is tightly and homeostatically regulated within a narrow band of 280-295 mOsm/kg of plasma,[8,9] just as is the blood pH and serum Na and K concentrations, to avoid a collapse of vital physiological function. Moreover, it is common in clinical practice to make a surrogate estimate for plasma osmolality using a formula including the circulating concentrations of Na, K, glucose and urea nitrogen, which comes within about 10 mOsm/kg of the true value as measured by osmometry[8].

We can get some indication of hydration, however, by assessing the total urinary volume and indicators of the resultant systemic regulation in the osmolality in the excreted urine (Uosm), which is constituted by the organic and inorganic solutes dissolved in that volume of urine.[5,7] Manz and Wentz, 2003[5] introduced the term “euhydration” to define the range of hydration state, defined by Uosm, consistent with normal function. Within that band, the Guatemalan preschoolers in this series have a relatively low Uosm in a comparative perspective.[3]

Since the children came from similar settings and had a common menu offering, it is notable how widely ranging were the variables of urinary concentration. This begins with the variation in urine volumes on the day of collection. This is determined not only by water intake, but also by the drives for excretion across the various routes. It should be remembered, moreover, that a common menu means cumulatively consistent offerings of food and beverages over a 40-day cycle, but on any individual collection day, the meals could be quite distinct from center to center. With that would follow different intakes of salt, protein and the diet’s contribution of the minerals of interest.

The SOSEP diet and subjects’ body size and composition are two major factors in the excretion of solutes in urine. It is useful to try to place the descriptive statistics on 24-h excretion of selected solutes in a context of publication on for daily urinary outputs in child populations from the comparative literature. The daily excretion values from Table 1 are expressed as medians, as none met criterion for Gaussian normality; for the purposes of comparative discussion, however, they have been converted to arithmetic means, the format used in all of the cited publications. With respect to the organic solutes, urinary urea is considered to reflec recent dietary protein intake; the mean of 525 mg/24 h is considerably lower than the ~3 g reported in 11 Japanese children below 4 y of age.[10] Uric acid is derived from the turnover of nucleic acids from both the exogenous diet and the turnover of endogenous tissues. Our mean uric acid value of 79 mg/24 h is about half that of the 175 mg from 44 Brazilian preschoolers.[11]

Our median full-collection Na was approximately 0.9 g, which would correspond to 2 g of salt (sodium chloride). This compares to 1.2 g in the same 11 Japanese preschoolers cited above.[10] Both of these daily urinary outputs were greatly surpassed by 6 year-olds in Finland, with a 1.6 g average.[12]

**Table 1:** Descriptive statistics of Urine volume, Usom and concentrations and daily excretions selected analytes.

| Variable (unit) | Median | 95% CI | min – max |
|---|---|---|---|
| Urine volume (mL/d) | 460 | 436-575 | 65-1670 |
| Uosm (mosm/kg) | 430 | 407-491 | 115-1102 |
| ***Solute concentration*** | | | |
| Uric acid (mg/dL) | 15.5 | 14.8-16.5 | 6.8-26.8 |
| Urea (mg/dL) | 100.0 | 76.3-117.2 | 0.02-283.0 |
| Na (mg/dL) | 183.0 | 172.2-210.3 | 19.3-441.6 |
| K (mg/dL) | 114.2 | 118.5-141.1 | 68.5-325.6 |
| Ca (mg/dL) | 6.3 | 6.2-8.7 | 0.6-34.4 |
| Mg (mg/dL) | 5.7 | 5.5-7.2 | 0.7-15.2 |
| Pi (mg/dL) | 21.9 | 23.6-31.9 | 2.35-75.15 |
| ***Daily excretion of solute*** | | | |
| Uric acid (mg/d) | 69 | 68-90 | 9-231 |
| Urea (mg/d) | 466 | 392-660 | 0.1-1914 |
| Na (mg/d) | 799 | 772-1040 | 58-2430 |
| K (mg/d) | 567 | 531-688 | 90-1500 |
| Ca (mg/d) | 27 | 29-46 | 3-206 |
| Mg (mg/d) | 26 | 25-35 | 1.4-94 |
| Pi (mg/d) | 111 | 105-145 | 5-411 |

Uosm=urine osmolality; Na=sodium; K=potassium; Ca=calcium; Mg=magnesium; Pi= inorganic phosphorous

**Table 2.** Coefficients of the third and final backward elimination multiple regression model for the dependent variable urine osmolality, presenting the urine solutes measured in the present study (uric acid, urea, Na, K, Ca, Mg, Pi) as independent variables.

| | | Unstandardized Coefficients | | Standardized Coefficients | | | 95.0% Confidence Interval for B | |
|---|---|---|---|---|---|---|---|---|
| No. | Model | B | Std. Error | Beta | t | Sig. | Lower Bound | Upper Bound |
| ***Dependent variable: Uosm*** ** | | | | | | | | |
| 3 | (Constant) | 142.810 | 18.649 | | 7.658 | 0.0001 | 105.481 | 180.139 |
| | Urea | -0.369 | 0.057 | -0.252 | -6.444 | 0.0001 | -0.484 | -0.254 |
| | Na | 0.095 | 0.007 | 0.538 | 13.525 | 0.0001 | 0.081 | 0.109 |
| | K | 0.047 | 0.010 | 0.155 | 4.666 | 0.0001 | 0.027 | 0.068 |
| | Mg | 0.535 | 0.157 | 0.140 | 3.407 | 0.001 | 0.220 | 0.849 |
| | Pi | 1.409 | 0.364 | 0.156 | 3.865 | 0.0001 | 0.679 | 2.138 |

Uosm=urine osmolality; Na=sodium; K=potassium; Mg=magnesium; Pi=inorganic phosphorus *Uric acid and Calcium were eliminated in the first two rounds of modeling

**$r^2$ = 0.951; $r^2$ = 0.975 (n = 64)

The corresponding K excretions in the three series are equally aligned with a respective: 0.61 g in Western Guatemala; 0.86 g in Japan; and 1.25 g in Finland. The lower Na intake for Guatemala is compatible with observations made with lithium-labeled salt by Melsa-Boonstra et al.,[13] who documented a 1.8 ± 0.6 g salt/d in rural boys aged 6 to 12 y.

Urinary Ca[11,14] and Mg[14] outputs in these citation was expressed as mg per kg body weight of the subject per 24 h. For comparative ends, then, we have used a median weight of 14.6 kg for our sample of preschoolers to unify the excretion units to adjust an arithmetic mean of 37.8 mg of Ca in full collections. This produces values of 2.60 mg/kg/d for Guatemalan preschoolers, which compared to 1.44 mg/kg/d for 125 Brazilian children in the 2 to 18 y range,[11] and 2.38 mg/kg/d for 52 healthy London children aged 1 to 15 y.[14] For Mg, our similarly adjusted mean urinary value of 2.0 mg/kg/d contrasts with the higher 2.82 mg/kg/d in 23 of the London children.[14] For all of these selected solute excretions compared, the failure to get complete collection in about half of the instances predisposes to a greater or lesser degree of underestimation for our series.

The primary task of our analysis for solute interactions was to tabulate association. With a total of 36 cross-variable correlations, up to two associations could be expected to be significant by chance alone. Thus, at least 20 of the correlation coefficients meeting the <0.05 criterion are likely to be truly significant. Furthermore, the pattern of associations was interesting. Three variables, Uosm, Na and K, showed significan cross-correlations in 7 of 8 instances 87.5%); in each case it was uric acid concentration that failed to associate at a significant level. Thereafter, in descending order, significant correlations were shown for Mg in 6 instances, Pi in 5, Ca in 4, and urinary volume in 3. Uric acid was the only solute of interest that showed no mutual associations whatsoever. Of the nine significant inverse associations seen, three involved urinary volume, and remainder were the entire series of significant relationships with urea concentration. The inverse volume relationships, with Uosm, Na and K are intuitively understandable, as a "dilution effect." i.e. more water lowers concentration of the electrolytes most associated with Uosm, and Uosm, itself. The inverse relationship with urea and osmolality, however, is not intuitively obvious. In fact, as stated, for prediction of plasma osmolality, urea is one of the positive determinants.[8,9] In order to verify the consistency of this indirect relationship between urea and Uosm, we generated Spearman partial correlations for seven subgroups (Table 3). Consistent with the direction at the total-sample level, inverse and significant associations were seen for each sex, for the sample polarized for age, and within each of the three different centers. In fact, the inverse relationship of urinary urea and Uosm follows the classical basic physiology of renal regulation of water balance.[15,16] In order to conserve water within the body, and hence excrete more concentrated urine, urea is more aggressively absorbed in the counter-current exchange in the terminal inner medullary collecting duct (loop of Henle) of the kidney.[17] Hence, less urea in the urine is at the basis of the generation of higher osmolality and vice versa.

We recognize a series of strengths and limitations in the present study. A major strength is that is addresses the age-group of young children, a population segment relatively ignored in the existing literature. Furthermore, it brings together the skilled analysis with modern osmolality equipment with an effort at quantitative collection of urine and the concurrent analysis of seven important solutes in human urine derived from the diet, endogenous tissue breakdown or both. Our children are offered a common and controlled dietary menu of both foods and beverage within the institutional center-day, which might theoretically narrow the sample-wide variance. At the same time,

Table 3: Full and partial Spearman correlation coefficients for the association of urea concentration (x-axis) and Uosm (y-axis)

| Correlation | r-value | P-value |
|---|---|---|
| Total (n= 65) | -0.742 | <0.001 |
| Girls (n= 32) | -0.772 | <0.001 |
| Boys (n= 33) | -0.658 | <0.001 |
| Older (n= 35) | -0.842 | <0.001 |
| Younger (n= 30) | -0.594 | 0.001 |
| Site A (n=19) | -0.651 | 0.003 |
| Site B (n=19) | -0.858 | <0.001 |
| Site C (n=27) | -0.767 | <0.001 |

Uosm=urine osmolality

the latter constitutes a contextual limitation, as that the study series is a convenience sample of children with unique dietary long-term homogeneity, generalization to the population at large would not be justified Furthermore, although the intention was to collect quantitative 24-h urine excretion on all collection occasions, an internal indicator[18] suggests that this was accomplished only in a slight majority of instances.[3]

In conclusion, a situation in which a define offering of an institutional menu and efforts at 24-h urinary collections converged in the study of systematic Uosm measurement; this has facilitated our gaining insights into the relationship of urinary excretion of selected solutes in the context of their relationship to Uosm, which is a proxy indicator for human hydration state. The most striking features overall in this group of relatively well-hydrated preschool children is the interindividual variation in excretion of the speci c species analyzed. The analyses allowed us to confirm the determinant roles in Uosm of urea and the principal electrolytes, Na and K and to appreciate a harmony in the collateral collinear associations with other excreted solutes.

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# Chapter 3

# Variation in Hydration Status within the Normative Range is Associated with Urinary Biomarkers of Systemic Oxidative Stress in Guatemalan Preschool Children

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# Variation in Hydration Status within the Normative Range is Associated with Urinary Biomarkers of Systemic Oxidative Stress in Guatemalan Preschool Children[1]

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[5] Abbreviations used: CeSSIAM, Center for the Studies of Sensory Impairment, Aging, and Metabolism; F2-Iso, 15-isoprostane F2t; SOSEP, Secretaría de Obras Sociales de la Esposa del Presidente (Secretariat of the Beneficial Work of the First Lady); Uosm, urinary osmolality; Uvol, urine volume; 8-OHdG, 8-hydroxy-deoxyguanosine.

**Authors' Responsibilities:**

*María-José Soto-Méndez: Planned the fieldwork, collected samples and performed laboratory analyses, analyzed data and wrote the manuscript. Laura Campaña-Martín and Victoria Martín-Laguna: performed laboratory analyses.*
*Concepción M. Aguilera and María D. Mesa: Supervised laboratory analyses and performed results interpretations.*
*Klaus Schümann and Ángel Gil: Designed the study and wrote the manuscript.*
*Noel W. Solomons: Designed the study, planned the fieldwork and wrote the manuscript.*
The authors report no conflict of interest.

**Abstract**

*Background:* Researchers have increasingly sought noninvasive methods to determine the health and nutritional status in humans. Easy and painless to collect, human urine is a source of noninvasive biomarkers. The potential mechanisms of any giardiasis-growth interaction have not been exhaustively explored.

*Objectives:* We aimed to explore the relation between systemic oxidative stress biomarkers excreted in urine and urinary osmolality (Uosm).

*Design:* The current trial was a descriptive, cross-sectional study. We collected seventy-eight samples of 24-h urine in preschoolers who

were attending daycare centers in the Western Highlands province of Quetzaltenango, Guatemala. After we measured the total urine volume (Uvol), the aliquot was stored for the later determination of Uosm as a hydration biomarker and to measure 15-isoprostane $F2_t$ (F2-Iso) and 8-hydroxy-deoxyguanosine (8-OHdG) as biomarkers of cellular oxidation with the use of ELISA assay kits in Spain. Descriptive statistics and linear (Spearman rank-order [$r_s$]) and nonlinear (goodness-of-fit) correlations were performed.

*Results:* Twenty-four hour Uvols ranged from 65 to 1670 mL, whereas the Uosm varied between 115 and 1102 mOsm/kg.

With respect to oxidative biomarkers, the 24-h urinary output of F2-Iso and 8-OHdG had median values of 748 and 2793 ng/d, respectively. The Uvol correlated inversely and significantly with the concentrations of both oxidative biomarkers (F2-Iso $r_s$ = 20.603, $P < 0.001$; 8-OHdG $r_s = 0.433$, $P < 0.001$), whereas the Uosm was correlated in a direct manner (F2-Iso $r_s = 0.541$, $P < 0.001$; 8-OHdG $r_s = 0.782$, $P < 0.001$) when analyzed as a concentration. Associations were weaker when they were analyzed as the total 24-h production.

*Conclusion:* Preschool children from the Western Highlands of Guatemala show strong correlations between hydration status measured through the use of Uosm and biomarkers of oxidative stress in urine. Thus, a relatively superior hydration status is associated with a quantitative reduction in urinary excretion of systemic oxidation products. This trial was registered at clinicaltrials.gov as NCT02203890.

*Key words:* Guatemala, hydration, oxidation, preschool children, urinary osmolality. Am J Clin Nutr 2015;102: Printed in USA. 

## Introduction

The Xela-Variance project, which is formally known as the Study on the Normative State and Inter- and Intra-individual Variation in Growth, Hematology, Hydration and Markers of Oxidation, Infection and Inflammation in Preschool Children With a Similar Dietary Intake, studies the variability in biomarkers from an assortment of different biological domains in preschool children living under precarious conditions in semi-urban and rural settings in the Western Guatemalan province of Quetzaltenango. All participants of the study attended a national system of daycare centers that offer a common menu.A previous report from this project has been published[1].

The study emphasized the use of as many noninvasive procedures and collections as possible to obtain diagnostic variables in saliva, feces, and spot and 24-h urine samples. The exception was a single 4-mL blood extraction that served as a reference for noninvasive hematologic and biochemical variables.

Urine is hygienically and abundantly available; its collection is noninvasive and not painful, and it permits the detection of a large number of biomarkers of human physiology. In the project in Quetzaltenango, these determinations included the urinary 24-h volume, bacteria and white blood cells, electrolytes, inorganic and organic solutes, creatinine, osmolality, and biomarkers of systemic oxidation. Urine can be collected either non quantitatively as a spot urine sample or quantitatively over a defined time period (such as 24 h). Admittedly, in children, it is easier to collect spot urines that are normalized for varying concentrations over the day. Such normalization uses creatinine concentrations that differ in response to varying renal function and the turnover of skeletal muscle[2–4].

Twenty-four-hour collections in free-living children are rarely reported although they have been consistently used in the Dortmund Nutritional and Anthropometric Longitudinally Designed study in Dortmund, Germany[5–7]. The group conducting the study pioneered creatinine-based methods to assess the completeness of quantitative collection in children [8]. A previous study at the Center for the Studies of Sensory Impairment,Aging, and Metabolism (CeSSIAM)[5(9)] measured urinary oxidative biomarkers, notably thiobarbituric acid reactive

substances and 8-hydroxydeoxyguanosine (8-OHdG), in healthy, free-living Guatemalan university students. The study documented the variation in these oxidative biomarkers as well as the biological associations even within the normal range of excretion. Because of the range of variance in urinary variables collected in the Xela-Variance study, we had the opportunity to explore these variables in an additional free-living population, this time in children. In addition,wewere able test an interesting hypothesis on the mode of interaction between systemic oxidation and hydration status. In this article, we present the results of this examination.

## Methods

### *Study design*

This descriptive, cross-sectional field survey presents results on the variation in hydration status and their association with variables on cellular oxidation.
This survey is part of a larger study called Xela-Variance[1].

### *Setting and subjects*

Guatemala has the highest prevalence of stunting in children.<5 y old in Latin America. The infant mortality rate is the third highest in the world[10].
This study was conducted in the Western Highlands in the province of Quetzaltenan-go, which is located 220 km from the capital at an altitude of 2357 m above sea level[11]. The Secretaría de Obras Sociales de la Esposa del Presidente (Secretariat of the Beneficial Work of the First Lady) (SOSEP) made 3 daycare centers (hogares comuni-tarios) available for our study. Center A was located in La Esperanza, which is a semi-urban area 2 mi (3.3 km) away from downtown Quetzaltenango. Center B was located in the outskirts of the city of Quetzaltenango in La Puerta del Llano, and Center C was situated in a rural area 15 mi (24 km) away from Quetzaltenango in La Estancia, San Martín Chile Verde. Children who were attending centers A and B were from the 2 different ethnic origins Mam-Mayan and Ladino; and children who were attending center C were exclusively of Mam-Mayan origin. However, some of the living habits, pastimes, and physical characteristics varied between centers.

*Exclusion Criteria:* Children were eligible for the study if they were attending the selected SOSEP centers and were 2–7 y old. Subjects had to have a ≥80% attendance record in the center during the study and be apparently healthy and without dietary restrictions related to the foods offered in the SOSEP menu. Children who did not adhere to the urine-collection routine, who refused to participate in the study, or whose parents did not sign the consent form were excluded.

*Ethical considerations:* The Human Subjects Committee of the CeSSIAM granted ethical approval for the study protocol. The director of the SOSEP system in Quetzaltenango authorized us to perform the study. A parent or guardian signed the written con-sent form. When it was necessary, and to provide all food items on the menu, study funds were used to complete the diet offered to the children.

### Urine collection, volume measurement, aliquots, and storage

In the last week of the 8-wk dietary cycle, we attempted to collect a 24-h urine sample in all subjects. We started the urine collection when a child arrived at the daycare center (between 0700 and 0800) with the SOSEP personnel assisting with the collection with the use of the BD evacuated tube (no. 364999) plastic 24-h collection container (Becton Dickinson and Co.). Parents were previously trained to continue the urine collection at home. The sample collection was finished after 24 h in the center. The collection process was repeated if incomplete. When the collection was

completed, we brought the urine sample to the laboratory where we measured the to-tal urine volume (Uvol) delivered in cylinders graded in milliliters (Becton Dickinson and Co.). The total urine sample was agitated to obtain homogeneous aliquots. One aliquot was stored at2808Cfor 37–46wk and sent to Granada, Spain, on dry ice to determine the Uosm and oxidative biomarkers.

## Anthropometric measures

Anthropometric determinations of weight were performed with the use of the calibrated Tanita Model BC522 digital scale (Tanita) and expressed as kg to the nearest 0.1 kg. Children were asked to remove their shoes for this process, and results were adjusted for clothing (a standard weight-for-clothing factor for the daytime clothes of girls and boys was subtracted).

## Laboratory analyses

*Measurement of urinary osmolality*

Analyses of the urinary osmolality (Uosm) were performed with the use of the Osmomat 030 osmometer (Gonotec) according to the manufacturer's specifications in Granada, Spain. The instrument works on the principle of freezing-point depression according to Peltier. Immediately before measurement, aliquots were mixed for ~ 3 min and 50 µL were transferred to the measurement vessel. Results are expressed in mOsm/kg urine.

*Measurement of urinary biomarkers of oxidative stress*

15-Isoprostane F2t (F2-Iso) and 8-OHdG are biomarkers of systemic oxidation and were determined with the use of ELISA as-say kits [catalog nos. EA84.102606 (Oxford Biomedical Research Inc.) and IM-KOGHS 040914E (JaICA, Nikken SEIL Co. Ltd.), respectively].

*Measurement of urinary creatinine*

Urinary creatinine concentrations were measured in Guatemala City in the Pharmacy School laboratory, University of San Carlos. These measurements were performed in a separate aliquot that was stored at refrigeration temperature for 2–4 d with the use of a standard colorimetric Jaffe-reaction method.

## Data handling and statistical analyses

SPSS software (version 20; IBM) was used to create a database. Data were checked for normality distribution, and descriptive statistics were expressed as the median, 95% CI, minimum, and maximum.
Associations of values among individual pairs of urinary variables were tested with the use of the Spearman rank-order coefficien for all 78 subjects.
Moreover, because of post hoc concerns surrounding the completeness of the urine collection, subanalyses were performed in the urine samples deemed to be more complete. At one level, we used the urine creatinine criterion of Remer et al.[8] to gau-ge the complete collection; at another level, we filtered urine samples on the basis of the expected minimal hourly urine output for children of .1.0 m/kg body weight[12].

## Results

*Characteristics of the population*

We successfully collected 24-h urine samples in 78 of 87 (90%; 45 boys and 42 girls). The mean age was 4 ± 1 y SD (range 2–6 y). We found no significant difference in age between sexes (P = 0.177).

*Descriptive statistics of Uvol, Uosm, and oxidative biomarkers*

Table 1 presents descriptive statistics [medians

(95% CIs) and ranges] of Uvol, Uosm, and oxidative biomarkers. The 24-h Uvol ranged between 65 and 1670 mL. The minimum value for Uosm was 115 mOsm/kg, and the maximum value was 1102 mOsm/kg. With respect to oxidative biomarkers, the 24-h urinary output of F2-Iso and 8-OHdG had median values of 748 and 2793 ng/d, respectively. Both biomarkers showed wide variations. With the use of volume and output data, we expressed concentrations for both biomarkers in ng/mL and further adjusted for creatinine, which resulted in an expression of ng/mg.

## Correlation of 24-h Uvol, Uosm, and quantitative excretion of oxidative biomarkers

Table 2 shows a correlation hemimatrix involving 24-h Uvol, Uosm, and total F2-Iso and 8-OHdG production. The Uvol was correlated significantly and negatively with Uosm (P = 0.001); it was also correlated significantly, but positively, with both oxidative stress biomarkers (P = 0.004 and <0.001, respectively). No significant correlation was shown between Uosm and F2-Iso (P = 0.163), whereas Uosm was correlated significantly with 8-OHdG (P <0.001). A significant mutual correlation between F2-Iso and 8-OHdG production was shown with the use of the Spearman test (P < 0.001).

*Goodness-of-Fit Regressions*

Because the Spearman test exclusively assumes linear relations, we explored whether a nonlinear association might show a stronger correlation. For this purpose, we ran the goodness-of-fit model exhaustively to generate a family of correlation coefficients. As shown in Figure 1A and Table 2, the nonlinear power

**Table 1:** Descriptive Statistics of Urine Volume, Osmolality and Oxidative Stress Biomarkers in Guatemalan preschool children (n=78).

| | MEDIAN | 95% CI | MIN-MAX |
|---|---|---|---|
| Uvol (mL/24 h) | 460 | 436, 575 | 65 – 1670 |
| Uosm (mOsm/kg) | 430 | 407, 491 | 115 – 1102 |
| **24-hour Production** | | | |
| F2-Iso (ng/24 h) | 748 | 771, 1034 | 113 – 3020 |
| 8-OHdG (ng/24 h) | 2793 | 2544, 3912 | 135 – 20244 |
| **Uncorrected Concentration** | | | |
| F2-Iso (ng/ml) | 1.75 | 1.93, 2.81 | 0.50 – 11.80 |
| 8-OHdG (ng/ml) | 5.69 | 6.26, 9.80 | 0.55 – 38.61 |
| **Creatinine-Adjusted Concentration** | | | |
| F2-Iso (ng/mg creatinine) | 5.8 | 6.2, 9.5 | 2.2 – 52.0 |
| 8-OHdG (ng/mg creatinine) | 18.2 | 18.6, 26.0 | 3.5 – 86.3 |

**Figure 1:** Scattergrams goodness-of-fit model between urine volume and total production of oxidative stress biomarkers in 24 h (n=78). Panel A: urine volume (Uvol) vs 15-isoprostane F2t (F2-Iso). Panel B: Uvol vs 8-hidroxydeoxyguanosine (8-OHdG).

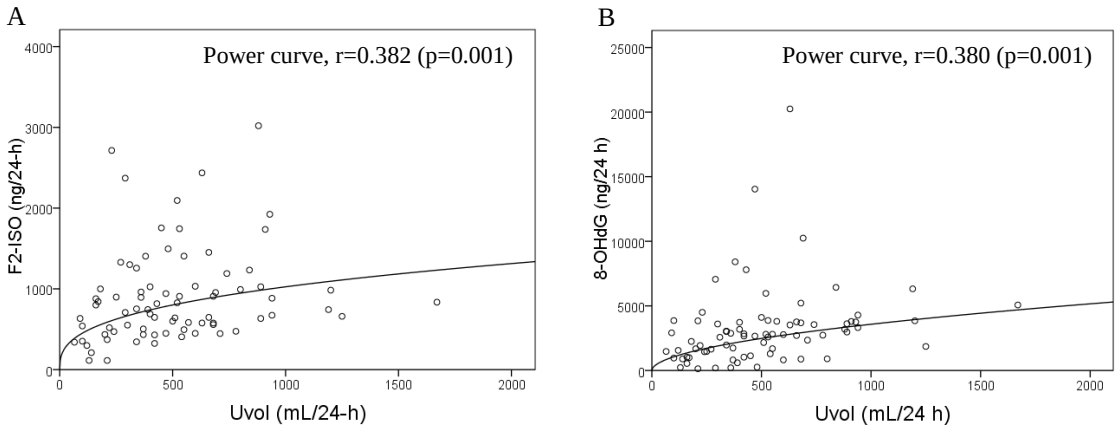
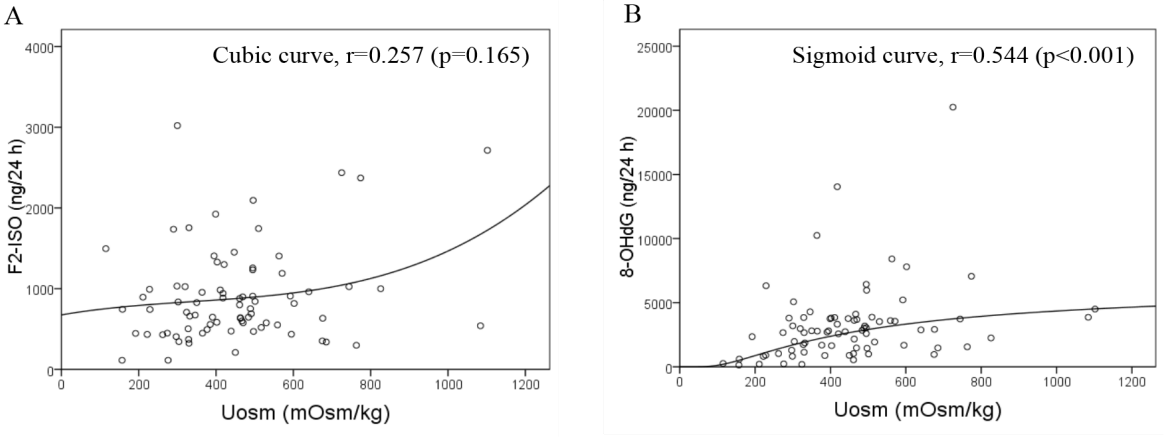



**Table 2:** Hemi-matrix of associations between urine volume, osmolality and 24-h production of oxidative stress biomarkers in Guatemalan preschool children (n=78).

| | | Uvol (ml/24hour) | Uosm (mOsm/kg) | F2-Iso (ng/24 h) | 8-OHdG (ng/24 h) |
|---|---|---|---|---|---|
| Uvol (ml/24 h) | $r_s$ value | 1.000 | | | |
| Uosm (mOsm/kg) | $r_s$ value | -0.363 | 1.000 | | |
| | p-value | 0.001 | | | |
| F2-Iso (ng/24-h) | $r_s$ value | 0.324 | 0.160 | 1.000 | |
| | p-value | 0.004 | 0.163 | | |
| 8-OHdG (ng / 24 h) | $r_s$ value | 0.421 | 0.387 | 0.413 | 1.000 |
| | p-value | <0.001 | <0.001 | <0.001 | |

**Figure 2:** Scattergrams goodness-of-fit model between urinary osmolality and total production of oxidative biomarkers in 24 h (n=78). Panel A: urinary osmolality (Uosm) vs 15-isoprostane F2t (F2-Iso). Panel B: Uosm vs 8-hidroxydeoxyguanosine (8-OHdG).



association between the total Uvol and F2-Iso 24-h production provided the highest r value (0.382), which was slightly higher than the $r_s$ values obtained with the use of the Spearman test (0.324). However, for 8-OHdG, the non-linear association had a substantially lower $r_s$ value than obtained with the linear assumption (0.380 vs 0.421, respectively) (Table 2 and Figure 1B).

Similarly, we tested the fit of non-linear associations for Uosm and oxidation biomarkers for F2-Iso (Figure 2A). However, even the best goodness-of-fit association failed to be significant. For 8-OHdG, this association presented an r = 0.544, which was higher than the $r_s$ value that was determined with the use of the Spearman test.

*Partial Spearman Correlations of Selected Subgroups*

The following 2 approaches were used to assign subgroups with greater likelihoods of having complete 24-h collections for additional post hoc (partial-correlation) analyses: one method was based on creatinine excretion[8], and the other method was based on the expected urine production[12]. With the creatinine criterion, 41 of 78 subjects (52%) were selected as having made a complete collection of urine; with the use of the renal physiologic urine excretion for body size approach, 53 of 78 subjects (68%) were judged to have a plausible Uvol. There were 38 individuals in common across the 2 subgroups.

When Spearman correlations of the hemi-matrix were generated with the use of only the 41 children who reached the Remer creatinine criterion[8] for the completeness of collection, there were major changes in certain strengths of associations (data not shown). The correlation coefficient of 24-h Uvol with Uosm rose by 92% to 0.694 compared with that for the inclusion of all subjects (Table 2). The relation of Uosm with F2-Iso remained virtually unchanged, whereas that with 8-OHdG declined by 62% to 0.146, which was an association that was no longer significant.

Conversely, when the correlation hemi-matrix was generated with the 53 subjects who had a plausible Uvol on the basis of body size, a general strengthening was seen throughout. The Uosm association with F2-Iso had an $r_s$ value of 0.274, which reached significance (P = 0.047) for the first time in a matrix analysis that was based on 24-h volume (Table 2). The comparable association with 8-OHdG also strengthened by 53%, compared with that for the inclusion of all subjects inclusion, to an $r_s$ value of 0.592 (P < 0.001).

**Correlation of Uvol, Uosm, and concentrations of oxidative biomarkers unadjusted and adjusted for creatinine**

Because the luxury of timed and quantitative 24-h urine collections may not be available under all circumstances, particularly not in children, we modeled the alternative approach of analyzing the data by the concentration after an ambient urine collection. Correlation coefficients are presented in a hemi-matrix in Table 3. With respect to the Uvol, the Spearman correlation with the F2-Iso concentration was inverse and highly significant; it was almost twice as strong as that seen with the total production (Table 2). The relation with

**Table 3:** Hemi-matrix of associations between urine volume, osmolality and concentration expression of oxidative stress biomarkers in Guatemalan preschool children (n=78).

| | | Uvol (ml/24 h) | Uosm (mOsm/kg) | F2-Iso (ng/mL) | 8-OHdG (ng/mL) | F2-Iso adj. for Creatinine (ng/mg) | 8-OHdG adj. for Creatinine (ng/mg) |
|---|---|---|---|---|---|---|---|
| Uvol (ml/24 h) | $r_s$ value | 1.000 | | | | | |
| Uosm (mOsm/kg) | $r_s$ value | -0.363 | 1.000 | | | | |
| | p-value | 0.001 | | | | | |
| F2-Iso (ng/mL) | $r_s$ value | -0.603 | 0.541 | 1.000 | | | |
| | p-value | <0.001 | <0.001 | | | | |
| 8-OHdG (ng/mL) | $r_s$ value | -0.433 | 0.782 | 0.542 | 1.000 | | |
| | p-value | <0.001 | <0.001 | <0.001 | | | |
| F2-Iso adj. for Creatinine (ng/mg) | $r_s$ value | -0.531 | -0.068 | 0.799 | 0.110 | 1.000 | |
| | p-value | <0.001 | 0.555 | <0.001 | 0.337 | | |
| 8-OHdG adj. for Creatinine (ng/mg) | $r_s$ value | -0.342 | 0.497 | 0.360 | 0.843 | 0.171 | 1.000 |
| | p-value | 0.002 | <0.001 | 0.001 | <0.001 | 0.133 | |

Spearman's rank-order test was used to determine correlations.
Uvol: urine volume; Uosm: urinary osmolality; F2-Iso: 15-isoprostane F2t; 8-OHdG: 8-hidroxydeoxyguanosine.

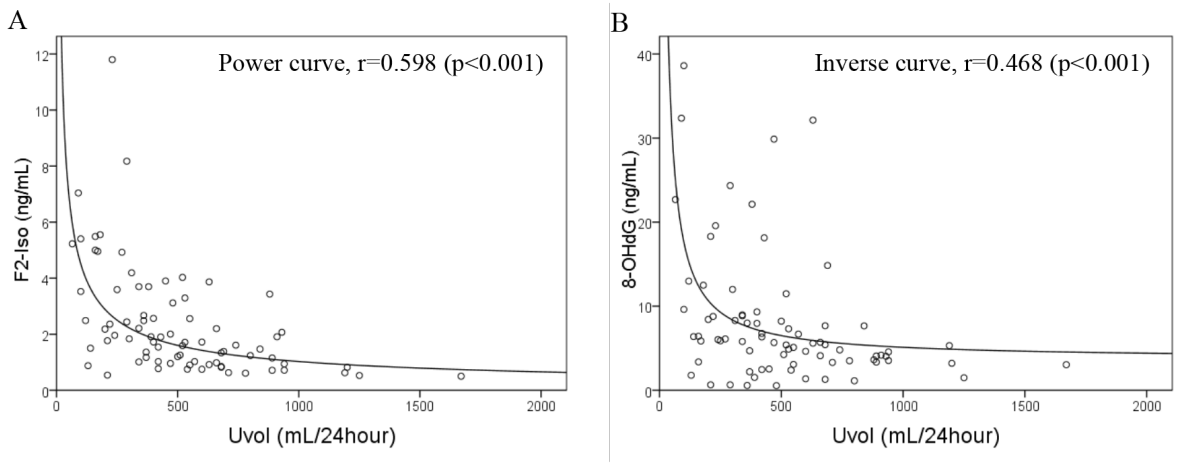
8-OHdG was also inverse but showed an almost identical Spearman $r_s$ value. In these analyses, osmolality and F2-Iso, as tested with the use of the goodness-of-fit, nonlinear approach, showed a significant positive correlation. For 8-OHdG, the correlation was positive and significant but showed an $r_s$ value that was almost twice as high as that obtained with the total production calculations. Finally, the intercorrelation of the 2 oxidative markers was positive and significant but higher than what was found with total 24-h outputs.

We also expressed Spearman correlations for the concentration value adjusted for creatinine (Table 3). As expected, the associations within each biomarker between the adjusted and nonadjusted values were of the highest order ($r_s$ = 0.799 for F2-Iso; $r_s$ = 0.843 for 8-OHdG). In no instance, across 7 pairs of associations, did adjustment for the urinary concentration lead to a stronger correlation than was seen with the nonadjusted concentration value. The association between Uosm and F2-Iso was significant when we used the Spearman test with a simple concentration, and it became non-significant after adjustment with the creatinine concentration. The correlation between the 2 oxidative stress biomarkers was significant (rs =0.542), but there was no significant correlation ($r_s$ = 0.171) after creatinine adjustment. No goodness-of-fit exploration was performed with creatinine-adjusted values.

*Goodness-of-fit regressions*

In Figure 3, we return to the goodness-of-fit exploration with the use of unadjusted concentrations of biomarkers.
With respect to the Uvol, power- and inverse-curve relations provided the best fit for the association with respective oxi-dative stress biomarkers. In the case of F2-Iso, the nonlinear power curve gave the best r value (0.598), the magnitude of which was virtually identical with the one obtained for the Spearman correlation $r_s$ (-0.603) (Table 3) but with the algebraic sign reversed. When we analyzed 8-OHdG, the respective correlation coe-fficients were similarly close in numerical values but with a slightly stronger inverse nonlinear assumption. When we looked at the nonlinear relations between both oxidative biomarkers and Uosm (Figure 4), we showed an r value of 0.703 with F2-Iso, which was higher than the rs of 0.541 shown with the Spearman test. By contrast, relations

**Figure 3:** Scattergrams goodness-of-fit model between urine volume and concentration of oxidative biomarkers in 24 h (n=78). Panel A: urine volume (Uvol) vs 15-isoprostane F2t (F2-Iso). Panel B: Uvol vs 8-hidroxydeoxyguanosine (8-OHdG).



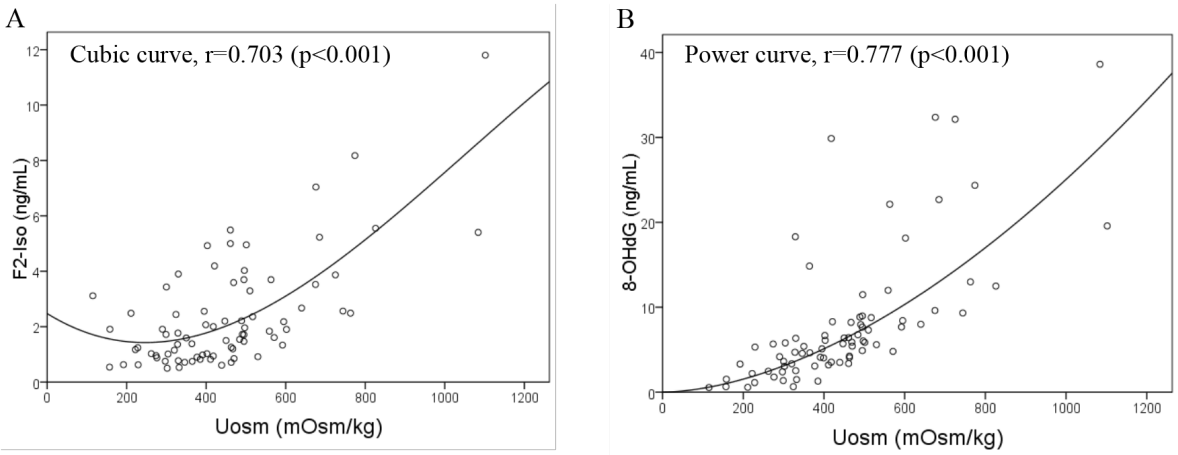

for 8-OHdG showed no superiority in association strength for either linear or goodness-of-fit associations.

*Partial Spearman Correlations of Selected Subgroups*

Partial-correlation analyses, which were performed in urine samples that were more likely to represent complete collections according to the Remer criterion[8] (n = 41) had a profound and positive impact on the strengths of associations that were based on concentrations of the biomarkers. This effect was most notable for the >50% increases in the correlation coefficient value with the 24-h Uvol, which was seen with the 8-OHdG concentration, both unad-justed and normalized for creatinine, than with the full series (Table 3). The Uosm relation with both oxidative stress biomarkers remained stable when unadjusted but rose 4-fold with the creatinine-adjusted values for F2-Iso. Also, within this subselected series, the various intercorrelations in biomarker concentrations improved markedly. For instance, the correlation between F2-Iso in the simple concentration with its creatinine-adjusted value rose from 0.799 to 0.900.

In contrast to what occurred when. The Uosm relation with both oxidative stress biomarkers remained stable when unadjusted but rose 4-fold with the creatinine-adjusted values for F2-Iso. Also, within this subselected series, the various intercorrelations in biomarker concentrations improved markedly. For instance, the correlation between F2-Iso in the simple concentration with its creatinine-adjusted value rose from 0.799 to 0.900. In contrast to what occurred whenthe plausible urine production criterion was applied[12] with 24-h volume interactions, in the current study, this criterion had a con-trasting effect in the same subgroup of 53 subjects. A Spearman correlation hemimatrix in this subgroup showed a general decline in the strengths of associations compared with those for full-matrix values in Table 3 (data not shown). Note that the correlation coefficient between Uosm and the F2-Iso concentra-tion diminished by 42% to 0.311, whereas the Uosm associations with

**Figure 4:** Scattergrams goodness-of-fit model between urinary osmolality and concentration of oxidative biomarkers in 24 h (n=78). Panel A: urinary osmolality (Uosm) vs 15-isoprostane F2t (F2-Iso). Panel B: Uosm vs 8-hidroxydeoxyguanosine (8-OHdG).




the 8-OHdG concentration and with its creatinine-adjusted concentration, respectively, both showed declines in the $r_s$ value by > 8%. A goodness-of-fit modeling was not performed with the partial-correlation groups.

## Discussion

These observations were made in the context of a larger study of Guatemalan preschool children in which efforts were made to collect 24-h samples of urine. Two classes of urinary analyses were combined in this evaluation. The assay for estimating human hydration Uosm[13] was joined with 2 excretory indicators of oxidative stress at the systemic level. One of these indicators is an isoprostane derived from the oxidation of fatty acids in the lipid membrane and circulating lipoproteins. The other indicator is a degradation metabolite of the nucleoside guanine derived from DNA. The 2 indicators represent different intracellu-lar sites for an attack by a free-radical action. Therefore, our studies revealed 2 interesting phenomena. First, the modest variation in hydration stage, which fluctuates well within the normal osmolality range, is often strongly associated with the excretion of both oxidative stress biomarkers. Second, the associations with the biomarkers for distinct cellular targets follow a parallel course with only a few notable exceptions. Existing data indicate F2-Iso a mounts are relatively stable within individuals but are widely variable across persons[14]. The amounts can be modified with modest interventions. For example, a 4-wk study with red wine and nonalcoholic red wine consumption in Dutch subjects with the use of 24-h urine collections demonstrated a rightward shift of the urinary biomarker concentration, albeit one that was still within the normative range of F2-Iso (157 pg/ mg creatinine) after red wine consumption; this amount compared with ~141 pg/mg creatinine with the control beverage[15]. Similar findings were revealed in another human trial that compared grape polyphenols supplementations in healthy adults with unsupplemented controls with significantl lower concentrations of F2-Iso (mean values: 520 vs. 473 pmol/mmol creatinine)[16]. With respect to cellular oxidation, a previous study at the CeSSIAM by Orozco et al.[9] established the synchronous rise and fall of 8-OHdG and thiobarbituric acid reactive substances, which are lipid-oxidation biomarkers that are similar to F2-Iso. Several other studies have shown coordination and parallel responses across these 2 general classes of urinary oxidative stress biomarkers[17– 21].

On the hydration side, a recent exploration from López-Plaza et al.[22] demonstrated that

healthy adults with adequate hydration status, as assessed with the use of bioelectrical impedance rather than with Uosm, presented lower concentrations on pro-inflammatory cytokines (serum interferon-g and IL-6 and IL-1 in the aqueous phase of stools). Such observations provide a certain degree of confirmation of the general principle that a mild variation in hydration status can be associated with changes in a functional biomarker

The origin of the variation in the hydration state of our children is not precisely known. Because the children had a common offering of meals and snacks, the intake of liquids across the sample could be expected to have a narrower range than that of peers who consume liquids exclusively within the family setting. However, liquids consumed at home and different physical activities as well as children of different body sizes receiving a uniform liquid offering would be candidate factors to produce a variation in Uosm from child to child. In comparative terms, these children in Guatemala were relatively well hydrated as shown in our earlier comparative review of Uosm values in Quetzaltenango compared with in reports from Europe and Israel[1]. Our median values of lower osmolality attest to a greater dilution of the urine[1].

We are also not certain of the influence affecting cellular oxidation in this population. A generic list of factors associated by Berk et al.[23], in relation to the close relationship of the environmental stimuli and inflammatio that may serve as a basis for oxidation, as well. These factors include “psychosocial stressors, poor diet, physical inactivity, obesity, smoking, altered gut permeability, atopy, dental cares, sleep and vitamin D deficienc .” The situations of the children from these poor communities in Guatemala leave open the possibility that one or several of these factors are present in the households, environments, or lives of our subjects.

What we are observing in the relation between Uosm and markers of oxidative stress is purely an association, and associations, per se, cannot provide definitive information on causality. Is the hydration state driving the oxidation state or vice versa? Or are common influences that determined both sates in respective directions? These queries would be the elements of a speculative discussion. The fact that nonlinear correlations were, in some instances, stronger than linear ones were attests to the complexity of the associations; this complexity would tend to discard the notion of an external influence affecting both variables. For example, in the current experience, we showed that F2-Iso was correlated significantly with Uosm when expressed as a concentration but was NS when expressed as total 24-h excretion. The methodologic issues that surround the quantitative completeness of individual urine collections over 24 h from young children impose themselves on the interpretation of the findings.

Logically, the strength of the associations between Uosm and the oxidative biomarkers should be comparable when expressed either as the total 24-h output or as a concentration; the former expression is based on the concentration multiplied by the 1-d Uvol. What could distort this relation, however, would be a failure to get complete collections and, therefore, an underestimation of biomarker excretion in a segment of the sample; this underestimation would have attenuated the strength of any true associations[24]. Evidence from both urinary creatinine excretion and net collected Uvols placed 48% and 32% of all collections in doubt of their quantitative completeness. As expected, the elimination of a source of misclassification by selecting concentrations from the more complete collections also improved the strength of the associations; this effect was true not only with the expression of total excretion but also with direct and adjusted concentrations. However, the simple concentration data, ignoring the total volume and with the use of the data from all 78 subjects, revealed all of the important biology in the osmolality oxidation relations in the initial analysis. Spot samples of urine (i.e., specimens from a

casual micturition) are often used in the estimation of hydration status when a quantitative collection is impossible, even in children[25, 26]. Some authors have criticized this spot-sample approach with the use of Uosm as invalid compared with a quantitative collection[27].

A recent study in adults, however, compared the timed components of spot urines with the osmolality of the pooled sample; casual samples in the afternoon best reflected the average across-day osmolality[28].

Even if incomplete, the more urine collected and pooled, the most representative should be the Uosm for ambient hydration. Granted that all urine samples were more than a casual single micturition, our experience points to the possibility of learning a great deal about urinary biomarkers from prolonged, but not necessarily totally quantitative, collections.

We acknowledge a series of strengths and limitations of the current study. The major strength was that the study adds additional experience to hydration research in children. A related strength, which could also be recognized in the context of limitations, is that we made the attempt to collect 24-h urine; almost one-half of the collections failed to include all of the urine produced over a full day. The reliance on Uosm, which is possibly the best human hydration biomarker, made our assertion of a hydration status more credible. The large variance in subjects in hydration and oxidation status favored the resolution of a mutual association. One of the limitations of this study was that we only had the resources to explore 2 among many possible urinary biomarkers of oxidation.

In conclusion, preschool children from the Western Highlands of Guatemala present strong correlations between hydration status measured through Uosm and biomarkers of oxidative stress in urine. The relation indicates that a relatively superior hydration status is associated with quantitative reductions in the urinary excretion of products of cellular oxidation. We should remember that the variance in both domains of interest is within the normal range of variation such that there is a strong interplay of hydration and oxidative stress in the daily context of normative health in indigenous communities in Western Guatemala.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The authors' responsibilities were as follows—MJS-M: collected and analyzed the data; MJS-M and NWS: planned the fieldwo k; CMA: organized the sample biochemical analyses and storage in Granada and coordinated the oxidative biomarkers analyses; LC-M and VM-L: performed the oxidative biomarkers analyses; KS, NWS, and AG: designed the study; MJS-M, KS, NWS, and AG: wrote the manuscript; and CMA, LC-M, and VM-L: approved the manuscript. This work will be used in partial fulfillment of the doctoral requirements that will allow the MJS-M to obtain the Ph.D. degree in the University of Granada, Spain. None of the authors reported a conflict of interest related to the study.


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# SECTION B

# INTERACTIONS WITHIN AND ACROSS BIOMARKERS OF OXIDATION AND INFLAMMATION


Sheny Romero, *Recolección de muestras*, La Puerta del Llano, Quetzaltenango, 2012

# Chapter 4

## Strong Associations Exist among Oxidative Stress and Anti-oxidant Biomarkers in the Circulating, Cellular and Urinary Anatomical Compartments in Guatemalan Children from the Western Highlands

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Submitted

*PLoS ONE*

# SUBMITTED

# STRONG ASSOCIATIONS EXIST AMONG OXIDATIVE SETRESS AND ANTIOXIDANT BIOMARKERS IN THE CIRCULATING, CELLULAR AND URINARY ANATOMICAL COMPARTMENTS IN GUATEMALAN CHILDREN FROM THE WESTERN HIGHLANDS

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NWS, KS, and AG designed the study.
NWS and MJSM planned the fieldwork
CMA, MDM, LCM and VML performed the analyses and processed results in order to be analyzed.
MJSM collected and analyzed data.
NWS, MJSM, KS and AG wrote the manuscript, and the rest of the authors approved it.
**Funded by** the Hildegard Grunow Foundation, and Department of Biochemistry and Molecular Biology II, University of Granada.

## Abstract

*Background:* A series of enzymes and non-enzymatic compounds act to pro-tect cells from uncontrolled propagation of free-radicals. It is poorly understood, thou-gh, to what extent and how their interaction is harmonized.

*Objectives:* To explore associative interactions among a battery of urinary and blood biomarkers of oxidative stress and enzymatic and non-enzymatic markers of the antioxidant defense system in low-income children.

*Methods:* For this cross-sectional descriptive study; urine, red cells, and plasma were sampled in 82 preschool children attending three daycare centers in Quetzaltenango Guatemala. Urinary oxidative stress biomarkers included F2-isoprostanes and 8-hydroxy deoxy guanosine.
Red cell enzyme activities included: catalase; superoxide dismutase; glutathione peroxidase and glutathione reductase.
Circulating non-enzymatic antioxidants included: retinol; α-tocopherol; β-carotene; and coenzymes Q9 and Q10.

*Results:* In a Spearman rank-order correlation hemi-matrix, of 55 paired combinations of the 11 biomarkers, 28 (51%) were significantly correlated among each other ($p \leq 0.05$), with the strongest association being retinol and alpha-tocopherol ($r = 0.697$, $p < 0.001$), and 4 (9%) showed a trend ($p > 0.5$ to $\leq 0.10$). F2-isoprostanes showed the greatest number of cross-associations, having significant interactions with 8 of the 10 remaining biomarkers. Goodness-of-fit modeling improved or maintained the r

value for 24 of the significant interactions and for one of the 5 borderline associations.

Multiple regression backward stepwise analysis indicated that plasma retinol, β-carotene and coenzyme Q10 were independent predictors of urine F2-isoprostanes.

*Conclusion:* Numerous significant associations resulted among biomarkers of oxidation and responders to oxidation. Interesting findings were the apparent patterns of harmonious interactions among the elements of the oxidation-antioxidation systems in this population.

## Introduction

The oxidation of substrates in the mitochondria, to generate ATP, is associated with a continuous of free radical formation[1,2]. At the same time, free radicals can damage cellular and organelle membranes, the cell nucleus, and protein chains. Free-radical action forms isoprostanes from lipids[3–6], 8-hydroxy deoxyguanisine from DNA[6–8], and carbonyl-amino acids from peptide chains[9]. Action of redox enzymes is part of the normal cellular metabolism in living organisms[10,11]. Thus, oxidative mechanisms are used to destroy microbes within macrophages[12,13]. Renal excretion of such split-products from lipids and nucleic acids converts them into urinary biomarkers of systemic oxidative stress that help to assess the protective reaction/response by antioxidant mechanisms[14,15].

The antioxidation system in the human organism is complex and geared to the suppression of uncontrolled oxidation while permitting essential and beneficial oxidation reactions[16]. A series of compounds, including dietary nutrients, have free-radical quenching properties or work in conjunction with enzymatic antioxidation reactions or both[17]. Retinol and their derivatives, ascorbate, and tocopherols, as well as diverse carotenoids and co-enzymes Q[18], are among the antioxidant nutrients. Their circulating concentrations roughly reflect the corresponding nutritional status of an individual.

Finally, a series of enzymes protect the cell from oxidation reactions involving molecular or atomic oxygen[19]. These include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR), which are the primary intercellular antioxidant enzymes that detoxify oxygen-containing free radicals produced during the normal aerobic respiration[20–22]. The zinc and copper-dependent SOD transforms the O2-. into $H^2O^2$ and $O2^{20}$, whereas CAT and GSR catalyze the reduction of $H^2O^2$ into H2O [21,23]. GPX works in concert with glutathione reductase, in a system that neutralizes hydrogen peroxide[22].

In a research project entitled “Study on the normative state and inter- and intra-individual variation in growth, hematology, hydration, and markers of oxidation, infection and inflammation in pre-school children with a similar dietary intake”, we collected concurrent data in a series of two urinary biomarkers of oxidative stress, four enzymatic biomarkers of the antioxidant defense system (ADS) in red blood cells (rbc), and five circulating antioxidant nutrients in preschool children in a governmental system of daycare centers. The degree, to which the interactions of biomarkers reflect biological harmonization is the central query of our companion article on the interaction of immunological biomarkers in this population sample[24], included as supplemental material (S1). We use parallel analytical approaches of cross-assocations with linear and non-linear correlations and backward elimination multiple-regressions to quantify the magnitude of interactions. We present here the result of an exploration of the mutual interplay and interactions of biomarkers of oxidative stress and ADS in the context of variable environmental and genetic situations, within the stabilizing influence of a common institutional dietary offering.

## Materials and Methods

*Study Design*

This study was a descriptive, cross-sectional field research on the variation and association among oxidative stress and antioxidant biomarkers. It is part of the larger research entitled: "Study on the normative state and inter- and intra-individual variation in growth, hematology, hydration, and markers of oxidation, infection and infla - mation in pre-school children with a similar dietary intake."

*Setting and Subjects*

Western Highlands of Quetzaltenango, located 136 miles from Guatemala City at 8717 ft above the sea level, were the setting for this research project[25]. Three daycare centers' attendees from the SOSEP (Secretaría de Obras Sociales de la Esposa del Presidente {Secretariat of the Beneficial Works of the First Lady}) system were assigned to participate in this study. Center A in La Esperanza was the semi-urban setting, located 2 miles away from downtown Quetzaltenango: Center B, in La Puerta del Llano was located in a marginal-urban setting in the outskirts of the city of Quetzaltenango; and Center C, was a rural setting situated 15 miles away from Quetzaltenango in La Estancia, San Martín Chile Verde. Almost all children attending the centers were of Maya indigenous ethnicity. However, some of the living habits, pastimes and physical characteristics varied between centers because of the infrastructure and the environment of each location.

*Recruitment and Enrolment*

*Inclusion Criteria:* To be enrolled in the study children had to be attending one of the selected SOSEP Centers, be aged 2 to 7 years, and maintaining an attendance of at least 80% during the 8 weeks of the fieldwork. Furthermore, subjects had to be apparently healthy and with no dietary restrictions related to the foods offered with the SOSEP menu.

*Exclusion Criteria:* Children who did not adhere to the urine collection routine, who refused to participate in the study or whose parents did not sign the consent form were excluded.

*Ethical Considerations:* The study protocol was approved by The Human Subjects Committee of the Center for the Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) and was registered at clinicaltrials.gov as NCT02203890. A parent or guardian signed the written consent form. The SOSEP's director for the Quetzaltenango area had authorized the study. The diet offered to the children was complemented when required, in order to provide all food items on the menu. STROBE statement for this article is included as supporting information file S2)

*Collection, Handling and Storage of Biological Samples*

We collected 24-h urine samples and a 5 mL blood sample during the last of the 8 study weeks. We started urine collection at each daycare center when a child arrived (between 7:00 and 8:00 a.m.) with SOSEP personnel assisting for the collection, using BD Vacutainer® No.364999 plastic 24-h collection container (Becton-Dickinson, New Jersey, USA). Parents continued the collection at home after training. Urine collection was fin - shed after 24 h in the center.
The collection process was repeated if suspected to have been incomplete.
When the collection was completed, we brought the urine to the laboratory, where the total urine sample was agitated in order to obtain homogeneity before aliquoting. One aliquot was stored at -80ºC for 37 to 46 weeks before being sent to Granada, Spain, on dry-ice for measurement of oxidative biomarkers.

An experienced phlebotomist extracted the blood using BD Vacutainer® 4 ml tubes with EDTA (No.367861) and Safety-Lok™ deposable needles (No.367281). Samples were centrifuged to separate red blood cells from plasma; both were stored in Nalgene® Cryogenic Vials (No.5000-0012) at -80ºC until shipment to the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, Granada, Spain in order to determine antioxidant enzymes activity in red blood cells and antioxidant nutrients in plasma.

*Laboratory Assays and Analyses*

Plasma retinol and alpha-tocopherol, ß-carotene, and coenzymes Q9 and Q10 (Co-Q9 and Co-Q10) were determined with the high-performance liquid chromatographic (HPLC) methods using a 100-microliter aliquot of plasma sample previously deproteinized with 1-propanol. Hemoglobin concentration was determined by use of Drabkin´s reagent (Sigma D5941). The final concentration of Hb was adjusted to 5 mg/ml for antioxidant enzymes analyses. Catalase (CAT) activity was determined using de Aebi[26] method. Superoxide Dismutase (SOD) was analyzed by spectrophotometry; presence of this enzyme in erythrocytes takes out the oxygen and inhibits the color development. Glutathione Reductase (GSR) was determined using the method of Carlberg and Mannervik[27]. Finally, Glutathione Peroxidase (GPX) was analyzed using the procedure developed by Flohé and Günzler[28].
Urinary biomarkers 15-isoprostane F2t (F2-Iso) and 8-Hydroxydeoxyguanosine (8-OHdG) were determined using ELISA assay kits (Oxford Biomedical Research, Inc., Catalog # EA84.102606, Michigan, USA and JaICA, Nikken SEIL Co., Ltd, Catalog# IM-KOGHS 040914E, Shizuoka, Japan, respectively)[28].

*Data Handling and Statistical Analyses*

Data were organized and recorded in an SPSS version 20.0 database in order to run all the statistical analyses. Normality of variables was assessed using the Kolmogrov-Smirnov test. Descriptive statistics are presented as median, 95% CI and range.
We ran the Spearman correlation coenfficients according to the distribution of the sample. When the Spearman test gave a correlation coefficient significant ($p < 0.05$) or with a tendency to be significant ($p > 0.05$ to $< 0.10$), we ran goodness-of-fit models of SPSS in order to detect any improvement in the relation. Multiple regression backward stepwise analyses were performed to develop models to predict values of the urine oxidative biomarkers F2-Iso and 8-OHdG from the antioxidant defense system parameters measured in the present study (SOD, CAT, GPX and GSR, and α-tocopherol, retinol, β-carotene and coenzymes Q9 and Q10) using the same software. Durbin-Watson statistics was used to assess whether the assumption of independent errors for the variables was tenable. ANOVA testing was done to determine whether the selected model was significantly better at predicting the outcome than using the mean as a “best guess”. The F represents the ratio of the improvement in the prediction as a result of fitting the model relative to the inaccuracy that still exists in the model. Variance inflation factor (VIF) and tolerance statistics were obtained to assess whether there was some co-linearity among the independent variables[30].

## Results

*Characteristics of the Participants*

Of the 87 children enrolled in the study as a whole, binary samples were available variously in from 78 to 82 cases. In this

binary sub-sample, 38 participants were girls, and 44 were boys. The ages ranged from 23 to 81 mo, with a mean of 55 ± 16 mo, and a median of 56 mo. Fig 1 illustrates the sex- and age-distribution of the children for each of the three day-care centers, and provides data on the median ages by sex.

*Descriptive Statistics of the Biomarkers Concentration*

Table 1 shows the median, 95% confidenc interval, and minimum and maximum values for each of the 11 biomarkers.

*Hemimatrix of Linear Spearman Correlations for Inter-Biomarker Associations*

Fig 2 shows the Spearman correlation coefficients for 55 binary inter-biomarker associations among the 11 variables including 2 urinary oxidative stress biomarkers, 4 rbc antioxidant enzymes and 5 plasma antioxidant compounds. Of these, 28 (51%) met the 5% statistical significance criterion. The strongest significance was found for the association between retinol and alpha-tocopherol ($r=0.697$, $p<0.001$) and the weakest between SOD and GSR ($r = 0.220$, $p = 0.048$).

*Comparison of Goodness-of-Fit Correlations for Inter-Biomarker Associations*

Table 2 lists the 28 significant binary associations as analyzed by Spearman rank-order correlation and the corresponding goodness-of-fit correlation coefficient with the appropriate curve form. In 24 cases of the series (86%), the r value was improved or remained the same, whereas in 4 cases

**Figure 1.** Characteristics of the Subjects, by setting and sex.

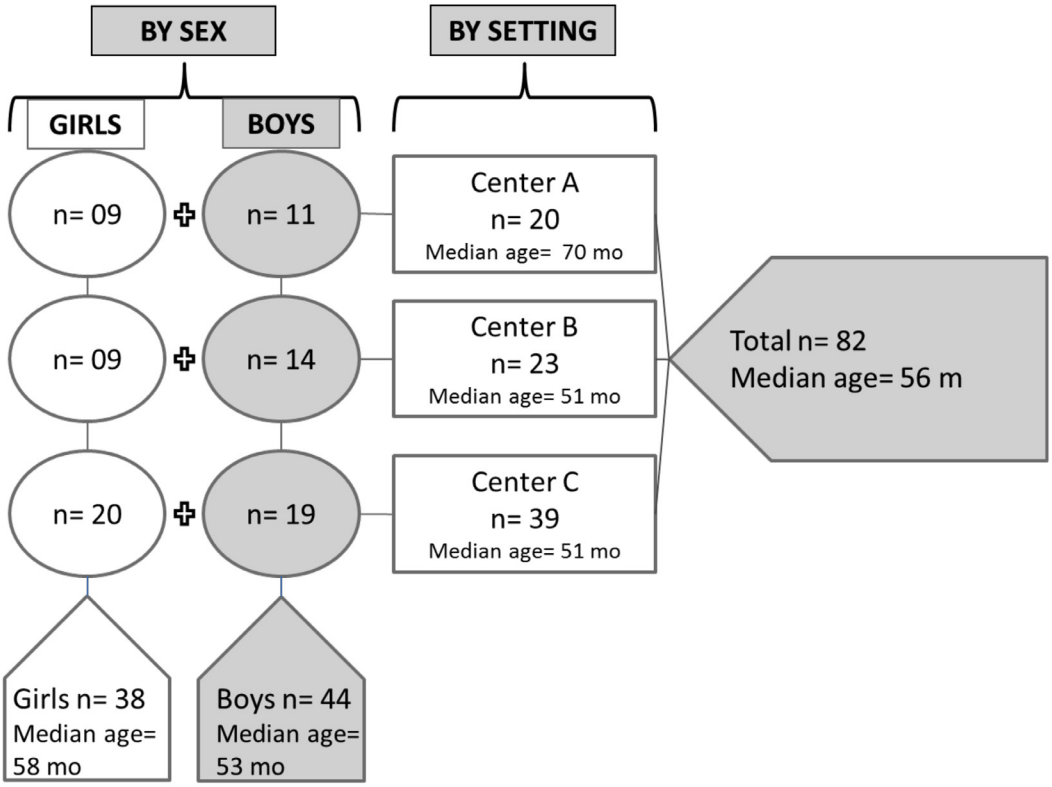


Table 1. Descriptive Statistics of the Biomarkers Concentration.

| Biomarker | N | Median | 95% CI | Min-Max |
|---|---|---|---|---|
| *Urinary oxidation biomarkers* | | | | |
| F2-Iso (ng/mL) | 78 | 1.75 | 1.93 – 2.81 | 0.50 – 11.80 |
| 8-OHdG (ng/mL) | 78 | 5.69 | 6.26 – 9.80 | 0.55 – 38.61 |
| *Antioxidant enzymes* | | | | |
| CAT Activity (nmol/seg/g Hb) | 82 | 6.68 | 6.46 – 7.05 | 4.31 – 9.91 |
| SOD Activity (U/g Hb) | 82 | 1.57 | 1.41 – 1.69 | 0.24 – 2.78 |
| GSR Activity (µmol/min/g Hb) | 82 | 2.82 | 2.67 – 2.93 | 1.79 – 4.06 |
| GPX Activity (µU/g Hb) | 82 | 6.05 | 5.57 – 6.41 | 0.00 – 11.99 |
| *Antioxidant nutrients* | | | | |
| Retinol (µg/dL) | 81 | 21.8 | 20.8 – 23.5 | 11.1 – 44.1 |
| α-tocopherol (mg/L) | 81 | 3.94 | 3.81 – 4.26 | 2.05 – 6.66 |
| β-carotene (mg/L) | 81 | 0.60 | 0.67 – 0.94 | 0.11 – 2.77 |
| Co-Q9 (mg/L) | 81 | 0.04 | 0.04 – 0.05 | 0.04 – 0.06 |
| Co-Q10 (mg/L) | 81 | 0.20 | 0.20 – 0.22 | 0.15 – 0.32 |

(14%) the goodness-of-fit r-value was lower than provided by Spearman analysis. The associations showing the greatest improvements for the strength of correlation were SOD and β-carotene (+43%) and retinol and β-carotene (+41%).

There were 5 correlation coefficients in the probability range of 0.051 to 0.100 including those between 8OHdG and SOD, CAT and retinol, GSR and retinol, β-carotene and retinol, and 8OHdG and GSR. Only the latter two associations in this series achieved significance at $p<0.05$ when applying the goodness-of-fit model

*Multiple Regression Models*

Table 3 shows the coefficients of the multiple regression models for the dependent variables urinary F2 isoprostanes and 8-hydroxy deoxy-guanosine using as independent variables the antioxidant defense system parameters measured in the present study (red blood cells activities of SOD, CAT, GPX and GSR, and plasma concentrations of α-tocopherol, retinol, β-carotene and coenzyme Q9 and 10). Plasma retinol, β-carotene, and Co-Q10 were independent predictors of F2-Iso, accounting for about 39% of the total variability, whereas plasma retinol and Co-Q10 were independent predictors of 8-OHdG accounting for 19% of the variability. Retinol and β-carotene in the first model and retinol in the second model had a positive relationship with the outcome, whereas Co-Q10 exhibited a positive relationship for both models.

Fig 3 illustrates 6 selected goodness-of-fit association curves showing the different curve-forms that appeared with the transformed regression model. These include three with cubic form and one each with exponential, sigmoid and linear forms as the result of the goodness-of-fit adaptation. Overall, the cubic configuration dominated with 18 (64%) of all transformations performed (data not shown).

Table 2. Comparison of Spearman and Non-Linear Correlation Coefficients in Inter-Biomarker Significant Associations.

| X-Axis | Y-Axis | Spearman r-value | Goodness-of-fit r-value | Best model curve form |
|---|---|---|---|---|
| F2-Iso | 8OHdG | .542 | .575 | Cubic |
| | Catalase | .468 | .539 | Cubic |
| | SOD | .256 | .349 | Cubic |
| | GSR | .347 | .357 | Cubic |
| | Retinol | -.444 | .538 | Exponential |
| | α-tocopherol | -.311 | .367 | Exponential |
| | β-carotene | -.540 | .541 | Cubic |
| | Co-Q9 | -.323 | .333 | Cubic |
| 8OHdG | Catalase | .242 | .273 | Cubic |
| | Retinol | -.283 | .294 | Cubic |
| Catalase | SOD | .276 | .282 | Linear |
| | GSR | .241 | .242 | Exponential |
| | β-carotene | -.341 | .347 | Linear |
| SOD | GSR | .220 | .235 | Exponential |
| | GPX | .362 | .295 | Cubic |
| | β-carotene | -.363 | .634 | Cubic |
| GSR | α-tocopherol | -.230 | .209 | Power |
| | β-carotene | -.274 | .271 | Power |
| GPX | Retinol | .300 | .387 | Cubic |
| | α-tocopherol | .280 | .344 | Cubic |
| Retinol | α-tocopherol | .697 | .719 | Cubic |
| | Co-Q9 | .373 | .453 | Cubic |
| | Co-Q10 | .503 | .539 | Cubic |
| α-tocopherol | β-carotene | .449 | .485 | Sigmoid |
| | Co-Q9 | .631 | .679 | Cubic |
| | Co-Q10 | .630 | .702 | Cubic |
| β-carotene | Co-Q9 | .493 | .478 | Cubic |
| Co-Q9 | Co-Q10 | .378 | .367 | Sigmoid |

*The r-value was improved or remained the same in 24 cases of the series, whereas the goodness-of-fit r-value was lower than Spearman's in 4 cases.

## Discussion

The underlying research hypothesis of this field survey was that the status of oxidative stress and other diagnostic domains would show substantial heterogeneity across the sample, despite the narrow age range and a common institutional menu. The findings amply confirm the notion of wide, inter-individual variance. At the second level, however, we observe broad, multiple and harmonious associations among antioxidant micronutrients, antioxidant enzymes and resulting free-radical damage to lipid and nuclear cells constituents. This suggests that the control of low-grade oxidative stress, indeed, behaves like an interactive system within the organism. Commenting on the related phenomenon of inflammation Berk et al.[31] include as candidate factors:

**Figure 2.** Spearman Correlation Coefficient Hemi-Matrix for Inter-Relationships of Biomarkers

| Biomarker | | F2-Iso (ng/mL) | 8-OHdG (ng/mL) | CAT Activity (nmol/seg/ g Hb) | SOD Activity (U/g Hb) | GR Activity (umol/min/g Hb) | GPX Activity (µU/g Hb) | Retinol (µg/dL) | α-tocopherol (mg/L) | β-carotene (mg/L) | CO-Q9 (mg/L) | CO-Q10 (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F2-Iso (n=78) | r-value | 1.000 | | | | | | | | | | |
| | p-value | | | | | | | | | | | |
| 8-OHdG (n=78) | r-value | 0.542 | 1.000 | | | | | | | | | |
| | p-value | <0.001 | | | | | | | | | | |
| Catalase Activity (n=82) | r-value | 0.468 | 0.242 | 1.000 | | | | | | | | |
| | p-value | <0.001 | 0.038 | | | | | | | | | |
| SOD Activity (n=82) | r-value | 0.256 | 0.206 | 0.276 | 1.000 | | | | | | | |
| | p-value | 0.028 | 0.079 | 0.012 | | | | | | | | |
| GSR Activity (n=82) | r-value | 0.347 | 0.219 | 0.241 | 0.220 | 1.000 | | | | | | |
| | p-value | 0.002 | 0.061 | 0.029 | 0.048 | | | | | | | |
| GPX Activity (n=82) | r-value | -0.079 | 0.064 | 0.011 | 0.362 | 0.010 | 1.000 | | | | | |
| | p-value | 0.505 | 0.589 | 0.923 | 0.001 | 0.929 | | | | | | |
| Retinol (n=81) | r-value | -0.444 | -0.283 | -0.193 | 0.016 | -0.206 | 0.300 | 1.000 | | | | |
| | p-value | <0.001 | 0.015 | 0.084 | 0.890 | 0.065 | 0.006 | | | | | |
| α-tocopherol (n=81) | r-value | -0.311 | -0.142 | -0.146 | 0.012 | -0.230 | 0.280 | 0.697 | 1.000 | | | |
| | p-value | 0.007 | 0.229 | 0.194 | 0.916 | 0.039 | 0.011 | <0.001 | | | | |
| β-carotene (n=81) | r-value | -0.540 | -0.098 | -0.341 | -0.363 | -0.274 | -0.048 | 0.205 | 00.449 | 1.000 | | |
| | p-value | <0.001 | 0.411 | 0.002 | 0.001 | 0.013 | 0.669 | 0.067 | <0.001 | | | |
| CO-Q9 (n=81) | r-value | -0.323 | -0.110 | -0.068 | -0.002 | -0.129 | 0.015 | 0.373 | 0.631 | 0.493 | 1.000 | |
| | p-value | 0.005 | 0.353 | 0.546 | 0.988 | 0.252 | 0.895 | 0.001 | <0.001 | <0.001 | | |
| CO-Q10 (n=81) | r-value | -0.005 | 0.154 | -0.118 | 0.135 | 0.010 | 0.106 | 0.503 | 0.630 | 0.132 | 0.378 | 1.000 |
| | p-value | 0.964 | 0.194 | 0.296 | 0.228 | 0.931 | 0.347 | <0.001 | <0.001 | 0.240 | 0.001 | |

The Spearman rank-order correlation coefficients Hemi-Matrix for mutual, cross inter-relationships of the 11 mea-sured biomarkers is illustrated within the 66 cells of pertinent reference. The dark-shaded cells represent the 11 auto-correlations. The remaining 55 cells illustrate the probability level for the corresponding Spearman r value. The 28 medium-shaded cells have statistically-significant associations, whereas the 27 clear cells have non-significant associations. Corresponding units of concentration of activity are given with the biomarkers along the horizontal axis; n values are given in the vertical axis.

"psychosocial stressors, poor diet, physical inactivity, obesity, smoking, altered gut permeability, atopy, dental cares, sleep and vitamin D deficien y." In our setting, all of the candidate factors except for obesity, smoking and sleep disturbance would be applicable. However, the environmental indoor smoke contamination of the Western Highlands homes can be substituted for tobacco smoke as an oxidative stress[32]. It was in fact, the scheme of environmental stressors that framed the rationale for our previous publication examining the interaction of inflammatory b iomarkers i n these same subjects[24] (**S1**).

Given that the subjects came from residential settings as described above, with high levels of poverty and low levels of hygiene, it would not be surprising to find values of the biomarkers mildly outside of the normative ranges. For the urinary biomarkers of oxidative stress there are no established normal values for children; regarding to F2-Iso using the normative range, adjusted for creatinine, given by the manufacturers in the kit (Source: Oxford Medical Research manual), 59 subjects (76%) are above the upper value, when similarly adjusted; also, all our subjects had higher values than the mean of 39 healthy adolescent controls at published earlier, when compared in common units[33]. When comparing our results to normative values for 8OHdG provided with the used kit (Source: JaICA manual), 70 of our subjects (90%) had values above the adult mean value as adjusted for creatinine concentration. Moreover, when compared to 14 healthy children aged 2 to 15 mo of age[34], 60 of our subjects (77%) were above their median creatinine-adjusted value. Thus, it seems that a mild elevation in both urinary markers of oxidation is the rule.

Red cell enzymes are all expressed in different units of reaction activity, but with all related to grams of hemoglobin in the hemolysate specimen. There is substantial literature on antioxidant enzymes in erythrocytes, and it would be inappropriate to select particular values as "normative" for reference. Important in diagnostic enzymology is that there is internal consistency within the series of interest.

We measured circulating concentrations of five a ntioxidant n utrients. With respect to the references system of[35], the normal range of plasma circulating retinol is 30 to

95µg/dL: in this context 75 of 81 samples (92%) would be considered low. This finding is surprising, as the most recent national nutrition survey[36] found the 6 to 59 mo population of Guatemala free of hypovitaminosis A; we suspect that there might be a calibration error in our analyses, but one that would not invalidate the relative rank-order among subjects in any way. The same reference source[35], provides the normative range from 5 to 20 mg/L for circulating levels of alpha-tocopherol; in our sample 64 of 81 samples (79%) are considered below the normal range.

The circulating levels of carotenoids are so dependent on the consumption of foods of plant origin for which no international references can or should be made. A previous longitudinal study of urban schoolchildren in Guatemala, conducted between February and May, documented a 3-fold increase in total carotene concentration as compared to other seasons, attributed to the mango season[37]. In the present study, blood was sampled from August through October, i.e. during the downslope of the annual ß-carotene cycle from native mangos.

According to Molyneux et al.[38], the normal range for Co-Q10 is 0.41 to 1.55 mg/L. With our values ranging from 0.15 to 0.32 mg/L, all subjects in this study would be considered to have low Co-Q10 status. However, with the reference range for Co-Q9 from 0.007 to 0.037 mg/L[38] and with our subjects' distribution 0.040 to 0.060 mg/L, our lowest value is still *above* the normative reference range.

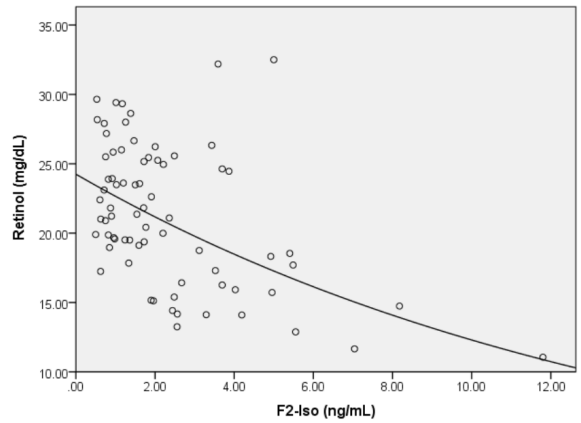
Multiple comparisons to evaluate interactions always run the risk of spuriously significant correlations. In our case, with a 5% probability criterion, we would have expected to find 2 and 3 correlations with a p value of <0.05 by

**Table 3.** Coefficients of the multiple regression model for the dependent variables F2-Iso and 8-OHdG, using as independent variables the antioxidant defense system parameters measured in the present study (SOD, CAT GPX and GSR, and plasma concentrations of α-tocopherol, retinol, β-carotene and Co-Q9 and Co-Q10).

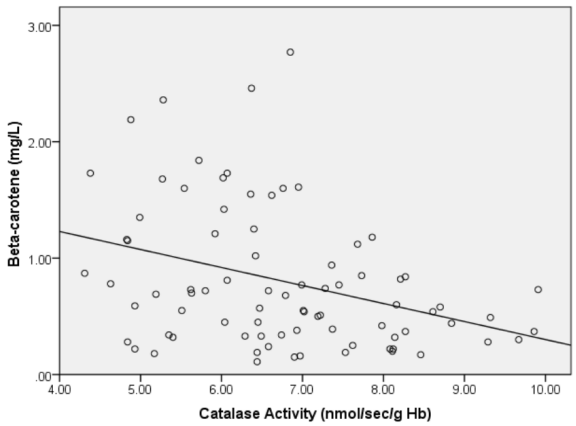
| No. | Model | Unstandardized Coefficients | | Standardized Coefficients | t | Sig. | 95.0% Confidence Interval for B | |
|---|---|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | | | Lower Bound | Upper Bound |
| ***Dependent variable: F2-Iso**** | | | | | | | | |
| 7 | (Constant) | 2.681 | 1.042 | | 2.572 | 0.012 | 0.601 | 4.761 |
| | Retinol (mg/dL) | -0.151 | 0.036 | -0.455 | -4.240 | <0.001 | -0.222 | -0.080 |
| | β-carotene (mg/L) | -1.007 | 0.267 | -0.372 | -3.769 | <0.001 | -1.540 | -0.474 |
| | Co-Q10 (mg/L) | 17.457 | 5.267 | 0.346 | 3.315 | 0.001 | 6.947 | 27.967 |
| ***Dependent Variable: 8-OHdG**** | | | | | | | | |
| 8 | (Constant) | 2.328 | 5.592 | | .416 | 0.678 | -8.825 | 13.482 |
| | Retinol (mg/dL) | -0.656 | 0.185 | -0.423 | -3.548 | 0.001 | -1.024 | -0.287 |
| | Co-Q10 (mg/L) | 95.208 | 28.777 | 0.395 | 3.308 | 0.001 | 37.815 | 152.602 |

*$r^2$ = 0.390; r = 0.624 (n = 72)
**$r^2$ = 0.190; r = 0.436 (n = 73)

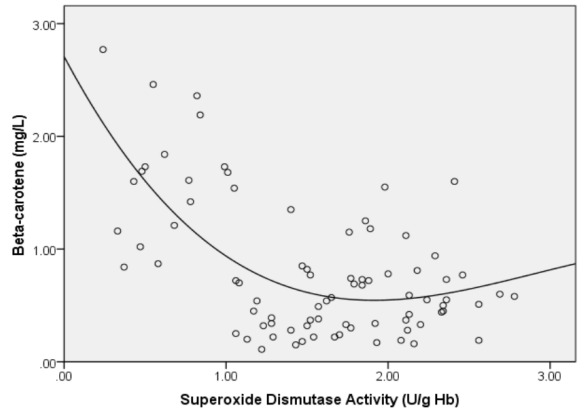
**Figure 3.** Selected associations between variables with the superimposition of the Goodness-of-Fit curve form and the correlation coefficient "r" for the model's equation.



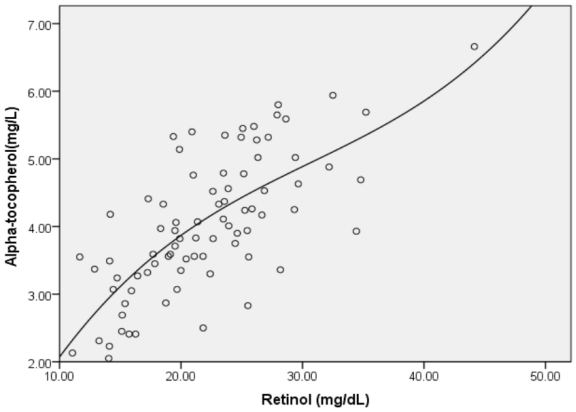

**Exponential curve r=0.538**



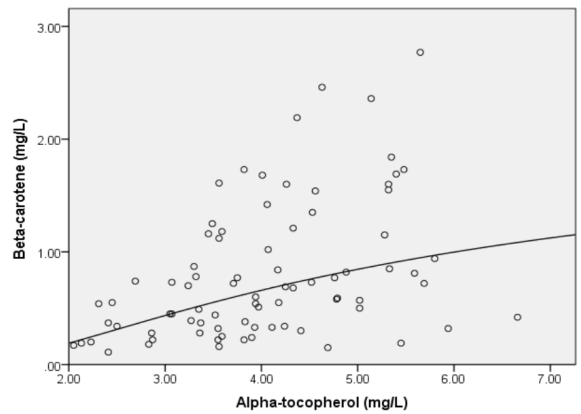

**Linear curve r=0.347**



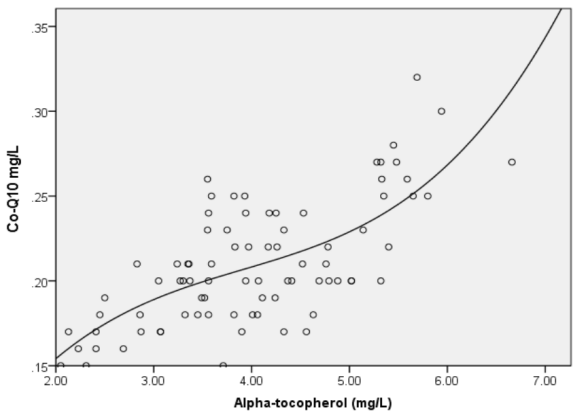

**Cubic curve r=0.634**




**Cubic curve r=0.719**




**Sigmoid curve r=0.485**




**Cubic curve r=0.702**

chance alone. With our finding of a total of 28, at least 25 interactions are likely to represent truly significant associations. In addition, the fact that the goodness-of-fit models are also significant and improved in their r value in 86% of the instances is additional evidence for the validity of the Spearman associations encountered to be truly significant, and not spurious

If we isolate the interactions by type of biomarker, we see a predominance of what could be called "auto-correlation", that is association within the same class, for example, vitamins with other vitamins and enzymes with other enzymes. Of even more interest, in a biological sense, might be the significant associations between biomarkers of *different* classes and origins, eg. urinary biomarkers of oxidative stress with selected plasma antioxidants (vitamins), urinary biomarkers with rbc enzyme activities involved in the ADS or vitamins with enzymes. Of all of the biomarkers, F2-Iso had the widest range of cross associations, with 8 of the 10 companion biomarkers; only GPX and Co-Q10 did not associate with F2-Iso concentration. Plasma retinol; β-carotene and Co-Q10 resulted as independent predictors of F2-Iso after running the backwards multiple regression model. Next in frequency was alpha-tocopherol, with 7 significant cross-associations. The multiple-regression model was also applied for 8-OHdG; and plasma retinol and Co-Q10 were the resultant independent predictors.

Clearly, excessive oxidation is suggested to be associated with an increase in the urinary biomarkers of oxidative stress F2-Iso and 8OHdG. Interpretation of the other biomarkers is not so straightforward. In theory, the response to oxidative stress could deplete the vitamin or affect the activities of enzyme biomarkers or produce a compensatory and protective increase. In the present experience, urinary excretion of oxidative biomarkers associates directly with the activity of antioxidant enzymes and inversely with the vitamin concentration. This association suggests that the enzymatic response is compensatory, whereas the antioxidant nutrients may be being consumed by the oxidation process. This interpretation is further confirmed by the fact the relationships than exist between ADS enzymes and non-enzymatic antioxidants, are universally inverse.

## Strengths and limitations of the study

We acknowledge certain strengths and weaknesses of design and execution of the study. The principal strength is that it deals with childhood, with children being of preschool age. In theory, the settings with similar dietary offering should control one important variable, and narrow overall variance. We were ambitious enough to attempt 24-h urine collection in the design for young children, with relative success in obtaining complete collections. We recognize, however, that we did not obtain full adherence in this effort due to the young age of the subjects. An additional strength is the possibility to relate a large variety of oxidative and antioxidant biomarkers of different classes and collected from different body fluids to each other. Finally, there are some inconsistences between the retinol status observed here and other experiences seen in Guatemala.

## Conclusion

In conclusion, the largely common menu offering at the daycare centers during 5 days per week was no guarantee of the nutritional or a physiological uniformity across a sample of low-income preschoolers. Nutritional biomarkers varied widely, with many falling below the cutoff for adequate status. Mildly elevated urinary biomarkers confirmed our assumption that the household and community environments generate more than usual oxidative stress. Most strikingly, however, are apparent patterns of harmonious interactions among the

elements of the oxidation-antioxidation systems within a setting that presents a variety of mild to moderate oxidative stress from the conditions of daily existence.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# Chapter 5

# Associations among Inflammatory Biomarkers in the Circulating, Plasmatic, Salivary and Intraluminal Anatomical Compartments in Apparently Healthy Preschool Children from the Western Highlands of Guatemala

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## ASSOCIATIOS AMONG INFLAMMATORY BIOMARKERS IN THE CIRCULATING, PLASMATIC, SALIVARY AND INTRALUMINAL ANATOMICAL COMPARTMENTS IN APPARENTLY HEALTHY PRESCOOL CHILDREN FROM THE WESTERN HIGHLANDS OF GUATEMALA

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**Abstract**

*Background:* Undernutrition and infla - mation are related in many ways; for instance, non-hygienic environments are associated with both poor growth and immunostimulation in children.

*Objectives:* To describe the distribution and central tendency of urinary osmolality (Uosm) for preschool children, and to assess the reproducibility of Uosm measurements after frozen storage and when determined in two different osmometers.

*Methods:* In this descriptive, cross-sectional study, samples of whole blood, feces, plasma and saliva were collected on the 8th and last week of observation among 87 attendees (42 girls and 45 boys) of 3 daycare centers offering a common 40-day rotating menu in Guatemala's Western Highlands. Analyses included white blood cell count (WBC), fecal calprotectin, and plasmatic and salivary cytokines including IL-1B, IL-6, IL-8, IL-10 and TNF-α. Associations were assessed using Spearman rank-order and goodness-of-fit correlations, as indicated, followed by backwards-elimination multiple regression analyses to determine predictor variables for IL-10 in both anatomical compartments.

*Results:* Of a total of 66 cross-tabulations in the Spearman hemi-matrix, 22 (33%) were significantly associated. All 10 paired associations among the salivary cytokines had a significan r value, whereas 7 of 10 possible associations among plasma cytokines were significant. As sociations across anatomical compartments, however, were rarely significant. IL-10 in both biological fluids were higher than corresponding reference values. When a multiple regression model was run in order to determine independent predictors for IL-10 in each anatomi- cal compartment separately, IL-6, IL-8 and TNF-α emerged as predictors in plasma (r2 = 0.514) and IL-1B, IL-8 and TNF-α remained as independent predictors in saliva (r2 = 0.762). Significant cross-interactions were seen with WBC, but not with fecal calprotectin.

*Conclusion:* Interactions ranged from robust within the same anatomical compartment to limited to nil across distinct anatomical compartments. The prominence of the anti-inflammatory cytokine, IL-10, in both plasma and saliva is consistent with its counter-regulatory role facing a broad front of elevated pro-inflammatory cytokines in the same compartment.

## Introduction

The immune system, in its complexity, is constituted of specialized cells with specifi secretory or functional roles. There are the rapid "innate" and the slower "adaptive" immune responses, highly integrated by hormonal signaling or cell-to-cell cross-talk[1].

These mobilize cellular elements, including, phagocytic, inflammatory and natural killer cells, among others, along with molecular components, such as hepatic acute-phase proteins and cytokines originating from a wide variety of tissues.

The cytokines produced by white blood cells constitute a series of "interleukins." Differentiation of thymic-derived helper cells produces subclasses supporting up-regulating, pro-infla matory cytokines (Th1) and counter-reacting, down-regulating anti-inflammatory cytokines (Th2)[1]. The Th1-directed acute-phase response directs a catabolic intermediary metabolism, tending toward poor tissue growth and wasting of nutrients[2].

Guatemala has the highest prevalence of under-five stunting in Latin America[3]. Poor linear growth begins in utero[4,5], and continues during the first 2 y of lif [6].

Stunting generates a series of adverse consequences not only in infancy and childhood, but also over the longer term; as summarized by Dewey and Begum[7]: "childhood stunting (was) linked with short adult stature, reduced lean body mass, less schooling, diminished intellectual functioning, reduced earnings and lower birth weight of infants born to women who themselves had been stunted as children."

In the context of the complexity of immune reactivity, linear growth impairment is associated with immunological alterations such as impaired gut-barrier function, reduced delayed-type hypersensitivity responses, atrophy of lymphatic tissue whereas the cytokine patterns seem to be skewed towards a Th2-response[8], as part of a well-described cytokine-induced or infection-induced malnutrition[9].

It has long been established in livestock and poultry[10,11] that unclean and contaminated environments retard body growth and weight-gain. Roura et al.[12] identified a cytokine-mediated immunological stress as the mechanism for this growth failure.

In 1993, Solomons, *et al.*[13] proposed an explanation for poor linear growth based on the theory of environmental contamination and poor utilization of nutrients. It has been proposed that decreasing infla - matory episodes will improve long-term outcomes on linear growth[14]. Interventions to prevent environmental enteropathy during infancy such as WASH (Water, Sanitation and Hygiene) in Kenya and Bangladesh or SHINE (Sanitation Hygiene Infant Nutrition Efficacy) in Zimbabwe suggest

that low-grade, chronic inflammation may impair infant growth and that reducing fecal-oral transmission of pathogenic microbes during infancy will reduce prevalence of stunting in developing countries[15,16].
A plausible mechanism for the direct interference with linear growth by infection comes from the work with an infected-mouse model[17], in which endogenous stress compounds (IL-1B, cortisol) interrupt the hormonal cascade to the epiphyseal growth plate that signals elongation of bone.
Hence, linear growth retardation is not only due to undernutrition or lack of nutrients; additionally, continuous inflammation of the body promotes the malabsorption and wasting of nutrients and dysregulation of skeletal growth.

In the course of a research project entitled "Study on the normative state and inter-and intra-individual variation in growth, hematology, hydration, and markers of oxidation, infection and inflammation in pre-school children with a similar dietary intake", we collected data on white blood cells, a biomarker of intraluminal intestinal inflamm tion, and a parallel series of selected cytokines in plasma and saliva among preschool children in a governmental system of daycare centers. We attempted to find any possible interaction among the inflammatory biomarkers from different anatomical compartments as an example of immunological cross-talk and reafferent integration of the system. We present here the findings from this exploration in young children within the context of variable environmental and genetic circumstances, within the potentially stabilizing and harmonizing influence of a common institutional dietary offering.

## Materials and Methods

### *Study Design*

The following descriptive, cross-sectional, field study on the variation and associations among variables related to inflammation is part of the larger undertaking entitled: "Study on the normative state and inter and intra-individual variation in growth, hematology, hydration, and markers of oxidation, infection and inflammation in pre-school children with a similar dietary intake."

### *Population and Setting*

The study was conducted in Guatemala in the Western Highlands Province of Quetzaltenango, known for its rural-based agrarian environment. With its capital city located 220 km from Guatemala City at 2357 m above sea level, Quetzaltenango Province has a majority indigenous population (60.6%) and an annual mean daily low temperature of 14.7°C, but varying from -12 to 25°C.
Study subjects were children attending daycare centers *(Hogares Comunitarios)* within the Secretariat of Beneficial Programs of the First Lady *(Secretaría de Obras Sociales de la Esposa del Presidente-SOSEP)* in three different settings: one semi-urban, one marginal-urban, and one rural. Each site had differences in its proportional ethnic make-up and the corresponding cultural customs and traditions. The rural site had an almost exclusively Mam-Mayan indigenous enrolment. SOSEP daycare centers offer a common, 40-day rotating menu. It is standardized in its recipes and provides four meals per attendance day.

### *Recruitment and Enrolment of Subjects*

Children from three daycare centers were eligible to enroll and be included in the analyses if they were: 1. attending one of the centers; 2. between 2 and 7 years-old; and 3. (post-hoc) had at least an 80% daily attendance record in the center during the time of the study. Moreover, they had to be apparently healthy and without restrictions related to the acceptance of the diet offered in the SOSEP

menu. Children whose parents or caregivers did not sign the consent form, or who did not adhere to the full fecal collection schedule were selectively excluded from the analyses.

*Ethical Considerations*

The SOSEP's director for the Quetzaltenango area authorized the study to be performed within the system. The Human Subjects Committee of the Centre for the Studies of Sensory Impairment, Aging, and Metabolism (CeSSIAM) granted ethical approval for the study protocol. A parent or guardian signed the written consent form after the purposes, benefits, inconveniences and risks of the procedures had been explained. Children gave a final assent at the moment of collection. As a collective benefit, any missing dietary items were subsidized by the study funds in order to provide all food items on the menu at all times, whenever the situation required. This study was registered at clinicaltrials.gov as NCT02203890.

*Anthropometric Measurements*

We performed anthropometric determinations during the last 7th week of each day-care center's 8-week period. Height was measured using a wooden wall stadiometer, and expressed in cm to the nearest 0.5 cm, with children standing without shoes and with their gaze in Frankfort plane. Weight was determined using a calibrated Tanita Model BC522 digital scale (Tanita, Tokyo, Japan); still with shoes removed. It was expressed in kg to the nearest 0.1 kg. An adjust- ment was made for clothing, subtracting a standard weight-for-clothing factor for girls' and boys' daytime clothes, respectively.

*Collection, Handling and Storage of Biological Samples*

In order to confirm the delivery of the 40-day rotating menu, we spent 8 weeks on each day-care center as an observation period from June to November, 2012, with sample collections beginning in July and extending to the end of the field activities. Whole blood assays (white blood cell count) were performed on the day of blood collection. Fecal samples were frozen-stored for from 3 to 16 weeks prior to analytic processing. Plasma and saliva samples, destined for cytokine assays, were frozen-stored for up to one year prior to the analyses in Spain.

*Blood (plasma and whole blood):* Blood samples were collected at each of the three centers during the last week of the 8-week study. A phlebotomist extracted the blood using BD Vacutainer 4 mL tubes, anticoagulated with EDTA (No.367861) in conjunction with Safety Lok deposable needles (No.367281) (Becton-Dickinson, NJ, USA). Five hundred microliters of the sample were separated in a tube and taken to La Democracia Hospital's clinical chemistry laboratory in order to obtain hemograms. The rest of each sample was centrifuged to separate red blood cells from plasma; the supernatant plasma was stored in Nalgene Cyrogenic Vials (No.5000-0012) (U.S. Plastics Corporation, Lima, OH, USA) in a -80°C freezer in the capital city prior to shipment to Granada, Spain, in order to measure plasma cytokines like interleu- kin-1-beta (IL-1B), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF-α).

*Fecal samples:* In the 7th week of the 8-week period, we supplied a container to the parents or caregivers to collect a fecal sample on the next morning, before bringing the child to the day-care center. Whenever the container was returned empty, we collected the sample if it was produced during the day; we repeated the process until we had all participants' samples. When samples were complete, they were taken to the local laboratory where we started the pre-preparation of the specimens to measure calprotectin; these were stored in a -20°C freezer, ready for biomarker assaying.

*Saliva:* On the day of blood collection, we also collected a saliva sample. Children were asked not to eat or drink anything during the two hours before saliva collection. Saliva was immediately stored in dry ice until storage in a -80°C freezer in the capital city and later shipped (again in dry ice) to Granada, Spain, in order to perform a parallel set of cytokine assays in both plasma and saliva.

*Laboratory Assays and Analyses*

*White blood cell count:* Analyses were performed in Quetzaltenango, Guatemala, at the La Democracia Hospital's clinical chemistry laboratory, using the Beckman Coulter AcT Diff Hematology Analyzer (Krefeld, Germany). White blood cell counts are expressed as quantity per $mm^3$ volume.

*Fecal Calprotectin:* Assays were performed in Quetzaltenango, Guatemala. ELISA assays were executed using the CALPRO Calprotectin ELISA Test from CALPRO AS (Lysaker, Nor- way). Catalog No. CAL0100. Concentrations were expressed in mg/kg of fecal sample. The detection limits were 25–2500 mg/kg, and a typical inter-assay CV was 3.7%.

*Plasma and Saliva Cytokines:* Samples were analyzed in Granada, Spain, using the MILLIPLEX MAP Human High Sensitivity Cytokine panel from Luminex Corporation (Missouri, USA) Catalog # HSCYTMAG-60SK for the five aforementioned classes of cytokines. The results were expressed in pg/mL. The cytokines of interest for both plasma and saliva were: IL-β, IL-6, IL-8, IL-10 and TNF-α. The minimum detection limit was 0.06, 0.20, 0.05, 0.48 and 0.07 respectively. The inter-assay CVs for plasma were 14.73%, 7.74%, 8.65%, 12.17% and 8.40%, respectively. For salivary cytokines CVs were 10.99%, 13.88%, 13.31%, 7.74%, 10.99% and 13.34%, respectively.

*Data Handling and Statistical Analyses*

The software SPSS Version 20 was used to create a database and run analyses. Descriptive statistics were expressed as the distribution in terms of median, 95% CI, and minimum and maximum. Associations of values collected at different points in time were tested by Spearman rank-order coefficient, as appropriate. We also ran the goodness-of-fit model to obtain correlation coefficients. In order to refine the predictive determination in the associations among the inflammation biomarkers, backwards-elimination multiple regression models were run to determine the parsimonious $r^2$ value. A probability level of 0.05 was accepted as statistically significant. STROBE statement for this article is included as supporting information file (S1 Checklist).

## Results

*Characteristics of the Participants*

Overall, 87 children had at least one inflammation datum Fig 1).

These included 42 girls and 45 boys. They had a median age of 55 mo, with a mean of 54 ± 16 mo and ranged from 23 to 81 mo. Fig 1 also disaggregates the sample by site and sex.
Table 1 presents the data on growth and nutritional status derived from the anthropometric measurements. Illustrated are the Z- scores for HAZ, WAZ and WHZ, and the respective prevalence of stunting, underweight and wasting for the entire sample and the distinct geographic sites. It was possible to make binary pairing of inflammation data for from 80 to 87 children, depending on the combinations (Fig 2).

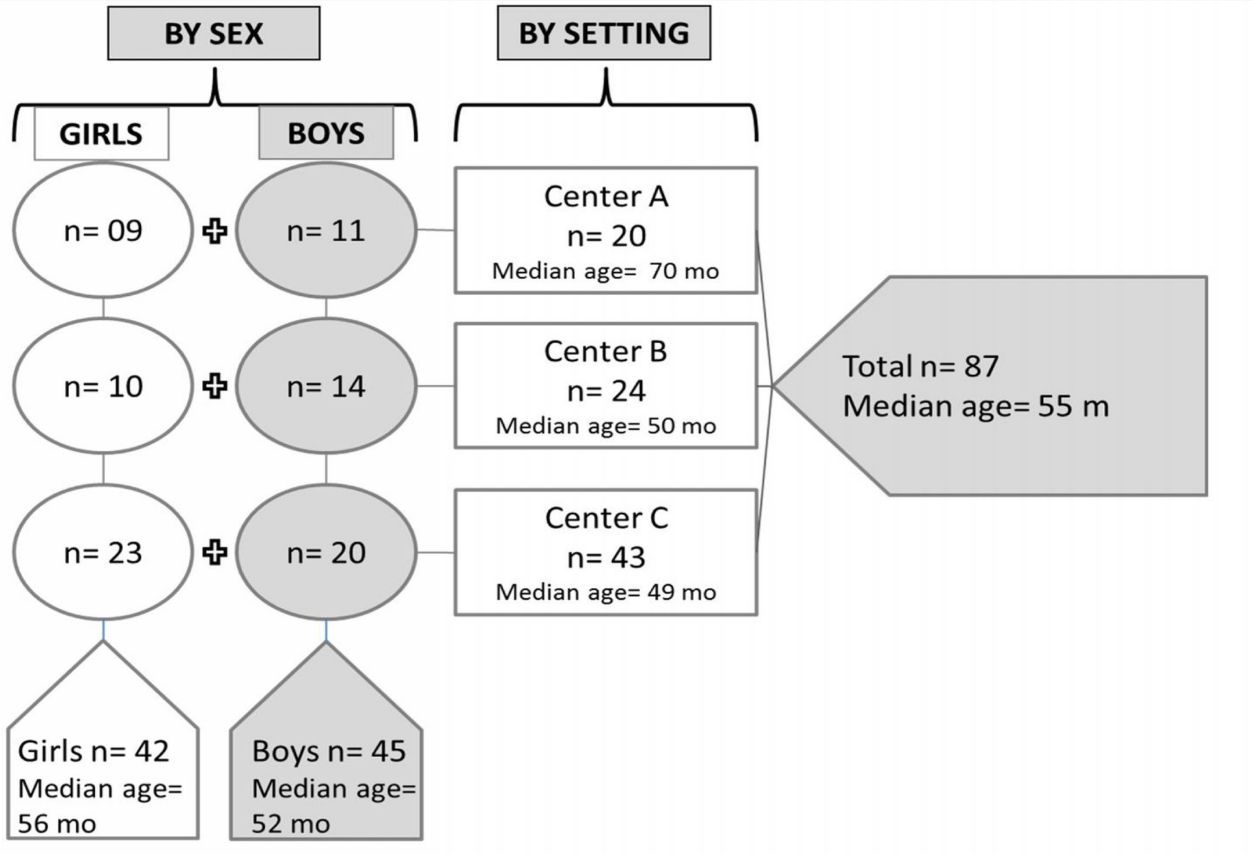
*Descriptive statistics of the inflammator biomarkers*

Illustrated in Table 2 are median, 95% CI, and limits for each of the 12 inflammatory biomarkers data set, grouped by origin: whole blood; feces; plasma; and saliva. In descending order, the concentrations for plasma values are: IL-10; TNF-α; IL-8; IL-6; and IL-1B. For saliva, corresponding values are: IL-8; IL-10; IL-1B; IL-6; and TNF-α. Only IL-8 and IL-1B had higher concentrations in saliva as compared to plasma, whereas IL-10; IL-6 and TNF-α had higher concentrations in plasma compared to saliva. In the final column of Table 2, we provide reference range values for normative values. A caveat is that the plasma and salivary cytokines, however, are from adult women, 40–50 years older rather than preschool subjects[18].

*Inter-Biomarker Associations by Linear (Spearman) Correlations*

Of a total of 66 cross-tabulations in the Spearman hemi-matrix (Fig 2), 22 (33%) were signifi antly associated at the level of p 0.05. In the face of multiple comparisons with this number of cross-correlations, however, one could expect that at least 3 would reach a probability level of 5% by chance alone. Hence, at least 19 of the associations are likely to be truly significant. The highest degree of linear association, with an r value of 0.871, was salivary IL-8 and salivary TNF-α. However, all 10 paired associations among the salivary cytokines had an r value >0.600 and a probability value of $10^{-3}$. The lowest r values still reaching the criterion for significance were a pair at 0.222 and 0.226 involving paired

Fig 1. Characteristics of the Subjects.




Characteristics expressed for the overall sample and disaggregated by setting and sex. This includes the respective numbers as well as the median age for each grouping. The data for girls and for the overall sample are included in the clear areas and those for boys and overall sample in the shaded areas.

**Table 1.** Median Height-for-Age, Weight-for-Age and Weight-for-Height of the Subjects, ove-rall and disaggregated by daycare center.

| | | Height-for-Age (stunting) | Weight-for-Age (underweight) | Weight-for-Height (wasting) |
|---|---|---|---|---|
| Setting | N | Z-score (% subnormal) | | |
| Total Sample | 87 | -2.39 (66%) | -1.37 (23%) | -1.00 (2%) |
| Semi-Urban (A) | 20 | $-1.61^{a}$ (35%) | $-0.96^{a}$ (0%) | -0.10 (0%) |
| Marginal-Urban (B) | 24 | $-2.42^{ab}$ (63%) | $-1.46^{b}$ (17%) | -0.16 (4%) |
| Rural-(C) | 43 | $-2.73^{b}$ (81%) | $-1.47^{b}$ (37%) | -0.07 (2%) |
| p-value | | 0.004* | 0.025* | 0.434* |

*Comparison among settings (last three rows) using the ANOVA test.
Values not sharing the same superscript were statistically different by the Bonferroni post-hoc test

**Table 2.** Descriptive Statistics of Biomarker Concentrations.

| Class | Biomarker | N | Median | 95% CI | Min-Max | Reference |
|---|---|---|---|---|---|---|
| Whole blood | White blood cells (thousands/$mm^3$) | 82 | 7.6 | 7.4 – 8.5 | 1.8 – 17.6 | 3.5 – 10.5 |
| Fecal | Calprotectin (mg/kg) | 87 | 57.5 | 69.1 – 126.9 | 10.0 – 950.0 | $<50^{x}$ |
| Plasmatic (pg/mL) | IL-1B | 82 | 0.9 | 0.9 – 1.2 | 0.3 – 5.7 | $0.37 – 1.25^{y}$ |
| | IL-6 | 82 | 3.7 | 3.8 – 6.1 | 1.6 – 44.2 | $0.52 – 1.89^{y}$ |
| | IL-8 | 82 | 4.5 | 4.5 – 5.6 | 1.5 – 13.4 | $6.5 – 13.8^{y}$ |
| | IL-10 | 82 | 52.2 | 52.9 – 86.5 | 17.0 – 608.1 | $0.60 – 2.70^{y}$ |
| | TNF-α | 82 | 7.5 | 7.5 – 8.7 | 4.43 – 18.0 | $0.94 – 2.64^{y}$ |
| Salivary (pg/mL) | IL-1B | 83 | 1.7 | 1.2 – 22.8 | 0.3 – 407.1 | $21.1 – 73.0^{y}$ |
| | IL-6 | 83 | 1.4 | 0.8 – 8.1 | 0.11 – 151.2 | $1.59 – 10.46^{y}$ |
| | IL-8 | 83 | 120 | 160 – 256 | 1.92 – 734.3 | $254 – 578^{y}$ |
| | IL-10 | 83 | 11.0 | 13.8 – 23.2 | 1.2 – 108.8 | $0.71 – 3.93^{y}$ |
| | TNF-α | 83 | 0.8 | 1.1 – 2.9 | 0.1 – 32.9 | $4.3 – 19.0^{y}$ |

x = manufacturer's suggested cut-off for normal calprotectin
y = published normative post-menopausal females' for cytokines [Browne et al., 2013], expressed as 25th and 75th percentiles

correlations of plasma IL-1B with two salivary cytokines; the notable exception was with salivary and plasmatic TNF-α.

*Inter-Biomarker Associations by Goodness-of-Fit Correlations*

Table 3 illustrates the 22 out of 66 Spearman rank-order correlations that reached the 0.05 p-value criterion for statistical significance, juxtaposed with the corresponding r value for the goodness-of-fi correlation. Also shown is the curve-form of the goodness-of-fit correlation. The magnitude of the r value improved (increase of >0.020 decimal points of improvement above the Spearman coefficient) in 9 instances (41%), with the greatest increment that of a 50% increase in the value of the association of IL-10 with IL-6 in plasma; r values remained relatively stable (change of -0.020 to +0.020) in 7 cases (32%); and the value declined (decrease of >0.020) in 6 (27%). The cubic curve-form was the predominant one, emerging in 7 regressions, followed by sigmoid and power curve-forms with 6 each. The remaining 3 curve-forms were a growth, a compound, and a logarithmic. In Fig 3, we selected 6 examples of the goodness-of-fit curves in a parallel representation: 3 from plasma and 3 from saliva. In each panel, IL-10 is on the x-axis distribution and the y-axis distributions are for IL-6 (top panels), IL-8 (middle panels) and TNF-α (bottom panels). Those involving IL-1B have been excluded for reasons of space. Compared across biological fluids of origin, IL-10 with IL-8 shows a cubic form in the goodness-of-fit correlation; the association was stronger in the salivary pair. Similarly, a common curve-form, power, was shared by IL-10 with TNF-α across fluids, again showing the stronger association.

**Figure 2.** Spearman Correlation Coefficient Hemi-Matrix for Inter-Relationships of Biomarkers.

| | | White Blood Cell Count | Fecal Calprotectin (mg/Kg) | Plasma IL-10 (pg/mL) | Plasma IL-1B (pg/mL) | Plasma IL-6 (pg/mL) | Plasma IL-8 (pg/mL) | Plasma TNF-α (pg/mL) | Salivary IL-10 (pg/mL) | Salivary IL-1B (pg/mL) | Salivary IL-6 (pg/mL) | Salivary IL-8 (pg/mL) | Salivary TNF-α (pg/mL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| White Blood Cell Count [n=82] | r-value | 1.000 | | | | | | | | | | | |
| | p-value | | | | | | | | | | | | |
| Fecal Calprotectin [n=87] | r-value | 0.084 | 1.000 | | | | | | | | | | |
| | p-value | 0.451 | | | | | | | | | | | |
| Plasma IL-10 [n=82] | r-value | 0.230 | 0.073 | 1.000 | | | | | | | | | |
| | p-value | 0.038 | 0.513 | | | | | | | | | | |
| Plasma IL-1B [n=82] | r-value | 0.065 | -0.017 | 0.419 | 1.000 | | | | | | | | |
| | p-value | 0.562 | 0.883 | 0.001 | | | | | | | | | |
| Plasma IL-6 [n=82] | r-value | 0.332 | 0.152 | 0.469 | 0.310 | 1.000 | | | | | | | |
| | p-value | 0.002 | 0.172 | 0.001 | 0.005 | | | | | | | | |
| Plasma IL-8 [n=82] | r-value | 0.074 | 0.006 | 0.584 | 0.200 | 0.194 | 1.000 | | | | | | |
| | p-value | 0.509 | 0.960 | 0.001 | 0.071 | 0.080 | | | | | | | |
| Plasma TNF-α [n=82] | r-value | 0.397 | 0.039 | 0.586 | 0.093 | 0.390 | 0.476 | 1.000 | | | | | |
| | p-value | 0.001 | 0.727 | 0.001 | 0.405 | 0.001 | 0.001 | | | | | | |
| Salivary IL-10 [n=80] | r-value | 0.032 | 0.076 | -0.002 | -0.226 | -0.075 | 0.044 | -0.030 | 1.000 | | | | |
| | p-value | 0.775 | 0.493 | 0.988 | 0.044 | 0.509 | 0.699 | 0.792 | | | | | |
| Salivary IL-1B [n=83] | r-value | 0.095 | -0.097 | -0.040 | -0.117 | 0.036 | -0.033 | -0.158 | 0.624 | 1.000 | | | |
| | p-value | 0.400 | 0.382 | 0.727 | 0.300 | 0.748 | 0.771 | 0.162 | 0.001 | | | | |
| Salivary IL-6 [n=83] | r-value | 0.141 | -0.031 | 0.081 | -0.060 | 0.181 | 0.123 | 0.083 | 0.676 | 0.735 | 1.000 | | |
| | p-value | 0.211 | 0.783 | 0.475 | 0.599 | 0.109 | 0.278 | 0.464 | 0.001 | 0.001 | | | |
| Salivary IL-8 [n=83] | r-value | 0.075 | 0.027 | 0.057 | -0.222 | 0.026 | 0.096 | -0.035 | 0.789 | 0.745 | 0.657 | 1.000 | |
| | p-value | 0.509 | 0.811 | 0.619 | 0.047 | 0.816 | 0.398 | 0.757 | 0.001 | 0.001 | 0.001 | | |
| Salivary TNF-α [n=83] | r-value | 0.101 | 0.017 | 0.022 | -0.200 | 0.040 | 0.030 | -0.043 | 0.740 | 0.780 | 0.616 | 0.871 | 1.000 |
| | p-value | 0.374 | 0.879 | 0.844 | 0.075 | 0.727 | 0.790 | 0.705 | 0.001 | 0.001 | 0.001 | 0.001 | |

The Spearman rank-order correlation coefficients Hemi-Matrix for mutual, cross inter-relationships of the 12 measured biomarkers is illustrated within the 78 cells of pertinent reference. The dark-shaded cells represent the 12 auto-correlations. The remaining 66 cells illustrate the probability level for the corresponding Spearman r value. The 22 medium-shaded cells have statistically-significant associations, whereas the 44 clear cells have non-significant associations. Footnotes: The units of expression for concentrations are provided in the superior captions on the x-axis. The numbers of individuals with analyzed values are provided in the left-hand column y- axis.

**Table 3.** Comparison of Spearman and Non-Linear Correlation Coefficients in Inter-Biomarker Significant Associations

| X-Axis | Y-Axis | Spearman r-value | Goodness-of-fit r-value | Best model curve form |
|---|---|---|---|---|
| White Blood Cells | Plasma IL-10 | 0.230 | 0.205 | Cubic |
| | Plasma IL-6 | 0.332 | 0.323 | Sigmoid |
| | Plasma TNF-α | 0.397 | 0.451 | Cubic |
| Plasma IL-10 | Plasma IL-1B | 0.419 | 0.383 | Sigmoid |
| | Plasma IL-6 | 0.469 | 0.703 | Cubic |
| | Plasma IL-8 | 0.584 | 0.624 | Cubic |
| | Plasma TNF-α | 0.586 | 0.538 | Power |
| Plasma IL-1B | Plasma IL-6 | 0.310 | 0.315 | Sigmoid |
| | Salivary IL-10 | -0.226 | 0.257 | Power |
| | Salivary IL-8 | -0.222 | 0.309 | Cubic |
| Plasma IL-6 | Plasma TNF-α | 0.390 | 0.378 | Sigmoid |
| Plasma IL-8 | Plasma TNF-α | 0.476 | 0.489 | Power |
| Salivary IL-10 | Salivary IL-1B | 0.624 | 0.686 | Growth |
| | Salivary IL-6 | 0.676 | 0.687 | Power |
| | Salivary IL-8 | 0.789 | 0.745 | Cubic |
| | Salivary TNF-α | 0.740 | 0.729 | Power |
| Salivary IL-1B | Salivary IL-6 | 0.735 | 0.713 | Sigmoid |
| | Salivary IL-8 | 0.745 | 0.707 | Logarithmic |
| | Salivary TNF-α | 0.780 | 0.872 | Cubic |
| Salivary IL-6 | Salivary IL-8 | 0.657 | 0.707 | Sigmoid |
| | Salivary TNF-α | 0.616 | 0.635 | Power |
| Salivary IL-8 | Salivary TNF-α | 0.871 | 0.854 | Compound |

*Associations by Backwards-Elimination Multiple-Regression modeling*

To evaluate the independence of the prediction in the hemi-matrix of binary inter-variable Spearman correlations (Table 4) by backwards-elimination multiple regressions, we anchored on the cytokine IL-10, as it was the only anti-inflammatory (Th2) cytokine assayed and would theoretically be a counter-weight to the other 4 (pro-infla - matory) cytokines in the series. Restricted to the domain of only the plasma cytokines, the regression modeling produced the par- simonious model with an $r^2$ value of 0.514, with IL-6, IL-8 and TNF-α remaining as predictor variables, after 2 models. When regression modeling was extended to include white blood cell count, which also had a significant association, the $r^2$ value remained unchanged; it strengthened further to an $r^2$ of 0.595, however, with all 11 complementary variables included in the modeling. The variables contributing determination within the model after serial modeling were plasma TNF-α, IL-6, IL-8; and salivary IL-1B. On the salivary 1L-10 side of the ledger, modeling with the remaining 4 salivary cytokines produced an $r^2$ of 0.762 in the final model, in which IL-1B, IL-8 and TNF-α remained as predictors after 2 models. Adding in further values from other, non-salivary biomarkers actually weakened the magnitude of the $r^2$ values of the resultant models.

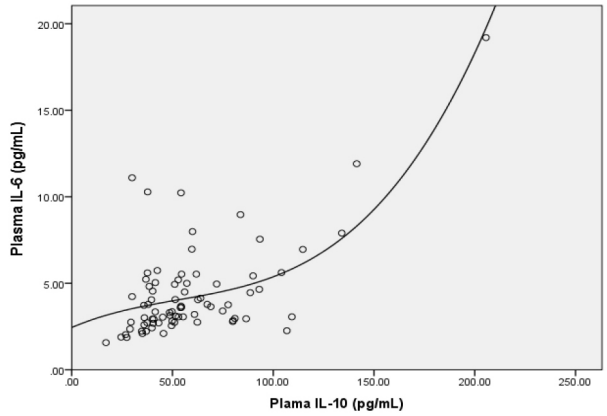
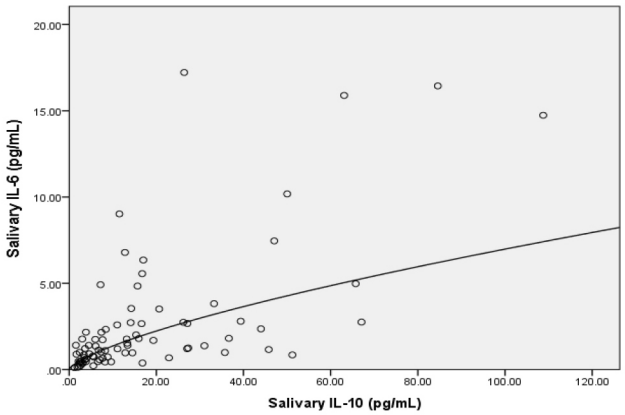
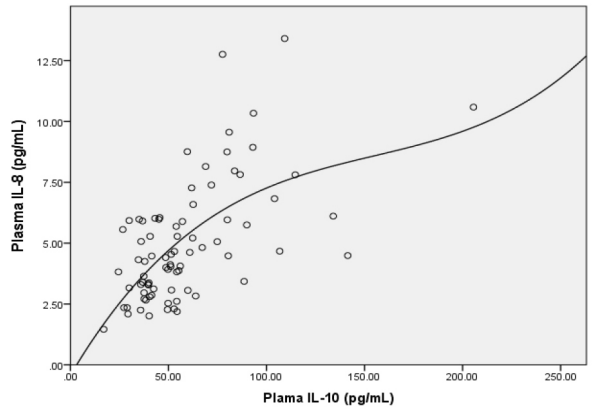
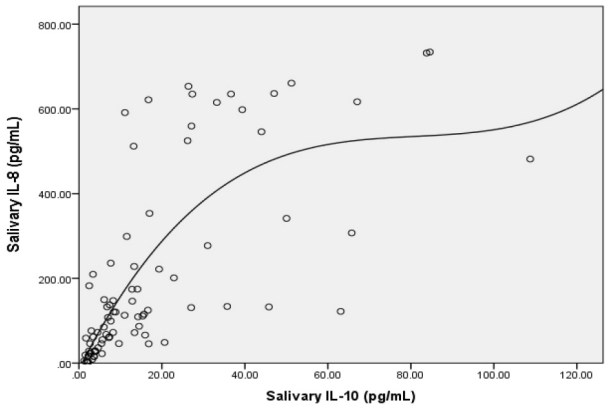
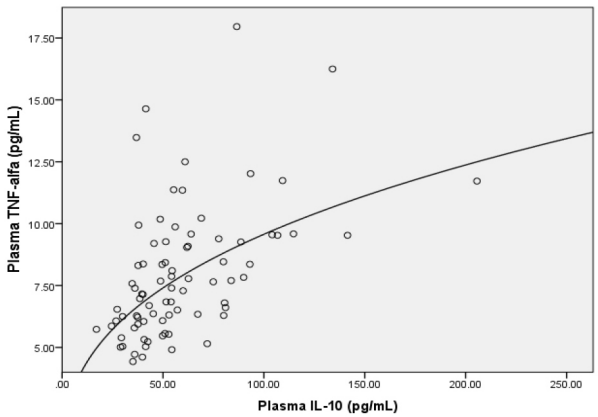
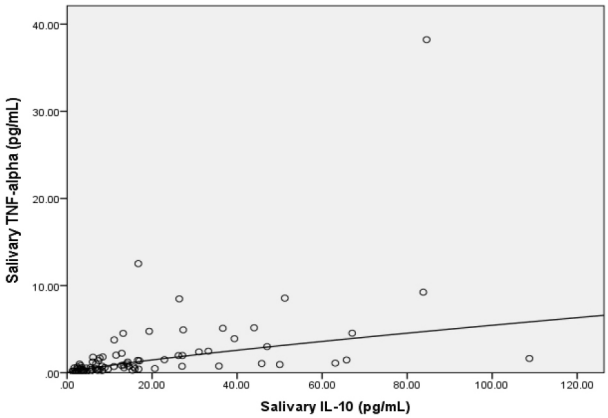
## Discussion

Poor-quality water[19] and rudimentary sanitation combine with indoor oven smoke [20], diverse parasites[21] to create conditions for abundant microbes and antigens to stimulate the immune systems of low-income residents of Guatemala. Our previous studies have shown evidence of immuno-stimulation. In urban children in Guatemala City, elevated C-reactive protein (CRP), α-1-acid glycoprotein (AGP) or both were elevated in 18% of blood samples collected for hematology[22]. Although these acute-phase protein biomarkers were omitted from this study, more sophisticated and modern diagnostic indicators (cytokines) took their place. The findings on growth (Table 1) showed variation by site, from extreme stunting to mild stunting across the centers. Underweight was milder, followed the pattern of de- creased stature. Wasting was virtually non-existent.

*Comparative analysis of biomarker distributions*

Leukocytosis is an elevation of the WBC above 10,500/$mm^3$. It is generally associated with infections, but also arises within the metabolic syndrome, coronary heart disease, and type 2 diabetes,[23]. On their day of collection, 9 subjects (11%) had an elevated WBC.
Calprotectin is a leukocyte-derived protein found in the cytosol of inflammatory cells that has been established as a sensitive marker of intestinal inflammatio [24,25].
We found 53 (61%) children with a calprotectin value >50mg/kg; this is almost twice the median value for a group of Ugandan children of a roughly comparable age and living in a correspondingly low- income setting[26].
As mentioned, cytokines are low-molecular-weight proteins that regulate immune responses, acting as mediators and messengers and are secreted by one cell to alter its own-another cell's-function[1]. Cytokines were analyzed in two compartments: plasma and saliva. For both anatomical compartments, our reference was the reported normative-range in post-menopausal women from New York from Browne et al.[18]. With regard to the plasmatic compartment, 19 (23%) of the subjects had levels of cytokines above the reference range for IL- 1B; this was 96% for IL-6; 0% for IL-8; 100% for IL-10 and 100% for TNF-α. Also, data from 223 normal-weight subjects from across Spain[27,28], which were

**Figure 3.** Selected Associations between Variables with the Superimposition of the Goodness-of-Fit Curve Form.

| PLASMATIC | SALIVARY |
|---|---|
|  |  |
| Cubic curve, r=0.703 | Power curve, r=0.687 |
|  |  |
| Cubic curve, r=0.624 | Cubic curve, r=0.745 |
|  |  |
| Power curve, r=0.538 | Power curve, r=0.729 |

Six examples of binary associations between inflammatory biomarker variables with application of the Goodness-of-Fit regres-sions are selected from among the 22 statistically- significant pairings in **Table 3** . They are chosen to illustrate the range of variation in curve form and strengths of correlation (r).

**Table 4.** Coefficients of the multiple regression model for the dependent variables plasmatic and salivary IL-10 as independent variables pro-in- flammatory cytokines, measured in the same body element (IL1B, IL-6, IL-8 and TNF-α).

| No. | Model | Unstandardized Coefficients B | Unstandardized Coefficients Std. Error | Standardized Coefficients Beta | t | Sig. | 95.0% Confidence Interval for B Lower Bound | 95.0% Confidence Interval for B Upper Bound |
|---|---|---|---|---|---|---|---|---|
| ***Dependent variable: Plasmatic IL-10**** | | | | | | | | |
| | (Constant) | -1.162 | 7.952 | | -0.146 | 0.884 | -16.999 | 14.675 |
| | Plasmatic IL-6 | 1.724 | 0.464 | 0.300 | 3.716 | 0.001 | 0.800 | 2.648 |
| 2 | Plasmatic IL-8 | 5.424 | 1.090 | 0.443 | 4.975 | 0.001 | 3.253 | 7.596 |
| | Plasmatic TNF-α | 3.098 | 1.011 | 0.273 | 3.063 | 0.003 | 1.084 | 5.113 |
| ***Dependent Variable: Salivary IL-10**** | | | | | | | | |
| | (Constant) | 4.318 | 1.351 | | 3.195 | 0.002 | 1.626 | 7.009 |
| | Salivary IL-1B | 0.223 | 0.041 | 0.644 | 5.390 | 0.000 | 0.141 | 0.306 |
| 2 | Salivary IL-8 | 0.057 | 0.006 | 0.719 | 9.609 | 0.001 | 0.045 | 0.068 |
| | Salivary TNF-α | -1.280 | 0.511 | -.344 | -2.504 | 0.014 | -2.298 | -0.262 |

*r2 = 0.514; r = 0.717 (n = 80); **r2 = 0.762; r = 0.873 (n = 79)

analyzed in the same laboratory with the same method and instruments can be used as reference values. For IL-6, the mean value for Spanish children of 4.4 pg/mL compares to our median value of 3.7 pg/mL; for IL-8, their result of 1.6 pg/ml mean of Spanish children is one-third that of the Guatemalan median of 4.5 pg/mL; and for TNF-α, the Spanish reference mean of 3.1 pg/mL is less than one half of our median of 7.5 pg/mL. Browne *et al*.[18] also provide normative values for the same cytokines in the salivary compartment in their study among postmenopausal women. In this anatomical compartment, 3 (4%) of our subjects have levels of cytokines above the reference range for IL-1B; this was 6% for IL-6; 14% for IL-8; 75% for IL-10; and 75% for TNF-α. In general, with some excep-tions, we could find modest to high rates of elevation when compared to reference values across the biomarkers from the 4 anatomical compartments.

*Mutual interactions of cytokines within and across anatomical compartments*

Of all of the biomarkers, IL-10 (plasmatic and salivary) and IL-8 (salivary) had the widest range of cross-associations, i.e. with 5 of the 11 companion biomarkers. If we isolate the interactions by type of biomarker, we see a predominance of what could be called "auto-correlation", that is association within the same compartment, specifically within salivary or plasmatic fluids. Of the possible ten cross-associations within the cytokines of the plasmatic compartment, 7 were statistically significant, whereas all ten possible correlations were significant in the salivary compart-ment. Of even more interest in a biological sense might be the significant associations between biomarkers of different anatomical compartments, e.g. plasma versus saliva, WBC versus feces, etc. The only significant correlations between anatomical compartments were those with plasma IL-1B with salivary IL-8 and with salivary IL-10. Goodness-of-fit models were run in order to improve the association with the best curve-form; strength of association improved in less than half of the significant correlations.

We also decided to run the backwards elimination multiple regression model with IL-10, the only anti-inflammatory (Th2) cytokine measured in each anatomical compartment. In each model, three variables emerged to be independent predictors, with IL-8 and TNF-α commonly present as predictors in models for both anatomical compartments.

*Within-individual correspondence of cytokines in plasma and saliva*

Because of the ease and innocuousness of saliva collection, especially for children, it has been recommended to exhaustively pursue its potential utility in field diagnosis[29]. In a survey of the salivary-cytokine literature, we find that the majority involve local conditions arising close to the salivary glands, such as oral inflammation[30,31] or head neck or oral, cancer[32,33]. Our observed dissociation of the cytokine response between the circulating pattern and that in the saliva, moreover, is neither unprecedented nor implausible.

In the present study, we found no significant plasmatic:salivary associations for any of the 5 cytokines measured in both anatomical compartments. Browne et al. [18], in post menopausal women, measured and associated the same 5 cytokines in both anatomical compartments with the same lack of significant association; their only finding of a significant plasmatic: salivary correlation was for IL-5.

With IL-6 as the only biomarker measured, Grisius et al.[34] and Minetto et al.[35] found no significant inter-compartmental correlation in healthy North American and Italian adults, respectively; this was also the experience of Fernandez-Botrán et al.[36], investigating emotionally-abused women, again with IL-6 as their only cytokine biomarker of interest. Among 8 cytokines, including the same 5 assayed in the present study, measured concurrently in plasma and saliva in healthy female adolescents, Riis et al. [37] found only IL-1B to reach a statistically-significant correlation between compartments. In 2013, Byrne et al.[38] evaluated 11 cytokines, including 4 of the 5 cytokines measured here, across the anatomical compartments in healthy Australian adolescents; they found significant associations only for IL-2, IL-12 and INF-γ, but for none of the cytokines measured in the present study. A final -and atypical-finding comes from patients with acute myocardial infarction in an Iranian hospital; among 4 cytokines measured in both the circulation and saliva, high inter-compartmental associations (all with p values of <0.001) were found for three: IL-2; IL-6; and TNF-α[39].

Despite the lack of individual correspondence of the cytokine concentrations, we applied them on a sub-group analysis. As a post-hoc exercise to further pursue the association of the systemic (plasma) and salivary compartments to reflect the aggregation of inflammation, we created a cumulative rank-order score for the 5 cytokines in each biological fluid

Separately, for each anatomical compartment, the sum of the rank (in ascending order) of each child in each of the 5 distributions: that is, the child with the lowest cytokine in each anatomical compartment was ranked as 1, and the individual with the highest value was ranked number 80, the number of children that had data for all cytokines. Thus, the combined composite scale could run from a high of 400 (the same individual was most inflamed for all 5 cytokines) to a low of 5 (the same individual was least inflamed for all 5 cytokines). As calculated, the individual cumulative rank-order score values ranged from 35 to 361 for the plasma biomarkers and 15 to 398 for the salivary. Even with this composite scoring of all cytokines, the Spearman correlation co- efficient for the plasma cumulative rank-order score with its salivary counterpart still showed an insignificant association ($r = -0.036$, $p = 0.749$).

Because circulating cytokines arise from adipose, hepatic and peripheral and tissue-fixed white blood cells[1], they would respond to systemic immuno-stimulation; by contrast, the salivary cytokines arise in salivary and oral sites governed by local events in and around the buccal cavity[40]. It would seem that systemic immune res-

ponse has low penetrance to the sites of salivary cytokines' origin.

*Hierarchical, group-wise relationships of cytokine biomarkers*

Although there is scant inter-compartmental correspondence at the individual level, that fact does not dismiss all of the possibilities. The question still remains as to whether salivary cytokines can be used as a proxy for those in the circulation on a group-wise basis. A survey in Sweden approached the utility of salivary cytokines as an epidemiological tool for screening for systemic diseases[41]. They considered tobacco smoking and 8 selected systemic disease conditions as self-reported by 1000 adults in southern Sweden. The cytokine biomarkers were IL- 1B, IL-6, IL-8 and TNF-α. For histories of heart surgery, heart disease, hypertension, diabetes and mental disorders, there were no associations with elevated salivary cytokines. Moreover, with salivary IL-6 and TNF-α, there were no association with any of the selected conditions. However, salivary IL-8 was elevated significantly in those reporting being smokers and having a history of tumors, bowel diseases and muscle and joint disorders. The latter was also associated independently with elevation of IL-1B. They suggest a certain utility of salivary cytokine biomarkers in population epidemiology. Plasma cytokines were not concurrently measured. Similar explorations of salivary cytokines with systemic diseases have been reported individually including: inflammatory bowel diseases[42]; pediatric type 1 diabetes[43]; obesity-related sleep apnea[44]; and cutaneous lichen planus[45].

Cytokines elevations were found for IL-8 among 90 salivary proteins after total body irradiation[46], similarly anti-inflammatory cytokine, IL-10, rose in saliva of airway-disease patients when exposed to thermal sulfur water bath therapy[47]. Finally, monitoring of diverse salivary biomarkers during 520 days of simulated space travel to Mars, showed no change in the 4 cytokines evaluated: IL-2, IL-6, TNF-α and INF-γ[48].

Research surrounding stress responses and the anti-inflammatory cytokine, IL-6 has been prominent. Reviewing the literature, Slavish and Graham-Engeland[49] conclude that the findings are currently inconsistent

Salivary IL-6 has been elevated in response to a social stress test[50], viewing disgusting visual images[51], spinning gyrations on a rotary device[35], and a psychiatric counseling visit[52]; this response was not seen, however, exercising to the point of exhaustion[35].

The elevation was also associated with active post-traumatic stress syndrome (PTSD) associated with spousal abuse in women[52]. In three situations in two studies, circulating IL-6 was simultaneously evaluated. The salivary and plasmatic responses corresponded with PTSD[52] and rotator stress[35], but not with exhausting exercise[35].

To examine this group-wise phenomenon in our own study setting, we calculated medians of the previously-described composite cytokine rank-order scores. We analyzed them by day-care site and by each body fluid (Table 5). Interestingly, in the sense of a common trend, the relative hierarchy was common across anatomical compartments, with the highest cytokine rankings clustered in the rural zone for both composites scores; the intermediate values are in the marginal-urban area; and the lowest rankings are seen in the semi-urban setting. The Kruskal-Wallis test, however, failed to find a significant difference among the sites.

*Strengths and limitations of the study*

We acknowledge certain strengths and weaknesses of design and execution of the study. The principal strength is that it deals with childhood, with children of preschool age, in a context of poor hygienic environment. Also related to young age are two collection methods, fecal and salivary, which are non-invasive and do not require the extraction of blood.

In theory, the settings with similar dietary offering should control one important variable, and narrow overall variance. An addi-

Table 5. Median Cytokine Cumulative Rank-Score by setting.

| Setting | N | Plasmatic Cytokines | Salivary Cytokines |
|---|---|---|---|
| | | median score (95% CI) | |
| Semi-Urban (A) | 20 | 172 (151 – 231) | 134 (105 – 217) |
| Marginal-Urban (B) | 38 | 186 (152 – 225) | 184 (162 – 233) |
| Rural-(C) | 22 | 227 (191 – 243) | 246 (194 – 260) |
| p-value | | 0.349* | 0.090* |

*Comparison among settings using Kruskal-Wallis test.

tional strength is the possibility to relate a large variety of inflammatory biomarkers from different anatomical compartments. A very important methodological strength is that we can compare our cytokine values with those generated with the identical multiplexing method[18], and, in the case of the Spanish children[27,28], a similar age-range and the same laboratory and equipment in Granada. The foremost limitation of the study is that our sample-size of 87 is modest, and the analyses by separate daycare site may have lacked statistical power to fully capture differences by geographical setting (Table 5). In addition, we were not able to measure the two most commonly used biomarkers of inflammation, CRP and APG, if not simply to relate them to the more sophisticated cytokine panels. Moreover, saliva is a viscous matrix. At least for an ELISA method, Dafar et al.,[53] found that extraction with sodium dodecyl sulfate (SDS) improved the detection of one of the cytokines of interest, salivary IL-8; whether this applies to the Milliplex method as well is not known. Thomas et al.[54] examined day-to-day fluctuation in salivary IL-1B, IL-6 and TNF-α finding coefficients of variation of up to 200%. As our design called for only a single day's collection, we can perhaps understand how the internal correlation among salivary cytokines could be so well coordinated while their associations with biomarkers in other anatomical compartments could be so poor. One could generalize this point to project that associations with factors external to the subjects might only be revealed with multiple repetitions of the measurements in the same individuals.

## Conclusion

Twelve biomarkers of inflammation from the whole blood, fecal, plasma and salivary compartments showed a diverse array of findings and interactions among low-income preschool children from the western highlands of Guatemala. In general, the indicators were elevated above reference levels, suggesting a response to the microbial and antigenic milieu of the poorly hygienic surroundings in which they live. The marker of fecal inflammation exhibited no interaction with any biomarker in any other domain. White cells showed a modest interaction, associating positively with three circulating cytokines. Similarly modest were the cross-associations for cytokines between plasma and saliva, with plasmatic IL-1B having two significant -and negative- associations. Within-compartment, however, the plasma and salivary cytokines showed a vigorous mutual interaction.

The prominence of the anti-inflammatory cytokine IL-10 in both plasma and saliva is consistent with its counter-regulatory role in the face of elevated pro-inflammatory cytokines.

## Supporting Information

S1 Checklist. STROBE Statement of items included in this cross-sectional study.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## Author Contributions

Conceived and designed the experiments: NWS KS AG. Performed the experiments: MERA CMA MCR. Analyzed the data: MJSM. Wrote the paper: MJSM NWS KS AG. Performed field wo k: MJSM MERA.

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# Chapter 6

# Interaction of Biomarkers of Oxidation and Antioxidation Defenses with those of Inflammation Status among Preschool children of the Western Highlands of Guatemala.

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In preparation

# IN PREPARATION

## INTERACTION OF BIOMARKERS OF OXIDATION AND ANTI-OXIDATION DEFENSE WITH THOSE OF INFLAMMATION STATUS AMONG PRESCHOOL CHILDREN FROM THE WESTERN HIGHLANDS OF GUATEMALA.

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**Abstract**

*Background:* Oxidative stress and inflammatory responses can arise from similar exposures, and are interrelated at the cellular level through nuclear factor kappa beta (NFkB). Interaction examples abound in animal models. What the associations would be for biomarkers of the respective classes of stress/response in free-living humans is minimally explored.

*Objective:* To determine the pattern and magnitude of paired associations of diagnostic biomarkers from oxidation and inflammation classes

*Methods:* A total of 23 biomarker variables from whole blood, plasma, saliva, urine and feces – 11 from an oxidation/anti-oxidation defense domain (F2-isoprostane and 8-hydroxy-deoxy-guanisine in urine; superoxide dismutase, catalase and glutathione peroxidase and reductase in red blood cells; and circulating retinol, ß-carotene, α-tocopherol, and co-enzyme Q9 and Q10) and 12 from the inflammatory domain (white blood cells; fecal calprotectin; and IL-1B, IL-6, IL-8, IL-10, and TNF-α in both plasma and saliva) were measured in up to 87 low-income preschool children attending three daycare centers in the Western Highlands of Guatemala.

*Results:* By pair-wise Spearman rank-order correlation coefficient analysis involving a 132-cell matrix, 13 statistically-significan associations (9.8%) were found, seven at a p value of <0.01. Goodness-of-fit models enhanced the strength of association in 11 instances. SOD and β-carotene were each associated with five and six inflammator biomarkers, respectively. Backward-elimination multiple-regression analyses with both as dependent variables showed the same two predictors, plasmatic TNF-α and saliary IL-8, with an r-value of 0.394 for SOD and 0.368 for β-carotene.

*Conclusion:* Significant associations in an inter-class context between paired, oxidation vs inflammation biomarker variables represent only a fraction, percentage-wise, of that seen with the same data, analyzed on an intra-class basis.

Key Words: *Oxidation - Inflammation - NFkB - Biomarkers – Guatemala*

## Introduction

All organisms are constantly subjected to external (environmental, dietary) or endogenous (metabolic, pathological) influe - ces that can incite an inflammatory response in the immune system,[1,2] or an excess generation of oxidative free radicals[3,4].
Fortunately, the state of immune response and oxidation can be counteracted and regulated by endogenous anti-inflammator and anti-oxdant mechanisms. Moreover, at the basic, cellular biological level, there are several points of convergence for the two classes of stress. Most notable is the NFκB transcription factor and its signaling pathway. It has been traditionally advanced that the level of oxidative stress in a cell is an inducer -or at least a facilitator- of NFκB's activation of cytokine-production[5]; this is a putative nexus between oxidation and inflammation
One can imagine biological scenarios in which, for instance, the mobilization of inflammatory cells to an infection focus would induce increased free-radical generation[6] or in which oxidative damage to cells or tissue would illicit the mobilization an inflammatory response[7]. This potential for bidirectional interaction has led to a large number of recent investigations in which the co-existence of oxidation and inflammation responses has been documented in rodent models[9-20] and human clinical studies[21-25]. Shei et al.[26] in reviewing human studies with diverse biomarkers comment: "Increased oxidative stress and inflammatory responses among individuals performing strenuous exercise, elite athletes, or military personnel have been consistently reported."
The key to the exploration of this situation in humans, as cited above, is the existence of detectable biomarkers of the damage induced by oxidation and in the responses to avert or reduce oxidative stress and to respond to an antigenic challenge in the immune system. On the one hand, for oxidation, parts of cell structures are fragmented with the release of metabolites such as thiobarbituric acid reactive substances (TBARS)[27,28] and F2-isoprostanes[29,30] from free-radical action on lipid membranes and lipoproteins, whereas 8-hydroxy-deoxy-guanosine (8-OHdG) and 5-hydroxy-methyluracil[31] arise from oxidation of DNA[32-34] and protein carbonylation from attacks on peptide structures[34,35]; the former two classes can be detected in urine specimens, whereas the latter class is detectable in blood samples. In addition, various nutrients, such as retinol, alpha-tocopherol, carotenoids, co-enzyme Q, zinc, copper, manganese and selenium participate in conjunction with the molecular elements of the anti-oxidant system composed of enzymes dedicated to neutralizing free radicals[36]. On the other hand, the acute and chronic inflammator response also can be gauged by a series of biomarkers from an elevation in the white blood cell count to increased complement-reactive protein (CRP)[37], and within the balance of an array of pro- and anti-inflammatory cytokine [38].
In a research project among up to 87 low-income preschool children attending three daycare centers in the Western Highlands of Guatemala, we measured a total of two biomarkers of oxidative stress, nine markers of anti-oxidative response, and 12 biomarkers of inflammation. The degree of mutual and harmonious interaction of these biomarkers, often from different anatomical compartments of the body, have been explored separately within their respective oxidation-related[39] and inflammation-relate [40] domains. These demonstrated extensive -but anatomically

selective- associations generally consistent with a *priori* expectations for mutual interactions. Guided by the intra-domain inquiries cited above, we have advanced our exploration in these preschoolers to the level of interactions of the respective biomarkers series, one with another. It was hypothesized that biomarkers of non-invasive collection, such as urinary oxidative biomarkers, might usefully serve as proxies for inflammation that require blood for assessment. We present here the results of this inter-class analysis.

## Subjects and Methods

### *Study design*

The following is a cross-sectional, descriptive, field study on the associations among variables related to inflammation and oxidation. The study is part of the larger project entitled: "Study on the normative state and inter- and intra-individual variation in growth, hematology, hydration, and markers of oxidation, infection and infla - mation in pre-school children with a similar dietary intake."

### *Recruitment of the Sample*

Children were elegible if they attended one of the three daycare centers made available to the study by the authorities of the day care system. Sujects had to be between two and seven years-old, and to have maintained at least an 80% attendance during the time of the project. Additionally, subjects had to be apparently healthy, with no restrictions for consuming the diet offered by the rotating menu of the SOSEP (Secretaría de Obras Sociales de la Esposa del Presidente "Secretariat of the Beneficial Works of the First Lady"). A parent or legal guardian signed an informed consent form. If a child did not adhere to the collection schedule for urine or feces, he or she was excluded from the analyses related to those specific biomarkers

### *Environmental Setting, Diet and Nutritional Status of Subjects*

The Western Highlands Province of Quetzaltenango was the setting of the study.
This rural-based agrarian area has its capital city located 136.7 miles from Guatemala City at 7733 feet above sea level. The province has an annual mean daily temperature of 14.7ºC, lower than the rest of the country and a majority of the population (60.6%) is of Mayan ascendancy. Each of the three settings of SOSEP-affiliated daycare centers had differences in the ethnic make-up, and its corresponding traditions and cultural customs; for example, almost exclusively Mam-Mayan children attended rural setting. The common, 40-day rorating menu offered by SOSEP daycare centers is standardized in its recipes and offers 4 meals per weekday[41].

### *Ethical Considerations*

The director of SOSEP for the Quetzaltenango project approved for the study to be conducted within the system. The Human Subjects Committee of the Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM) granted the study protocol ethical approvation. The purposes, benefits, and conveniences, and the risks of the procedures where explained in the written consent form, signed by parent or guardian. At the moment of collection, the children gave a final agreement (assent) as well. A benefit for the participants was the provision to the center by the study of any dietary items unavailable for a given mealplan as listed on the 40-day rorating manu. This study was registered at clinicaltrials.gov as NCT02203890.

### *Collection, Handling and Storage of Biological Samples*

*Plasma and whole blood:* Blood was extracted by an experienced phlebotomist

using Vacutainer® 4 mL tubes with EDTA (No. 367861) and Safety-Lock ™ diposable needles (No. 367281) both form Becton-Dickinson (Franklin Lakes, NJ, USA). Blood samples were centrifuged to separate plasma from red blood cells and both components were stored at -80ºC in order to be shipped to the Center of Biomedical Research, University of Granada, Granada, Spain in to perform laboratory analyses and determine antioxidant enzymes, antioxidant nutrients and plasma cytokines.

*4-hour Urine:* 24-h urine samples were collected during the 8th week of the study. Collection started at each daycare center when the child arrived with SOSEP personnel's assistance, using the Vacutainer® (No.364999) plastic 24-h collection container (Becton-Dickinson, Franklin Lakes, NJ, USA). Collection was continued by parents at home during afternoon and night, and finished in the center after 24 hours; the process was repeated if reported to have been incomplete.
The urine was brought to the laboratory and homogenized before aliquoting. The aliquots were stored at -80ºC and sent to the Center of Biomedical Research, University of Granada, Granada, Spain for determination of oxidative biomarkers.

*Saliva:* We collected saliva the same day of the blood collection, on the 8th week of the study. Saliva was stored in a -80oC freezer until shipment to Granada University for saliva cytokines determination.

*Feces:* A fecal sample for calprotectin determination was collected on the 7th week of the period. A stool-cup container was supplied to parents or caregivers to make the collection the next morning before bringing the child to each of the centers. When the container returned empty from the home, a sample was collected at the daycare center during that day. Samples were processed and stored in a -20ºC freezer to perform analyses until we had all subjects' samples.

*Determination of Oxidative Stress and Anti-Oxidant Biomarkers*

Analyses to determine the two urinaty biomarkers of oxidative stress -15-isoprostane F2t (F2-Iso) and 8-Hydroxydeoxyguanosine (8-OHdG)- were performed using ELISA assay kits No. EA84.102606 (Oxford Biomedical Research Inc., Rochester Hills, MI, USA) and No.IM-KOGHS040914E (JaICA, Nikken SEIL Co., Ltd., Shizouka, Japan), respectively.
Drabkin's reagen D5941 (Sigma, St. Louis, MO, USA) was used to determine hemoglobin (Hb) concentration. The final Hb concentration was adjusted to 5 mg/mL for antioxidant enzymes analyses.
The Aebi[42] method was used to perform catalase (CAT) activity determination; superoxide dismutase (SOD) was determined by spectrophotometry[43]; gluthathione reductase was analized using the Calberg and Mannverik[44] method; and gluthathione peroxidase (GPX) was determined using the method developed by Flohé and Günzler[45].
High-performance liquid chromatographic (HPLC) method was performed in a 100 µL plasma sample in order to measure the plasma antioxidant nutrients (retinol, alpha-tocopherol, β-carotene, and coenzymes Q9 and Q10)

*Determination of Inflammatory Biomarker*

*White blood cell count:* White blood cell count (WBC) determination was perfomed at La Democracia Hospital's clinical biochemistry laboratory, in Quetzaltenango Guatemala, using the Coulter AcT Diff Hematology Analyzer (Beckman, Krefeld, Germany). WBC is expressed as quantity per mm3 volume.

*Fecal calprotectin:* The calprotectin ELISA test No. CAL0100 (Calpro AS, Lysaker, Norway) was used to determine calprotectin concentrations in Quetzaltenango Guatemala. Concentrations are expressed in mg/

kg of sample. Detection limits are 25-2500 mg/kg, and the typical CV was 3.7%.

*Plasma and saliva cytokines:* The Milliplex® MAP Human High Sensitivity Cytokine panel No.HSCYTMAG-60SK (Luminex Corp., St. Louis, MO, USA) located in the University of Granada, Spain, was used to analyze plasma and saliva samples in order to determine the following five cytokines: IL-1β, IL-6, IL-8, IL-10 and TNF-α. The minimum detection limits were 0.06, 0.20, 0.05, 0.48 and 0.07, respectively. The inter-assay CVs for plasma cytokines were 14.73%, 7.74%, 8.65%, 12.17% and 8.40%, respectively; and for salivary cytokines were 10.99%, 13.88%, 13.31%, 7.74%, 10.99% and 13.34%, respectively.

*Data Handling and Statistical Analysis*

A database was created in the SPSS version 20 software (Chicago, IL, USA). The Spearman rank-order test was used to determine associations between the variables from oxidation and infla - mation classes. The goodness-of-fit modeling was used to obtain correlation coefficient and different curve forms. The backwards-eleminitation multiple regression models were performed in order to determine the predictive determination in associations among the variables that presented correlations between the two classes. A probability level of ≤0.05 was accepted as statistically significant

## Results

*Characteristics of the Population*

82 children, 38 girls and 44 boys (median age 56 mo), deliver samples for one or more of the required determinations in this analysis. Figure 1 also dissagregates sample by site. It was possible to make pairing correlations of inflammation and oxidation data for 74-82 children, depending on the combinations of anatomical compartments in the full-matrix.

*Magnitude and Pattern of Significant Inter-Class Associations:*

A matrix of 132 paired-variable cells from the 11 oxidation and anti-oxidant-defense biomarkers on the X-axis and the 12 inflammator biomarkers on the Y-axis is shown in Figure 2. Each cell contains a Spearman rank-order correlation coefficient, a probability value and a number of paired values for the two variables. Overall, 13 inter-class associations, one-tenth of the maximal possibility, reached the 0.05 probability criterion for significanc (Table 1). A total of three variables from the oxidation-antioxidation series (SOD, retinol, beta-carotene) showed one or more significant cross-associations, while the remaining 8 were unassociated. Conversely, only two inflammatory variables (salivary IL-1B and IL-6) did not show significant associations, with the remaining 10 having a significant level of association. The Spearman correlation coefficients (r value) ranged from a low of 0.226 (p=0.042) for plasma retinol with plasma IL-6 to a high of 0.363 (p=0.001) for beta-carotene with plasma TNF-α.

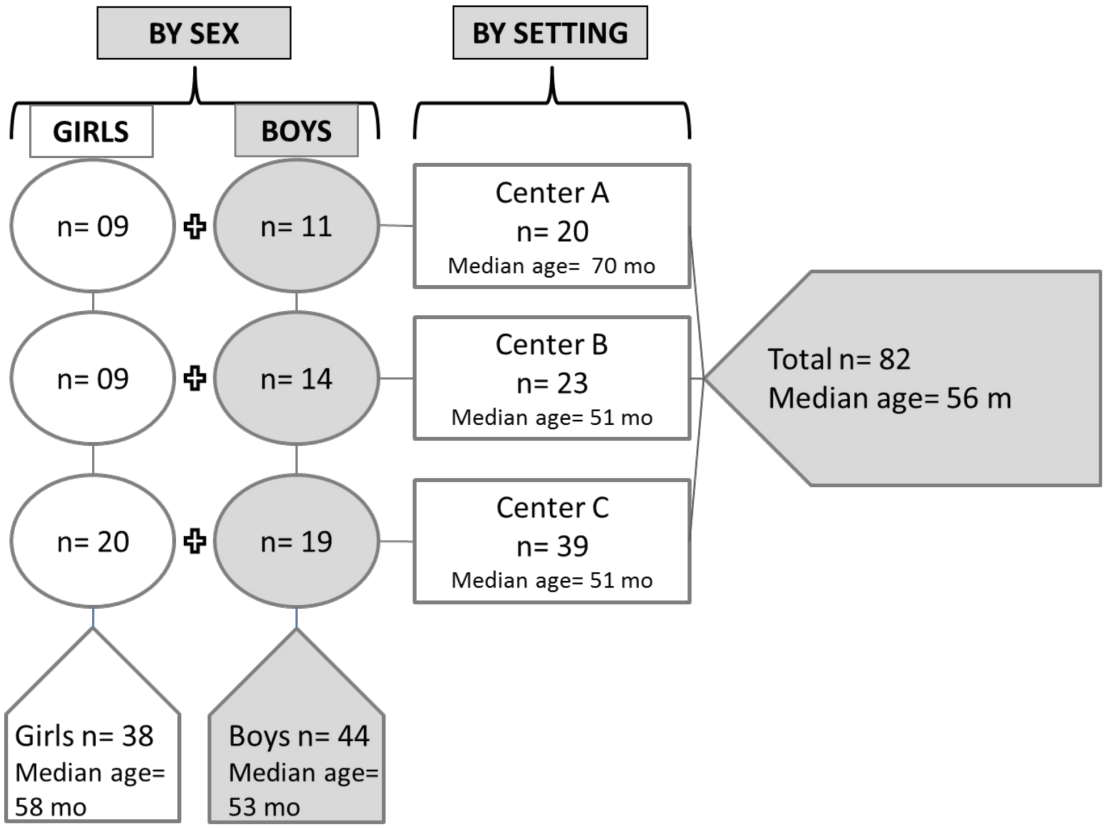
In terms of the pattern as viewed from the X-axis projection onto the Y-axis, there were two major “shafts” with five significant associations subtended by SOD and six by beta-carotene, and one “couplet” related to retinol (Figure 2).

As viewed from the Y-axis projection onto the X-axis, there are three couplets, with plasma and salivary TNF-α and salivary IL-8. Five additional inflammatory biomarkers projected a single significant association with an X-axis variable (Figure 2).

*Amplification by Goodness-of-Fit Correlations:*

For the 13 pairings with a significant association by the Spearman rank-order perspective, we generated a goodness-of-fit correlation coefficient. There is no *a priori*

Figure 1: Characteristics of the samples dissagregated by setting and sex.




guidance as to which variable in the pair should be considered independent or dependent. With non-linear regressions, the assignment of independence for variables makes a difference, such that goodness-of-fit correlations were generated for both alternative configuration of the significant associations. The strength of the both r values, as well as the curve form of the association, is shown in Table 1. The magnitude of the r value was enhanced in 6 instances. The most common curve forms were power and exponential. The value of the r value for the goodness-of-fit correlation increased beyond the corresponding Spearman value by 10% or more in 11 of 26 associations as illustrated by the asterisks in Table 1. This occurred for bidirectional orientations of the association for five specific pairings.

Graphic visualizations of the six strongest associations by goodness-of-fit are illustrated in Figure 3.

*Backward-Elimination Multiple Regression Analysis*

The correlation matrix offered two "shafts" from the reflection of the oxidation variables on the inflammatory variables. This obligated our backward-elimination multiple regression analysis to treat the oxidation-related variable as the dependent variable and assessing how many potential *independent* variables remained significant through running sequential modeling (Tables 2 and 3). Both with SOD (Table 2) and beta-carotene (Table 3), the regression modeling eliminated all but two variables through the first three rounds, retaining the same two cytokine biomarkers -plasmatic TNF-α and salivary IL-8- into the 4th model.

## Discussion

The environment and conditions under

**Figure 2.** Spearman Correlation Coeficient Full-Matrix for Relationships between Biomarkers of Oxidation and Antioxidation, and Inflammation.

| | | F2-Iso (ng/mL) | 8OHdG (ng/mL) | Catalase Activity (nmol/seg/ g Hb) | SOD Activity (U/g Hb) | GR Activity (umol/min /g Hb) | GPx Activity (µU/g Hb) | Retinol (mg/L) | α-tocopherol (mg/L) | β-carotene (mg/L) | CO-Q9 (mg/L) | CO-Q10 mg/L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Wihite Blood Cell Count** | r-value | .015 | -.051 | .005 | .149 | .080 | .018 | .059 | -.005 | -.322** | .0449 | .122 |
| | p-value | .901 | .664 | .967 | .182 | .475 | .871 | .599 | .967 | .003 | .691 | .276 |
| | N | 74 | 74 | 82 | 82 | 82 | 82 | 81 | 81 | 81 | 81 | 81 |
| **Fecal Calprotectin e (mg/Kg)** | r-value | -.019 | .054 | -.205 | -.147 | .014 | .062 | -.227* | -.209 | -.108 | -.173 | .037 |
| | p-value | .871 | .637 | .064 | .188 | .901 | .583 | .042 | .062 | .337 | .123 | .743 |
| | n | 78 | 78 | 82 | 82 | 82 | 82 | 81 | 81 | 81 | 81 | 81 |
| **Plasma IL-10 (pg/mL)** | r-value | .119 | -.063 | .203 | .091 | .007 | .001 | -.019 | .021 | -.288** | .010 | .062 |
| | p-value | .314 | .594 | .067 | .415 | .948 | .989 | .868 | .849 | .009 | .931 | .581 |
| | n | 74 | 74 | 82 | 82 | 82 | 82 | 81 | 81 | 81 | 81 | 81 |
| **Plasma IL-1B (pg/mL)** | r-value | -.166 | -.090 | -.007 | -.239* | -.107 | -.084 | .031 | .156 | .205 | .179 | -.055 |
| | p-value | .156 | .443 | .948 | .030 | .340 | .454 | .787 | .166 | .066 | .110 | .624 |
| | n | 74 | 74 | 82 | 82 | 82 | 82 | 81 | 81 | 81 | 81 | 81 |
| **Plasma IL-6 (pg/mL)** | r-value | .056 | .025 | .156 | .021 | .110 | .134 | -.226* | -.065 | -.183 | -.082 | -.126 |
| | p-value | .636 | .834 | .162 | .850 | .325 | .230 | .042 | .567 | .103 | .469 | .261 |
| | N | 74 | 74 | 82 | 82 | 82 | 82 | 81 | 81 | 81 | 81 | 81 |
| **Plasma IL-8 (pg/mL)** | r-value | .017 | .068 | -.059 | .277* | -.134 | .068 | -.017 | .053 | -.198 | .083 | .086 |
| | p-value | .887 | .565 | .598 | .012 | .231 | .543 | .881 | .638 | .076 | .464 | .447 |
| | N | 74 | 74 | 82 | 82 | 82 | 82 | 81 | 81 | 81 | 81 | 81 |
| **Plasma TNF-alfa (pg/mL)** | r-value | .115 | .048 | .071 | .294** | .126 | .065 | .051 | -.006 | -.363** | -.068 | .039 |
| | p-value | .331 | .687 | .526 | .007 | .259 | .564 | .651 | .959 | .001 | .546 | .730 |
| | N | 74 | 74 | 82 | 82 | 82 | 82 | 81 | 81 | 81 | 81 | 81 |
| **Salivary IL-10 (pg/mL)** | r-value | .137 | .028 | .158 | .199 | -.006 | .022 | -.101 | -.193 | -.290** | -.028 | -.065 |
| | p-value | .240 | .813 | .162 | .077 | .960 | .849 | .375 | .089 | .009 | .809 | .569 |
| | N | 75 | 75 | 80 | 80 | 80 | 80 | 79 | 79 | 79 | 79 | 79 |
| **Salivary IL-1B (pg/mL)** | r-value | .003 | -.057 | .066 | .034 | -.185 | .140 | .081 | .128 | .035 | .176 | .059 |
| | p-value | .980 | .627 | .560 | .763 | .100 | .217 | .479 | .263 | .760 | .121 | .607 |
| | n | 75 | 75 | 80 | 80 | 80 | 80 | 79 | 79 | 79 | 79 | 79 |
| **Salivary IL-6 (pg/mL)** | r-value | .085 | .024 | .212 | .174 | -.056 | .125 | -.017 | .011 | -.185 | .121 | -.046 |
| | p-value | .467 | .837 | .059 | .122 | .620 | .268 | .883 | .922 | .103 | .289 | .686 |
| | n | 75 | 75 | 80 | 80 | 80 | 80 | 79 | 79 | 79 | 79 | 79 |
| **Salivary IL-8 (pg/mL)** | r-value | .140 | .040 | .178 | .263* | -.115 | .032 | -.107 | -.134 | -.310** | .057 | -.104 |
| | p-value | .233 | .731 | .115 | .019 | .310 | .775 | .348 | .239 | .005 | .620 | .360 |
| | n | 75 | 75 | 80 | 80 | 80 | 80 | 79 | 79 | 79 | 79 | 79 |
| **Salivary TNF-alfa (pg/mL)** | r-value | .138 | .032 | .175 | .262* | -.117 | .033 | -.107 | -.132 | -.307** | .058 | -.104 |
| | p-value | .237 | .783 | .121 | .019 | .302 | .774 | .347 | .247 | .006 | .610 | .362 |
| | n | 75 | 75 | 80 | 80 | 80 | 80 | 79 | 79 | 79 | 79 | 79 |

Shaded cells present significant corrrelations.

which the subjects of this study live would be conducive to both inflammation and oxidation. At the nexus of this combined inquiry is the litany of candidate factor of Berk et al.[46]: "psychosocial stressors, poor diet, physical inactivity, obesity, smoking, altered gut permeability, atopy, dental cares, sleep and vitamin D deficiency"; with the exception for obesity, smoking and sleep disturbance, this listing would seem applicable to our children's Guatemalan setting. Moreover, they are candidates for inciting oxidation as well, along with smoke contamination in under-ventilated homes[47]. In fact, in the study of mutual associations among the 11 oxidative stress and anti-oxidant defense indicators[39], 51% of the pairings across the cells of the correlation hemi-matrix were statistically significant. In the similar study among the 12 inflammatory biomarkers[40], 33% of the correlation hemi-matrix had significant Spearman r values.

Given these numerous and logically-integral within-domain interactions previously found in these children with the same set of biomarkers reexamined here, we anticipated finding a rich array of interaction across domains, as well. In the present study, however, only 9.8% of the 132 cells in the full-matrix showed statistically-significant associations. Thus, for this varied litany of biomarkers, the within-domain associations examined earlier[39,40] were 3- to 5-times more abundant than are the across-domain correlations explored here.

We, of course, have been cognizant of the penchant for multiple comparisons to generate significant associations mere-ly by chance. With a probability criterion of 5% among 132 cells in the matrix, one could expect an overall number of six to seven associations occurring at the $p \leq 0.050$ level, with one of these being at the $p \leq 0.010$. Applied to the 13 significant

Table 1. Comparison of Spearman and Non-Linear Correlation Coefficients in Inter-Biomarker Significant Associations with Ascending Order by Spearman values

| Oxidation variable | Inflammation variable | Spearman rank-order r-value | Goodness-of-fit r-value | Curve form | Goodness-of-fit r-value | Curve form |
|---|---|---|---|---|---|---|
| | | | Oxidation as Independent (x-axis) variable | | Inflammation as Independent (x-axis) variable | |
| SOD | Plasmatic IL-1β (n=82) | -0.239 | 0.217 | Sigmoid | 0.292 | Cubic |
| | Plasmatic IL-8 (n=82) | 0.277 | 0.276 | Cubic | 0.280 | Cubic |
| | Plasmatic TNF-α (n=82) | 0.294 | 0.335 | Quadratic | 0.334 | Power |
| | Salivary IL-8 (n=80) | 0.263 | 0.360 | Power | 0.409 | Sigmoid |
| | Salivary TNF-α (n=80) | 0.262 | 0.216 | Power | 0.254 | Cubic |
| Retinol | Calprotectin (n=81) | -0.227 | 0.170 | Exponential | 0.211 | Cubic |
| | Plasmatic IL-6 (n=81) | -0.226 | 0.268 | Cubic | 0.249 | Power |
| β-carotene | WBC (n=81) | -0.322 | 0.292 | Cubic | 0.250 | Cubic |
| | Plasmatic IL-10 (n=81) | -0.288 | 0.270 | Power | 0.286 | Sigmoid |
| | Plasmatic TNF-α (n=81) | -0.363 | 0.342 | Power | 0.348 | Sigmoid |
| | Salivary IL-8 (n=79) | -0.310 | 0.432 | Exponential | 0.452 | Cubic |
| | Salivary IL-10 (n=79) | -0.290 | 0.317 | Exponential | 0.427 | Inverse |
| | Salivary TNF-α (n=79) | -0.307 | 0.227 | Exponential | 0.248 | Cubic |

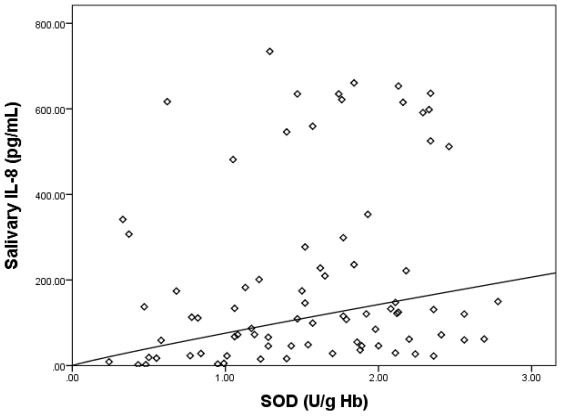
associations found, six had probability values between p≤0.050 and p=0.010, with the remaining seven at a level of p<0.010. In probabilistic terms then, almost all of the lower-grade associations could theoretically be attributable to chance alone, whereas only one of the higher-grade associations is likely to have occurred by chance.

From the standpoint of the oxidation series, both of the associations with circulating retinol were of the lower-order; of the fiv associations with SOD, four were of the lower-order; but of the six associations with beta-carotene, all were of the higher-order. This is bolstered by operation of the backward elimination multiple regression for explanation of beta-carotene by infla - matory biomarkers; the fact that 11 of the strength of the goodness-of-fit regressions were stronger than their corresponding Spearman correlation coefficients further fortifies the likelihood of validity around this analyte.

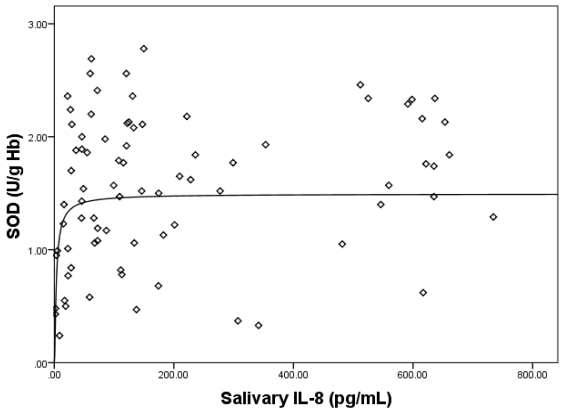
The theoretical basis for inter-class interaction between biomarkers of oxidative and inflammatory natures is strong, much stronger in fact than the number of interactions we find in the present findings from human diagnostic biomarkers. One explanation could be the luxury of experimental designs in laboratory animals where the proof of interaction is more abundant.

Anatomical considerations, however, could be important in the human context. It is not hard to imagine that white cells produced in the bone narrow or calprotectin produced from white cells in the gut would have difficult interaction with the circulating and salivary compartments. Alpha-tocopherol is localized on lipid frameworks within the

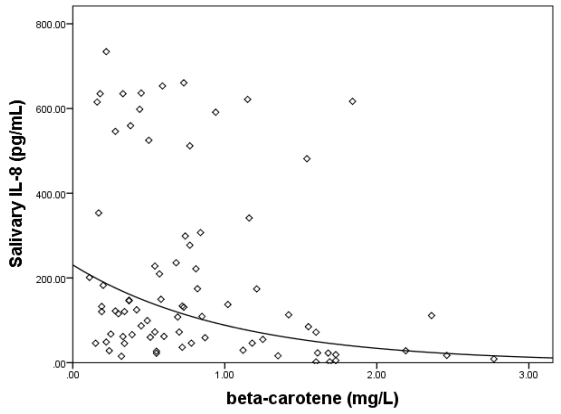
Figure 3: Selected Associations between Variables of Oxidation and Inflammation with the Superimposition of the Goodness-of-Fit Curve Form.



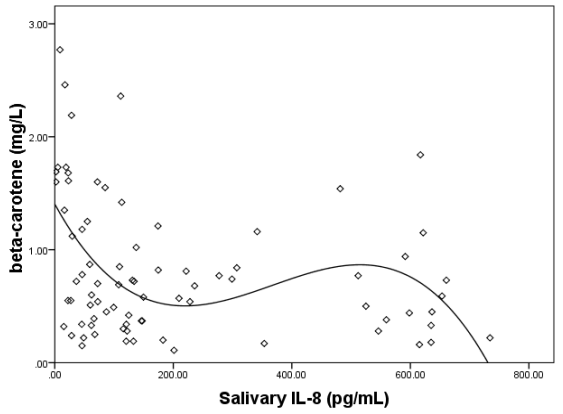

Power curve, r = 0.360



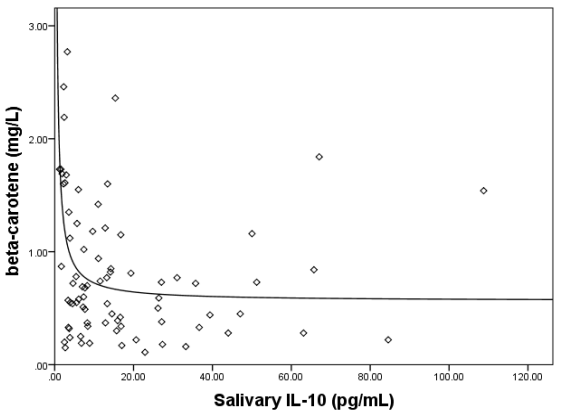

S-curve, r = 0.409



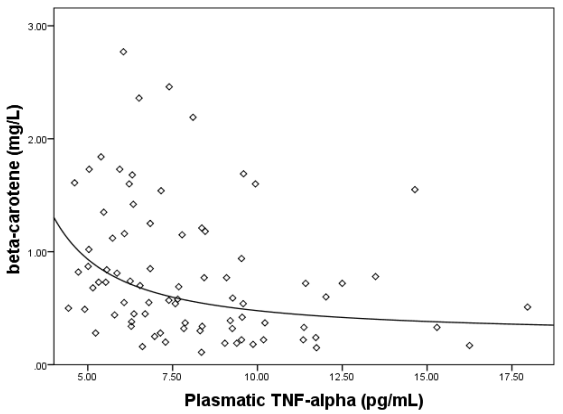

Exponential curve, r = 0.432




Cubic curve, r = 0.452




Inverse curve, r = 0.427




Sigmoid curve, r = 0.348

cell or outside the cell[48]. The antioxidant enzymes reflected in the red cell membrane have their own specific localization within the nucleated cells of the body. It is really the degree to which the biomarkers we measure firmly reflection tissues and cell levels that any interactions would be detectable.

In terms of construct validity and established biology supporting the inflammatio (independent) on oxidation (dependent) axis, beta-carotene levels would primarily be determined by intake of food-sources, and it can have a stark seasonal variation in Guatemala[49]. In terms of its circulating fluctuation, plasma vitamin A is known to be reduced in inflammatio [50], so it is plausible that beta-carotene could be displaced by cytokine activations as modeled in Table 2. SOD is important for the scavenging of superoxide radicals and converting them to peroxide or molecular oxygen; it is inducible when the intracellular superoxide density is enhanced[51]. The inflammatory response at the cellular level may provoke cellular oxidation explaining the multiple-regression results for SOD as the dependent variable (Table 3).

In terms of the oxidation (independent) on inflammation (dependent) axis, beta-carotene displayed a negative association with white cell count and all cytokines, including IL-10 (Figure 2). TNF-α is a pro-inflammator cytokine; IL-8.is a chemokine attractant that mobilizes immune cells and hence falls on the pro-inflammatory side of the continuum; and IL-10 is an anti-inflammatory cytokine[38]. Beta-carotene has an immune-modulating potential as both intact molecule[52] and through its conversion through apocarotenals[53,54]; but it also exhibits primary anti-oxidant properties[55,56], which might be relevant to the observed associations.

The decline in IL-10 associated with increasing beta-carotene might best be postulated to be an indirect, reactive decrease in concentration accompanying the primary depression of the pro-inflammatory series. Finally, no plausible and cohesive scenario for increased SOD activity eliciting a positive response from IL-8 and TNF-α, as seen for both the plasmatic and salivary compartment, readily comes to mind; therefore, the inflammation-on-oxidation axis, discussed above, is the only logical interaction format. This study has assorted strengths and certain recognized weaknesses. This represents one of the few studies in preschool children focused on oxidative and infla - matory biomarkers, many of which coming from non-invasive collection methods. Overall, the number of biomarkers, at 23, is large and diverse. This study has the perspective of two prior analyses earlier[39,40] in which a robust proportion of biomarkers showed mutual interactions when analyzed within domains. An inherent limitation is that of interpreting the meaning of statistically-significant associations in terms of causality in the absence of longitudinal or intervention studies. Indeed, some or all associations could be related to a common response to external factors that act comparably as both oxidative and inflammatory stressors[26].

## Conclusions

Both oxidative and inflammatory stress biomarkers show wide variation -but relative elevation- in low-income and deprived preschoolers of predominantly Mayan ascent sharing a highly uniform dietary offering. There are abundant candidate environmental factors to explain the oxidant and immune alterations[46]. As compared to our previous work with interactions among the biomarkers within the domains of oxidation[39] or of inflammatio [40], the finding here are nowhere as robust or harmonic when examined across the two domains. In an applied sense, the promise of findin a reliable proxy marker from one area to provide information of the other is nowhere to be realized. As a practical matter, we can eliminate 90% of the cells in the matrix

**Table 2.** Coefficients of the multiple regression model for the dependent variable β-carotene versus the independent variables of inflammatory biomarkers (WBC, plasmatic IL-10 and TNF-α, and salivary IL-8, IL-10 and TNF-α)

| No. | Model | Unstandardized Coefficients | | Standardized Coefficients | | | 95.0% Confidence Interval for B | |
|---|---|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | t | Sig. | Lower Bound | Upper Bound |
| *Dependent variable: β-carotene* | | | | | | | | |
| | (Constant) | 1.446 | 0.209 | | 6.915 | 0.000 | 1.029 | 1.862 |
| 4 | Plasmatic TNF-α | -0.060 | 0.023 | -0.276 | -2.585 | 0.012 | -0.106 | -0.014 |
| | Salivary IL-8 | -0.001 | 0.000 | -0.259 | -2.427 | 0.018 | -0.001 | 0.000 |

$r^2 = 0.136$; $r = 0.368$ (n = 79)

**Table 3.** Coefficients of the multiple regression model for the dependent variable red cell supe-roxide dismutase as versus independent variables of inflammatory biomarkers (plasma IL-1B, IL-8 and TNF-α; and salivary IL-8 and TNF-α)

| No. | Model | Unstandardized Coefficients | | Standardized Coefficients | | | 95.0% Confidence Interval for B | |
|---|---|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | T | Sig. | Lower Bound | Upper Bound |
| *Dependent variable: β-carotene* | | | | | | | | |
| | (Constant) | 0.776 | 0.221 | | 3.515 | 0.001 | 0.336 | 1.216 |
| 4 | Plasmatic TNF-α | 0.079 | 0.024 | 0.339 | 3.232 | 0.002 | 0.030 | 0.128 |
| | Salivary IL-8 | 0.001 | 0.000 | 0.220 | 2.097 | 0.039 | 0.000 | 0.001 |

$r^2 = 0.155$; $r^2 = 0.394$ (n = 80)

(Figure 2) as bases of biological cross-talk. Beta-carotene and SOD, on the oxidation side, and salivary IL-8 and IL-10 and plasmatic TNF-α, on the inflammation side, stand out for the consistency in their associations. The salivary cytokines, moreover, have the great advantage in children of their non-invasive collection procedure. Since associations do not impute causality, however, the inherent directionality of the interactions remains obscure. However, dynamic situations of experimental, environmental or clinical changes in oxidation or inflammation states in human subjects could provide the settings to infer more about any directions of causality in these interactions.

Measuring the selected biomarkers emerging here within these opportunities provides a potential next stage of inquiry.

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# SECTION C

# INTESTINAL HEALTH

María José Soto, *Listos para el evento*, Quetzaltenango 2012

# Chapter 7

## Normative Fecal Calprotectin Concentrations in Guatemalan Preschoolers are High Relative to Children Reported Elsewhere

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# NORMATIVE FECAL CALPROTECTIN CONCENTRATIONS IN GUATEMALAN PRESCHOOLERS ARE HIGH RELATIVE TO CHILDREN REPORTED ELSEWHERE

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**Author's responsibilities as follows:**

María-José Soto Méndez: Planned the fieldwork, collected samples and performed laboratory analyses, analyzed data and wrote the manuscript.
María-Eugenia Romero-Abal: Collected samples and performed laboratory analyses.
Klaus Schümann: Designed the study and wrote the manuscript.
Ángel Gil: Designed the study and wrote the manuscript.
Noel W. Solomns: Designed the study, planned the fieldwork and wrote the manuscript.

All the authors revised and approved the final manuscript. None of the authors reported any conflict of interest related to the study.

Study registered at *clinicaltrials.gov* as **NCT02203890.**

## Abstract

*Background:* Calprotectin is a fecal marker of intra-intestinal inflammation derived from activated enteric neutrophils and macrophages. It is useful as a clinical marker in inflammator bowel diseases; furthermore, it may have a role in public health epidemiology.

*Objectives:* To describe the distribution of fecal calprotectin in Guatemalan preschool children sharing a common institutional diet; to relate it collectively to pediatric distributions in other geographic settings, and individually to concomitant indicators of intestinal infection or colonization and other descriptive features of the child.

*Methods:* Fecal samples were collected in 87 subjects, aged 2-7 y across 3 daycare centers sharing a common institutional menu, but from different ecological settings. Stools were examined, variously by routine light microscopy, quantitative egg counts, and a Giardia antigen test, for microbiological diagnosis, as well as an ELISA assay for fecal calprotectin (CalproLab$^{TM}$).

*Results:* The median fecal calprotectin value was 58 mg/kg, with a mean of 98±136 mg/kg and a range from 10 to 950 mg/kg; 61% of values were above the manufacturer's cut-off for elevated concentration and 51% exceeded an

age-adjusted criterion.
There were no associations for sex, age, growth indicators, or fecal microbiological findings by microscopy or ELISA assays, alone or in combination.
The central tendency (mean or median) and distribution were generally shifted to the right in relation to comparable reports from children across the world literature.

*Conclusion:* Although specific low-grade intestinal infections do not define calprotectin subgroups, right-shifted fecal calprotectin status in this population may reflect a general and diffuse stress of adverse environmental sanitation.

Keywords: *Calprotectin - Intestinal Infla - mation – Giardia intestinalis - Preschool Children – Guatemala*

## Summary Box

*What is known?*

- Fecal calprotectin is a protein derived from neutrophils and tissue macrophages in the gut, and is known as a useful clinical marker in inflammatory bowel diseases
- Viral and bacterial diarrheal pathogens have been associated with increased calprotectin responses.

*What is new?*

- Children raised in low-income environments in Guatemala present a constellation of intestinal microbial burden, and environmental enteric dysfunction as a consequence.
- Low-income preschoolers in Guatemala manifest the highest central-tendency calprotectin values so far reported from developed or developing countries.
- High prevalence of enteric inflammatio must be considered as a factor for the poor linear growth in Guatemala.

## Introduction

Fecal calprotectin is a protein derived from neutrophils and tissue macrophages in the gut. It is a low-molecular-weight, 36 kilodalton, dimeric peptide, with two calcium-binding sites of a molecular weight related to the S-100 series; it can constitute 30 to 60% of cytosolic proteins of an intestinal neutrophil. It is recognized under several synonyms including S1008 and S1009 [1,2].
It is considered to be involved in antibacterial action and cellular chemotactic activities[3], and has an apoptotic effect on exogenous cells[4]. Calprotectin is well-recognized in the area of diagnostics, where it provides a non-invasive biomarker of the inflammatory status of the intestinal tract.

The intestinal exudate that forms in inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, gives rise to a fecal excretion of calprotectin that permits its differentiation from non-inflammatory functional bowel disorders, and allows monitoring of flares and remissions in the activity of IBD[5-8]. In addition, calprotectin increases in the feces in cystic fibrosi [9,10], cow's milk protein allergy[11] and celiac disease[12]. In neonates and throughout the first year of life, fecal calprotectin is higher than later in life[13], and is nurtured by the exclusivity of breast feeding[14,15]; nevertheless, intestinal disease can push calprotectin even higher in the infancy year[13]. In fact, normative values for fecal calprotectin follow an upward course throughout the first 4 years of life, stabilizing out beyond the 48$^{th}$ month[16].

Variable levels of fecal calprotectin have been documented in samples of free-living children in the small number of reports available in the literature[17-23], with only one from a low-income developing country, namely, Uganda[23]. In Chinese infants, fecal calprotectin levels were higher in a rural milieu, as compared to urban, and negatively associated with linear growth[24]. Finally, viral and bacterial diarrheal pathogens have been associated with increased calprotectin responses[25]. In a project ba-

sed in three government-subsidized daycare centers in the Western Highlands of Guatemala, we collected fecal samples for assessment of fecal calprotectin levels and its relation to the evidence available on intestinal protozoa and helminthes infestation. We present here the results of this nested survey.

## Subjects and Methods

### *Geographical Setting and Sites*

The study was conducted in the city of Quetzaltenango, the capital of the province of Quetzaltenango, located at about 2600 meters above sea-level. The provincial population is primarily of Mayan ascendancy, with a minority of mixed European-indigenous ancestry (mestizo or ladino), the latter concentrated in urban areas. Homes of low-income families are often of rudimentary constructions, poorly ventilated with indoor cooking stoves. Household piped water and electricity have become universal, but sewage disposal remains unsatisfactory.

The observations, monitoring and collections were conducted in three local units of the Secretariat of Social Programs of the First Lady (SOSEP for the initials of the original Spanish: Secretaria de Obras Sociales de la Esposa del Presidente).

They sponsor a nation-wide system of daycare centers (Hogares Comunitarios) providing low-income families with the opportunity for caretakers to work outside of the home while their preschool children receive care, instruction, recreation and four repasts. The feeding is based on a standard 40-day rotating menu, cycling every 8 work-weeks, consisting of a breakfast, mid-morning snack, lunch and afternoon snack.

The sites of the study were either inside -or close to- the urban limits of Quetzaltenango, all within a 20-km radius. Center A is in a semi-urban setting in a suburb, La Esperanza, of the main city.

Center B is situated in a marginal-urban area of the city itself, in the barrio of La Puerta del Llano.

Finally, Center C is located in a truly rural, agricultural zone in the hamlet of San Martín Chile Verde.

### *Enrolment of Subjects*

Children, aged 2 to 7 y, attending the three previously-described SOSEP daycare centers in metropolitan Quetzaltenango, Guatemala were sought for enrolment into the study. Most were of Mayan indigenous ascent, with a minority of ladino origin.

### *Exclusion Criteria*

Children were excluded if they did not attend one of the 3 selected daycare centers at least 80% of the center's working days during the 40-day within the study period, or whose parents refused to sign the consent form. Eligible subjects were apparently healthy and with no restrictions in consuming the foods and beverages offered within the SOSEP menu.

### *Ethical considerations*

Ethical approval was granted by the Human Subjects Committee Center for the Studies of Sensory Impairment, Aging, and Metabolism. A parent or legal guardian signed a written informed consent form after the nature, purpose, risks and benefits were explained. Authorization was also obtained from the district director of SOSEP for the Quetzaltenango area. A physician responded to the findings of the diagnostic tests (e.g. hemogram, stool and urine tests), and delivered deworming treatment along with medical prescription, e.g. for oral iron supplementation or antibiotics for urinary tract infection.

### *Anthropometric Measurements*

Children's height was measured in the 7$^{th}$ week of data-collection using a wall stadiometer to the nearest 0.5 cm; with children standing in an erect posture without shoes with their gaze in the Frankfort plane. Weight was measured without shoes, but with normal daily attire, to the nea-

rest 100 g on a calibrated Tanita® Model BC522 digital scale (Tanita, Tokyo, Japan).

*Collection of Fecal Specimens*

Two samples of feces were collected; the firs during the 7th, and the second one during the 8th week of the cycle. Feces collected during the 7th week of the cycle were used to determine Fecal Calprotectin and Giardia intestinalis. Specimens collected during the 8th week were used to perform the fecal microscopy, light microscopy (Kato-katz), and a second determination of Giardia intestinalis. Feces were immediately processed, homogenized, and stored in a -20ºC freezer when analyses could be performed after storage.

*Fecal Microbiology and Giardia intestinalis Assays*

For the fecal microscopy, a conventional microscopic preparation of feces was made in the clinical laboratory of the La Democracia Hospital in Quetzaltenango, immediately after collection. An experienced clinical microscopist examined the specimens to identify and report helminth ova, motile protozoan trophozoites and protozoan cyst forms. They were reported in qualitative terms of present or absent in the fecal smear.

The Kato-Katz quantitative fecal helminthes egg count method using sedimentation and light microscopy was performed by a member of the research team (MERA). Only a total of 80 samples had the proper consistency to perform the quantitative, Kato-Katz thick-smear technique. A standard preparation of fecal samples with malachite green was set to dry under a hood, and then covered with a cover-slip to produce a permanent prep. Light microscopy was performed on a UNICO® microscope (model G380LED, Dayton, NJ, USA), examining the entire surface of the 1.0 specimen. The total number of worm eggs counted is multiplied by a factor of 10 for equivalency to the ova per gram of stool.

*Giardia intestinalis* prevalence and intensity were derived in Guatemala using the ProSpecT-Giardia-EZ microplate ELISA assay (Oxford, Cambridge, UK) and read on a co-analyzer STAT FAX® 303-plus ELISA plate reader (Awareness Technologies, Inc., Palm City, FL, USA). The absorbance value was used as a proxy for the intensity recognizing that values higher than 300 nm optical density (O.D.) cannot be further resolved.

*Measurement and interpretation of fecal calprotectin*

Concentration of calprotectin in the fecal samples from the 7th-week collection was measured using the CalproLab™ Calprotectin ELISA Test Kit (CALP-0170, Calpro AS, Oslo, Norway) following the manufacturer's instructions for previous preparation, storage and determination by ELISA procedure. Absorbance was read at an O.D. of 450 nm on the STAT FAX® 303-plus ELISA reader.

We have used two established criteria for abnormal elevation in calprotectin concentration. The first is that of >50 mg/kg of feces, derived from the package insert and used generically in the literature. The second are age-adjusted concentration criterion derived from of the 97.5th percentile of healthy Norwegian childrten stratified by age-groups up to 4 years. The authors present their criteria as follows: "As a result, three cut-off levels were established based on the 97.5% percentiles of FC in different age groups: 538 mg/kg (1 < 6 months), 214 mg/kg (6 months < 3 years) and 75 mg/kg (3 < 4 years)." (16). For our subjects below 48 months, >75 mg/kg was applied as an alternate criterion.

*Data Handling and Statistical Analysis*

Descriptive statistics of median, mean and standard deviation, and minimum to maximum limits were calculated for the assorted variables. For calprotectin, with a non-Gaussian distribution, median is the preferred expression, but the arithmetic mean was computed for comparison with some literature reports. Other variables were characterized with central tendency

expressions appropriate to the normality of the distribution. The Mann-Whitney U-test and Kruskall-Wallis test were used for non-parametric analysis of the medians between or among groups, and Student's t test for parametric analysis of means within a normal distribution.

Associations among variables were assessed using the Spearman rank-order correlation coefficient

Z-scores for weight-for-age (WAZ), weight-for-height (WHZ) and height-for-age (HAZ) were calculated as growth indicators using the WHO Anthro software[26] for children aged up to 59 mo, and Anthro Plus software[27] for children 60 mo and older.

## Results

### *Characteristics of the Subjects*

Table 1 provides the descriptive statistics of mean, standard deviation, median and minimal and maximal limits for age, height; and the anthropometric growth indicators WAZ, WHZ, and HAZ for the 87 children, and disaggregated for the 42 female and 45 male participants. In general, the children were very short in both absolute (cm) and relative (by the HAZ growth indicator) terms. More than half of the subjects would be classified as stunted, with an HAZ below -2 SD. The rate of underweight was considerably lower, with the median WAZ of -1.4. There was virtually no wasting, with the WHZ median value close to the 0.00 Z-score level.

### *Descriptive Statistics of Fecal Calprotectin*

The final column of Table 1 provides the descriptive statistics of calprotectin concentrations for the whole sample of 87 preschoolers, as well as the subgroups of 42 females and 45 males. The aggregate calprotectin concentration had a median of 57 mg/kg, with an arithmetic mean of 98±136 mg/kg and a min-max range of 10 to 950 mg/kg. There was no significant difference in concentrations by sex between the median concentration (p=0.417). In terms of diagnostic interpretation, using the criterion of the manufacturer (CalproLab®) for active inflammation, as in an inflammatory bowel disease, >200 mg/kg of stool, only 9 children (10%) would be classified with severe inflammation. Using the manufacturer's generic criterion for "non-inflamed of <50 mg/kg", applicable to all ages, 32 subjects (37%) were so classified, leaving 55 (63%) in moderate or severe intestinal inflammation categories. The newer -and age-specific- Norwegian classificatio [16] uses a normative cut-off for children less than 48 mo of age of <75 mg/kg, leaving the <50 mg/kg for 48 mo or older. Partitioning our 36 children under 4 years old, and the remaining 51 above that age for application of these age-specific cut-offs, the recalculated aggregate rate of elevated fecal calprotectin declines to 51%.

### *Association of Fecal Calprotectin with Sex, Anthropometric Status and Site*

Table 2 compares median fecal calprotectin across anthropometric status categorical groups by the Mann-Whitney U test where applicable. The distribution for WHZ was too narrow and normal to be compared in this way. None of the probability values reached the 5% criterion for statistical significance. For the age in mo and all of the anthropometric Z-score indices, associations with individual calprotectin were calculated as the Spearman rank-order correlation coefficient. Once again, the p values did not achieve statistical significance

Descriptive statistics for fecal calprotectin across the three daycare centers are shown in Table 3. As it can be seen, there was a 9 mg/kg gradient in calprotectin concentration from semi-urban to rural setting, but without statistical significanc (p=0.499).

Table 1. Characteristics of the subjects by age, height, growth indicators, and fecal calprotectin concentration.

| Sex | | Age (mo) | Height (cm) | WHZ | WAZ | HAZ | Calprotectin (mg/kg) |
|---|---|---|---|---|---|---|---|
| **Total (n=87)** | Mean±SD | 54.5 ± 16.4 | 95.9 ± 10.0 | -0.6 ± 0.8 | -1.4 ± 0.9 | -2.3 ± 1.0 | 98 ± 136 |
| | Median | **54.5** | **95** | **-0.1** | **-1.4** | **-2.4** | **57** |
| | Limits | 22.5 – 81.1 | 74 – 115 | -2.2 – 1.3 | -3.4 – 1.6 | -4.6 – 1.4 | 10 – 950 |
| **Female (n=42)** | Mean±SD | 57.0 ± 15.3 | 95.4 ± 10.0 | 0.0 ± 0.7 | -1.6 ± 0.9 | -2.6 ± 1.0 | 81 ± 85 |
| | Median | **56.3** | **94.5** | **-0.1** | **-1.4** | **-2.5** | **56** |
| | Limits | 29.4 – 80.5 | 74.0 – 115.0 | -2.1 – 1.3 | -3.4 – 0.7 | -4.6 – -0.4 | 10 – 395 |
| **Male (n=45)** | Mean±SD | 52.2 ± 17.1 | 96.3 ± 10.1 | -0.1 ± 0.8 | -1.3 ± 0.8 | -2.1 ± 1.0 | 114 ± 169 |
| | Median | **52.1** | **96.0** | **-0.2** | **-1.3** | **-2.2** | **57** |
| | Limits | 22.5 – 81.1 | 77.0 – 112.0 | -2.2 – 1.3 | -3.0 – 1.6 | -4.5 – 1.4 | 10 – 950 |
| | p-value | 0.175* | 0.668** | 0.267** | 0.141** | 0.016** | 0.417* |

*Across sexes comparison using Mann Whitney U-test. **Across sexes comparison using student t-test. WHZ: weight-for-height Z-score; WAZ: weight-for-age Z-score; HAZ: Height-for-age Z-score.

Table 2. Fecal calprotectin concentrations compared by and across age and growth indicators.

| | Median (95% CI) Fecal Calprotectin (mg/kg) | | p value[1] | Spearman $r_s$ value | p value[2] |
|---|---|---|---|---|---|
| **Age** | **<60 mo (n=51)** | **≥60 mo (n=36)** | | | |
| | 63 (71,136) | 55 (36,144) | 0.196 | -0.136 | 0.209 |
| **WHZ** | n.a. | n.a. | | | |
| | n.a. | n.a | – | 0.132 | 0.222 |
| **WAZ** | **Not underweight (n=67)** | **Underweight (n=20)** | | | |
| | 55 (60,123) | 59 (47,193). | 0.709 | -0.006 | 0.954 |
| **HAZ** | **Not stunted (n=30)** | **Stunted (n=57)** | | | |
| | 56 (48,86) | 58 (71,157) | 0.649 | -0.108 | 0.319 |

WHZ: weight-for-height Z-score; WAZ: weight-for-age Z-score; HAZ: Height-for-age Z-score.
n.a. = not applicable because there is no variance in the classification of "wasted" by WHZ.
[1]Probability value for the Mann-Whitney U test. [2]Probability value for the correlation coefficient.

Table 3. Fecal calprotectin concentrations compared by the site and setting of collection.

| Site | Setting | Fecal Calprotectin (mg/kg) | | |
|---|---|---|---|---|
| | | Median | 95% CI | Limits |
| Center A (n=20) | Semi-urban | 64 | 64, 117 | 15 – 270 |
| Center B (n=24) | Marginal-urban | 61 | 21, 192 | 10 – 950 |
| Center C (n=43) | Rural | 55 | 58, 135 | 10 – 630 |
| | **p value*** | 0.499 | | |

*Independent samples Kruskal-Wallis test.

*Description of the microbiological and parasitological status of the population*

In terms of prevalence of helminthes, the two methods were combined; only 12 (15%) of the 80 subjects with a fecal sample suitable for analysis had any type of ova. Regarding prevalence and intensity, specifically by Kato-Katz technique, *Ascaris lumbricoides* was found in 9 samples, in three of which *Trichuris trichiura* was also found; 3 additional specimens had only hookworm species. The concentration of nematode eggs was modest, ranging from 30 to 13,320 per gram of feces.
The second approach was non-quantitative fecal smear for helminth ova and protozoal trophozoites and cysts, as performed by the microscopist of the collaborating hospital laboratory.

One or another of the reportable fecal protozoa and helminths for the laboratory *(Ascaris lumbricoides, Hymenolepis nana, Giardia intestinalis, and Entamoeba histolytica)* alone or combined with other reportable pathogenic or non-pathogenic parasites was found in 32 of 87 stool specimens submitted. The third mode of diagnosis was for fecal G. intestinalis with an ELISA method based on a giardial antigen. A total of 39 of 87 subjects tested (45%) were positive for an asymptomatic giardiasis by this method. In sum, cumulatively, across the three modalities of microbiological diagnosis, 68 subjects (78%) had one or more findings recorded in the data-base

*Interaction of fecal calprotectin with Fecal Microbes and Pathogens*

We performed several analyses between the two domains of fecal diagnosis: calprotectin concentration and microbiology.
The subgroup comparisons are presented in Table 4 based on prevalence. We also compared the median fecal calprotectin concentration in four comparative formats: any helminth eggs vs none (p=0.676); any light-microscopic findings vs none (p=0.775); Giardia positive vs Giardia negative by antigen assay (p=0.983); and any microbial prevalence vs none (p=0.770). None reached statistical significance. Finally, the median 54 mg/kg of the 40 individuals who had more than one microbiologic species diagnosed across the microscopic and antigen-based examinations with the median 60 mg/kg for the 47 remaining subjects with one or no species detected. The p value was 0.565.
We next moved from the interaction in the binary sense of positive or negative for parasitic infection (prevalence-based analysis) in relation to elevated or normal calprotectin, to explore the possibility an examination based on a graded association involving the individual burden of parasites (intensity-based analysis). For helminth burden, we took advantage of the quantitative ova counts by the Kato-Katz microscopic technique. Only the 12 subjects with helminth eggs were included in this sub analysis; a non-significant rank-order correlation (r=0.168, p=0.602) was found between egg count and calprotectin values. For the Giardia burden, we use an intensity index previously used in a thesis performed at CeSSIAM to assess protozoa burden with growth[28]. This involves using the optical density values (O.D). for absorbance in the ELISA giardial antigen test as a continuous, scaling variable. Using all available assays (n=87), a non-significant Spearman correlation (r=0.112, p=0.450) was found between the proxy for antigen burden and the amount of calprotectin in the stool.

## Discussion

Technology has emerged to allow for accessible diagnostic assessment of intestinal inflammation using a number of fecal biomarkers: lactoferrin, S100A12 and calprotectin[8,9,29,30]. The latter is the most widely available and the most thoroughly

studied. We took advantage of a field study conducted in preschool children to measure fecal calprotectin among various useful variables of growth, nutritional status and intestinal health in order to construct a descriptive and comparative profile for this age-group living in low-income circumstances in the region.

Apart from the specific illnesses associated with the activation of inflammatory cell in the intestine, graded dietary and environmental stimuli may be responsible for the higher or lower concentrations of fecal inflammatory biomarkers. In a geographic, comparative sense, we were able to identify 7 pediatric series in at-large populations or healthy-controls in clinical studies in the literature (Table 5). The age variation makes direct comparisons among sites difficult to interpret. They are not perfectly age-matched across the spectrum, a factor of consideration, given the age-sensitive calprotectin biology in early childhood[16]. The median value of 55 mg/kg for our older children is not substantially different from Norwegian children of 5 y[9]; however, our distribution is shifted to the right, with a high value of 950 mg/kg and 11 individuals exceeding the maximal concentration of 176 mg/kg seen in 5-year-olds in Norway. A comparison of the median of all 87 of our subjects with the medians for the broadest age-ranges for UK[17,22], Italy[21] and Sweden[20] finds both our median and distribution of calprotectin values displaced to the right. As compared to the only other tropical country in the series, Uganda[23], the comparison may be the most difficult of all, as it is heavily loaded with children who are both younger and older than the 2-7 age-range of the Guatemalan series. What we can see in Table 5 is a median fecal calprotectin concentration of 28 mg/kg, about half of our 55 mg/kg value when comparing their 159 subjects above the age of 4 y with our 36 children older than 5 y.

As with the reference literature (data not shown), our study failed to find differences between sexes in calprotectin status. Age is clearly a factor, with decreasing fecal calprotectin with increasing age, at least through 4

**Table 4.** Fecal calprotectin concentrations compared by different diagnostic criteria for micro-bial infestation of the intestinal tract.

| Median (95 CI) fecal calprotectin concentration in mg/kg | | p value* |
|---|---|---|
| **Positive** | **Negative** | |
| Any helminth eggs (n=12) | No helminth eggs (n= 68)[1] | |
| **51 (27, 149)** | **60 (70, 141)** | **0.676** |
| Any microscopic finding (n=32) | No microscopic finding (n=55) | |
| **59 (40, 162)** | **55 (66, 127)** | **0.775** |
| *Giardia* by ELISA (n=48) | No *Giardia* by ELISA (n=39) | |
| **55 (63, 131)** | **58 (48, 150)** | **0.983** |
| Any microbial finding[2] (n=68) | No microbial finding[2] (n=19) | |
| **59 (67, 120)** | **55 (31, 216)** | **0.770** |

*Comparison using Mann Whitney U test 1= seven stool samples lacked the texture to make a suitable Kato-Katz preparation. 2= helminth eggs and/or protozoa by light microscopy examination and/or giardia antigen positivity

Table 5. Comparison of central tendencies for fecal calprotectin in free-living populations of children.

| Study | Site | Subjects | n | Median | Mean (mg/kg) | SD | Range |
|---|---|---|---|---|---|---|---|
| **Bunn (2001)[17]** | UK | 1.5–15 y | 31 | 2.1 | – | – | – |
| **Olafsdottir (2002)[18]** | Norway | <1 y | 27 | – | 277 | 109 | – |
| | | 1–13 y | 24 | – | 40 | 28 | – |
| **Rugtveit & Fagerhol (2002)[19]** | Norway | 1 y | 20 | 67 | 177 | 243 | 15–900 |
| | | 2 y | 19 | 64 | 127 | 118 | 6–407 |
| | | 5 y | 15 | 49 | 81 | 47 | 6–176 |
| **Fagerberg et al. (2003)[20]** | Sweden | 4–6 y | 27 | 14 | – | – | – |
| | | 7–17 y | 117 | 28 | – | – | – |
| **Berni Canani et al. (2004)[21]** | Italy | 1–17 y | 67 | 28 | – | – | – |
| **Bremner et al. (2005)[22]** | UK | 5–14 y | 7 | 16 | – | – | – |
| **Hestvik et al. (2011)[23]** | Uganda | <1y | 54 | 249 | – | – | – |
| | | 1–<4 y | 89 | 75 | – | – | – |
| | | 4–<12 y | 159 | 28 | – | – | – |
| **Soto-Méndez, et al. (present study)** | Guatemala | 2–<5 y | 51 | 63 | 104 | 117 | 10–630 |
| | | 5–<7 y | 36 | 55 | 90 | 160 | 10–950 |
| | | 2–7 y | 87 | 58 | 98 | 136 | 10–950 |

or 5 y[16,18,19,23] . We found a numerical tendency with a 10 mg/kg variation across medians above and below 5 years, but without statistical significance

An experience in China found increased evidence of intestinal inflammation with decreased height-for-age Z-scores in a cohort within the first year of life[24]. The truly unique nature of these highland preschoolers is their degree of linear growth retardation or stunting, consistent with the position of Guatemala, as the nation with the highest under-five stunting rate in the Western Hemisphere (49.8%)[31].The total and sex-specific HAZ values in Table 1 reflect this reality. Overall, stunting was present in 57 of the 87 participants, for a global prevalence of 66%.

The respective HAZ values indicate stunting rates of the respective sites resolves to: Center A, 36%; Center B, 63%; and Center C, 81% (data not shown).

The same Chinese study[24] found more rural location correlated with more elevated fecal calprotectin in infants.

As described, the three sites of the study had a rural to urban gradient.

Our findings fail to confirm any such association within this geographic continuum in our preschoolers (Table 3). On the basis of the literature review in the Table 5, the fecal calprotectin concentration for our Western Guatemala subjects seems to be distributed generally to the right as compared to the experience for children in developed countries, and even compared to a series from Uganda, in East Africa.

We interpret this as stronger background inflammatory response in the intra-intestinal milieu. The explanation may reside in the contextual factors from the Western Highlands of Guatemala. A burden of intestinal parasitosis is evident, represented primarily by protozoan organisms of both commensal and pathogenic nature, especially *G. lamblia.* We grant that we were unable to find differential or gradient relationships with prevalence or intensity of in-

festation and colonization after exhaustive analysis (Table 4). The prevalence of both of the aforementioned classes, however, attests to a profound fecal -oral transmission among the children. That helminthic infections and their egg counts, such as common roundworm and hookworm, are so modest is commensurate with the altitude of over 2600 meters and intermittent de-worming efforts of which the children -or their family members -are benefici - ries. Although specific associations with neither the presence nor intensity of parasite infestations and fecal calprotectin concentrations were evident, the higher concentration of fecal calprotectin in Guatemalan supposes a greater inflammator state in the intestine. The frequent hosting of parasitic intestinal species sets the present children apart from those in the other studies listed, with the exception of Uganda. In an aggregate, -but not graded manner, there is a great deal of abnormal intestinal colonization. Moreover, although not documented in these children, environmental enteric dysfunction (EED)[32,33] is likely to be prevalent in these preschool children. As early as the 1970s, Rosenberg et al.[34] documented the conditions for EED (then known as tropical enteropathy) in Guatemalan children. Less than pristine intestinal health in a diffuse sense is undeniably the situation of the study sample.

*Strengths and Limitations of the Study*

With 87 subjects, this study ranks third in terms of total sample size among the comparative studies listed in Table 5, and has the second largest sample of children under 7 years of age after the series in Uganda[23]. This is both a strength and a weakness, as the overall narrow age range of 5 years limits its comparability with the more widely aged groups in the pediatric literature. A further strength is an epidemiological focus with a systematic collection of fecal samples to describe a preschool group in yet a second developing country. The subjects in the rural-urban Chinese report[24] were infants. As a cross-sectional, point-in-time study with a single stool sample for each individual, we are able to construct a distribution, but we have no understanding of day-to-day within-individual variation, especially in those individuals with fecal calprotectin values in excess of 200 mg/kg.

## Conclusions

In comparison with other countries, developed or developing, in which at-large populations of children have been samples for fecal calprotectin, our central-tendency values are the highest so far reported. Five of 10 or 6 of 10 subjects had an elevated calprotectin concentration in the stool, depending on the diagnostic criterion, and 1 in 10 had a level corresponding to clinically-significant, active inflammatory disease of the bowel; all of this is seen in their free-living setting between home and day-care attendance. Although we have been unable to associate the individual burden of *G. lamblia* or the fecal microorganisms from light microscopy in a gradient fashion with the individual calprotectin level, this constellation of intestinal microbial burden across the population might condition a generalized chronic inflammatory condition. Any interference with absorption or retention of essential nutrients derivative from this prevalence of enteric inflammation must be considered as a factor, potentially remedial, for the poor linear growth at the core of the Guatemalan public health agenda.

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# Chapter 8

# Interaction of *Giardia intestinalis* and Systemic Oxidation in Preschool Children in the Western Highlands of Guatemala

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# INTERACTION OF GIARDIA INTESTINALIS AND SYSTEMIC OXIDATION IN PRESCHOOL CHILDREN IN THE WESTERN HIGHLANDS OF GUATEMALA

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*The authors report no conflicts of interest.*

## Abstract

*Background:* Guatemala is a country with the highest prevalence of stunting in under-5 children in the Americas, with a national average of 49.8%. Asymptomatic intestinal colonization with Giardia intestinalis is common in Guatemalan preschoolers and has been implicated as a factor in linear growth retardation. The potential mechanisms of any giardiasis-growth interaction have not been exhaustively explored.

*Objectives:* The aim of the present study was to describe urine oxidative stress biomarkers and erythrocyte antioxidant enzyme activity, and to explore any association with prevalence or intensity of *G intestinalis* infection in preschoolers attending 3 government-subsidized day care centers in the Guatemalan Western Highlands.

*Methods:* Samples of feces, urine, and red blood cell (RBC) hemolysate were collected in a total of 74 preschoolers enrolled in 3 day care centers. Giardia prevalence and a proxy index for intensity were assessed by enzyme-linked immunosorbent assay (ELISA). Urinary biomarkers of oxidative damage to DNA (8-hydroxydeoxyguanosine [8-OHdG]) and to lipid (F2t 15-Isoprostane [F2-Iso]) were measured by ELISA. The erythrocyte activity of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GSR), and glutathione

peroxidase (GPX) were measured by respective spectroscopic substrate-based reaction assays.

*Results:* Median values of RBC CAT activity ($P = 0.016$) and urine F2-Iso ($P = 0.023$) differed between children who were positive (n = 39) and negative (n = 35) for *Giardia*. Similarly, *G intestinalis* intensity was significantl and positively associated with urinary F2-Iso (r = 0.446, $P < 0.001$), RBC SOD (r = 0.283, $P = 0.014$), and RBC CAT (r = 0.260, P = 0.025).

*Conclusion:* The optical density reading of the fecal ELISA assay for G intestinalis has potential as a proxy for the intensity of infestation. In this respect, there exists an association of this intensity with indicators of the systemic oxidation.

Keywords: *antioxidant enzymes, biomarkers of oxidative stress, Giardia intestinalis, Guatemala, preschool children*

## Summary Box

*What is known?*

- Giardia intestinalis is a protozoon that causes giardiasis because of poor hygienic environment in low income countries.
- Asymptomatic intestinal colonization with Giardia intestinalis is common in Guatemalan preschoolers.
- Little is known about the relation of oxidative stress and antioxidation activity with this infection.

*What is new?*

- Associations between red blood cell antioxidant enzymes, urine oxidative stress biomarkers, and giardiasis were found in this population.
- Oxidation was also related to the intensity of asymptomatic giardiasis.
- Oxidation may represent a potential mediating factor in growth impairment associated with the intensity of Giardia colonization in the intestine.

## Introduction

Growth retardation has been identified as the highest priority issue in the public health agenda for the Republic of Guatemala in Central America[1]. The nation ranks highest in the Western Hemisphere, with the prevalence of 49.8% in children between 6 and 50 months in the most recent national survey[2]. Moreover, the stunting rates are the highest in the rural areas, and indigenous Guatemalans are more stunted at all ages than their nonindigenous compatriots[3,4]. Environmental factors in addition to dietary deficiencies have been imputed as determinants of linear growth retardation[5,6], but the nature and mechanisms of their influence on growth are incompletely understood.

The literature is variable in terms of a negative influence of intestinal infection with *Giardia intestinalis* on growth. The earliest confirmation of an adverse effect actually came from Guatemala, in which children with the greatest time excreting Giardia cysts in early life were the shortest in stature[7,8], found relatively higher infections of *G intestinalis* in Guatemalan children with moderate malnutrition based on weight. In our review of the literature since 2004, the majority of studies confirmed an adverse association of *Giardia* infection and linear or ponderal growth[9–19], whereas a small minority did not detect any association[20,21].

With a common database for fecal assays for *G intestinalis* and battery of biomarkers of oxidation or the antioxidative system in urine, plasma, and erythrocytes in 74 preschool children in an environment, that is, Guatemalan day care centers, with proven predisposition to transmission of the protozoa[22], we tested the hypothesis of an association of asymptomatic giardiasis and oxidative stress. We present here the findings related to this exploration of associations.

## Methods

*Geographical and Ecological Setting*

The Western Highlands in Quetzaltenango, located 220 km from Guatemala City at 2657m above sea level, were the setting for this research project. It is a generally agricultural region with a temperate climate. Principle crops include maize, coffee, and fruit. The economy is based on agriculture, livestock, commerce, finances and tourism.

Three day care centers in different locations were assigned by the Secretaría de Obras Sociales de la Esposa del Presidente (SOSEP, Secretariat of Beneficial Works of the First Lady) for availability to our study. The semiurban center (center A) was located in La Esperanza, 2 miles away from downtown Quetzaltenango, with all of the basic services and 80% of indigenous attendees. The marginal urban setting (center B) was located in the outskirts of the city of Quetzaltenango in La Puerta del Llano, with great majority of indigenous attendees, most of whom were children of families that moved from the rural areas to run small business in markets; they were living in overcrowded conditions, some of them without basic sanitary services. The rural setting (center C) was situated 15miles away from Quetzaltenango proper in La Estancia, San Martín Chile Verde; 100% of the children attending this center were indigenous preserving their original customs. Although the services at the distinct sites were common and equivalent; the varying ethnic mixture and environmental circumstances produced variation in some of the living habits, pastimes, and physical characteristics among attendees of the different centers.

*Enrollment of Subjects*

Eligible children had to be attending 1 of the selected centers and be 2 to 7 years old; they had to have ≥80% attendance during the 8 observation-collection weeks. Subjects had to be apparently healthy and with no restriction related to the menu offered by the SOSEP system. Children were excluded from particular analyses when they did not adhere to the urine, fecal, or blood collections. Full exclusion resulted when they declined to participate in the project or the caregivers did not sign consent form.

Ethical approval was granted for the study protocol by the human subjects committee of the Center for the Studies of Sensory Impairment, Aging, and Metabolism (CeSSIAM). The SOSEP's director for the Quetzaltenango area gave authorization to realize the study. To provide all of the food items on the menu, the diet offered to the children was complemented when required by funding from the investigators.

*Collection of fecal, urinary and red cell samples*

Two samples of feces were collected: the first during the seventh and the second during the eighth week of the cycle. Feces were immediately processed and homogenized, and an aliquot of ~2 g was stored in a -20ºC freezer to be analyzed for G intestinalis by an enzyme-linked immunosorbent assay (ELISA) method. We attempted to collect a 24-hour urine sample during the same eighth and final observation-collection week.

Urine collection was started when a child arrived at the day care center (from 7:00 AM to 8:00 AM); SOSEP personnel and investigators started the collection at each day care center using Vacutainer® plastic 24-hour collection container (no. 36499, Becton-Dickinson, Franklin Lakes, NJ, USA). Parents continued the collection at home. The sample collection was finishe after 24 hours at the center. The collection process was repeated if incomplete.

Red blood samples were collected after an extraction of whole blood by an experienced phlebotomist using BD Safety Lok® diposable needles (no. 367281, Becton-Dic-

kinson, Franklin Lakes, NJ, USA) and Vacutainer® 4-mL tubes with ethylenediaminetetraacetic acid (EDTA) (no. 367861, Becton-Dickinson, Franklin Lakes, NJ, USA). Samples were centrifuged to separate red blood cells (RBCs) from plasma; both were stored in criogenic vials (no. 5000-0012, Nalgene Nunc International Corp., Rochester, NY, USA) at -80ºC until shipment to the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, Granada, Spain, to determine antioxidant enzyme activity in RBCs.

*Measurement of the fecal Giardia intestinalis concentration*

*Gi* prevalence and intensity were derived in Guatemala using the ProSpecT® Giardia EZ Microplate Assay (no. 245896 Remel Inc., Lenexa, KS, USA). The absorbance value was used as a proxy for intensity recognizing that values > 300 O.D. cannot be further resolved[19].

*Measurement of urinary biomarkers of oxidation*

The frozen aliquots of urine in cryogenic tubes were shipped on dry ice to Granada. Measure of oxidative damage to DNA was based on the 8-hydroxydeoxyguanosine [8-OHdG] assay (JaICA, Nikken SEIL Co., Ltd, Catalog# IM-KOGHS 040914E, Shizouka, Japan) and that to lipid by 15-Isoprostane $F^{2t}$ F2-Iso) (no. EA84102606, Oxford Biomedical Research Inc, Rochester Hills, MI, USA).

*Measurement of erythrocyte biomarkers of oxidation*

The red cell hemolysates were shipped in the cryogenic tubes on dry ice to Granada. Hemoglobin concentration was determined by use of Drabkin´s reagen (no. D5941, Sigma, St. Louis, MO, USA). The final concentration of Hb was adjusted to 5 mg/mL for antioxidant enzymes analyses. Catalase (CAT) activity was determined using de Aebi method[23]. Superoxide dismutase (SOD) was analyzed by spectrophotometry[24]. Glutathione reductase (GSR) was determined using the method of Carlberg and Mannervik[25]. Glutathione peroxidase (GPX) was analyzed using the procedure developed by Flohé and Günzler[26].

*Data handling and statistical analysis*

SPSS Version 20 (IBM, Chicago, IL) was used to create a database. Descriptive statistics were expressed as median, 95% confidence interval, and minimum and maximum. Association of values was tested using the Spearman rank-order coefficient. Comparisons between positive and negative for *Giardia intestinalis* infection subjects were done using Student *t*-test or Mann-Whitney *U* test as appropriate. A probability level of ≤0.05 was accepted as statistically significant and of ≤0.10 as indicative of a trend.

## Results

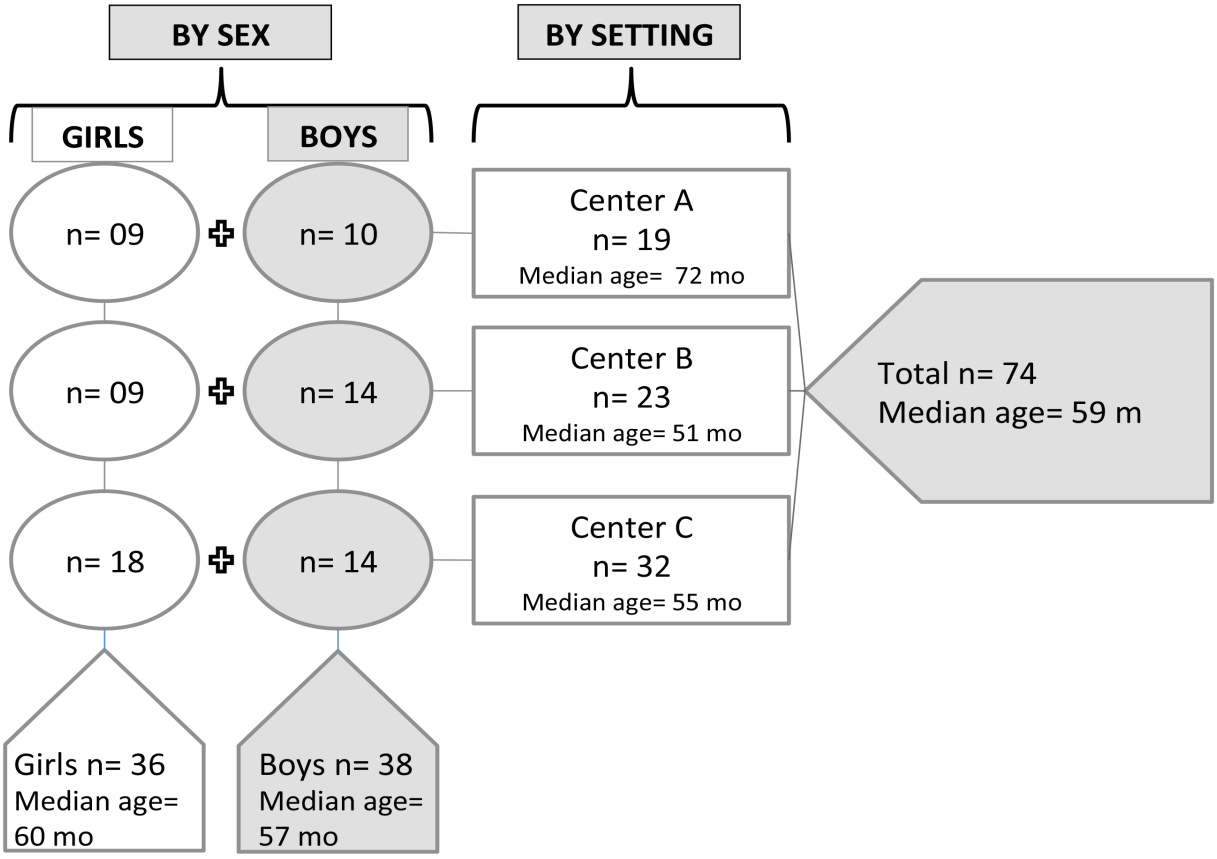
*Characteristics of the Sample*

Of the 87 children enrolled in the study, 74 (85%) were sampled for both fecal antigens and the array of urinary and red cell oxidation biomarkers; 36 were girls, and 38 were boys.
The ages ranged from 23 to 81 months, with a mean of 5815 months and a median of 59 months. Figure 1 illustrates the partition of the children by sex and age in relation to the specific day care centers and provides data on the median ages by sex.

*Pattern of intestinal colonization with Giardia intestinalis*

Based on the cutoff criterion of positivity and negativity for *giardia* antigen in the

**Figure 1.** Characteristics of the sample.




ELISA assay, 35 subjects were negative (47%) and 39 were positive (53%). With respect to the distinct daycare center sites, the *Giardia* prevalences were: A, 16%, B 78%, and C, 56%, respectively. There was a significant difference in prevalence by site ($P < 0.001$ by 3 x 2 $\chi^2$).

*Distribution of biomarkers of oxidative stress*

The median, 95th confid nce intervals and the minimum and maximum values are provided in Table 1. There are no cut-off criteria established for normative excretion amounts or red cell enzymes for children in this age group. Interestingly, 8-OHdG and F2-Iso median values were different between sexes ($P = 0.03$ and $P = 0.04$, respectively), even when calculating the total amount excreted per day and adjusting to weight. The median daily excretion values for the former biomarker were 3.2 µg/d in boys and 2.1 µg/d in girls ($P = 0.008$), whereas for the latter were 1.0 µg/d and 0.6 µg/d ($P < 0.001$), respectively.

*Interaction of Giardia infection and the oxidation-antioxidation system*

As shown in Table 2, the *Gi*-positive children has significantly higher urinary concentrations of F2-Iso ($P < 0.001$) and rbc GSR ( P =0.019). Correspondingly, there was a trend, with p values of <0.10, for the comparison of infected and uninfected subjects for higher rbc CAT (p=0.062) and rbc SOD ($P = 0.067$). Only urinary 8-OHdG and rbc GPX failed to show any association with giardiasis status. With respect to intensity, using the O.D. absorbance values of the ELISA test as a proxy for intensity of infection, Table 3 provides the Spearman correlation coefficients for the crude ab-

Table 1: Descriptive statistics for the urinary and enzymatic biomarkers of oxidation.

| Biomarker | Median | 95% CI | Min – Max |
|---|---|---|---|
| 8-OHdG (ng/mL) | 5.69 | 6.18, 9.80 | 0.55 – 38.61 |
| F2-Iso (ng/mL) | 1.72 | 1.86, 2.78 | 0.50 – 11.80 |
| CAT (nmol/seg/g Hb) | 6.73 | 6.47, 7.10 | 4.31 – 9.91 |
| SOD (U/g Hb) | 1.53 | 1.36, 1.67 | 0.24 – 2.78 |
| GSR (umol/min/g Hb) | 2.86 | 2.67, 2.95 | 1.79 – 4.06 |
| GPX (μU/g Hb) | 6.06 | 5.51, 6.40 | 0.00 – 11.99 |

sorbance data with the values for the respective urinary and enzymatic biomarkers. Significant positive associations were found between higher O.D. and urinary F2-Iso (<0.001), RBC CAT (P = 0.025), and RBC SOD (P = 0.014).

## Discussion

The United Nations, through its operative agencies, is committed to addressing the challenge of "Zero Hunger"; highest among the operative priorities is the reduction in linear growth retardation (stunting)[1].

Guatemala ranks highest in prevalence of stunting in the Americas[2].

As cited, a subset of studies in Guatemala[7,8] and elsewhere[9-19] provide evidence for an association between *Giardia* infections and inferior growth. As in our previous evaluations of the prevalence of asymptomatic intestinal colonization with this protozoa in Guatemalan schoolchildren[22,27], we found an abundant prevalence of antigen-positive samples among the stools collected in the three daycare centers in the SOSEP system in the Quetzaltenango province. Moreover, the three study sites differed in their average height-for-age Z-score (HAZ) status, with stunting rates varying from 35% in center A to 63% in center B to 81% in center C (unpublished findings)

Our results are consistent with findings of Basu, et al.[28] who also documented a higher excretion of both biomarkers in adult male subjects. We speculate that behavioral traits of boys at this age may produce greater exposure to oxidative stress or that the susceptibility to oxidation is influence by sex. In a previous study at CeSSIAM, the use of the O.D. in the ELISA readings of the *Giardia* fecal antibody assay showed an inverse correlation with height. Since the reaction intensity for absorbance truncates at an O.D. of 3.0, we postulated with expansion of the range, perhaps through dilution of high-response samples, an even better rank-ordering could be achieved[19]. Nevertheless, using the entire range that the ProSpecT-Giardia-EZ microplate permits, another detectable biological association, namely absorbance versus oxidation biomarkers, shows the potential validity of the former as an indicator of the intensity of *Giardia* infestation. We find heretofore unrecognized associations between rbc antioxidant enzymes and urine oxidative stress biomarkers and the prevalence and intensity of asymptomatic giardiasis in our study population. With respect to oxidative exposures and urinary biomarkers, a greater excretion would logically signify a greater systemic oxidation. For enzyme activity, the interpretation is not so straight-

Table 2: Comparison for oxidation biomarkers between subjects with positive and negative diagnostic for Giardia intestinalis (Gi) (n=74).

| Oxidation Biomarker | Statistics | Negative for *Gi* | Positive for *Gi* | p-value |
|---|---|---|---|---|
| 8-OHdG (ng/mL)Ŧ | Median | 5.3 | 6.1 | 0.526 |
| | 95% CI | 5.0 / 10.6 | 5.7 / 10.6 | |
| | Min - Max | 0.6 - 38.6 | 0.6 – 32.4 | |
| F2-Iso (ng/mL)Ŧ | Median | 1.0 | 2.4 | <0.001 |
| | 95% CI | 1.2 / 2.0 | 2.2 / 3.7 | |
| | Min - Max | 0.5 - 5.4 | 0.8 – 11.8 | |
| CAT (nmol/seg/g Hb)* | Median | 6.4 | 7.0 | 0.062 |
| | 95% CI | 6.0 / 6.9 | 6.6 / 7.5 | |
| | Min - Max | 4.3 - 9.9 | 4.8 - 9.9 | |
| SOD (U/g Hb)Ŧ | Median | 1.2 | 1.6 | 0.067 |
| | 95% CI | 1.1 / 1.6 | 1.5 / 1.8 | |
| | Min - Max | 0.2 - 2.8 | 0.4 - 2.6 | |
| GSR (umol/min/g Hb)Ŧ | Median | 2.4 | 3.0 | 0.019 |
| | 95% CI | 2.4 / 2.8 | 2.8 / 3.2 | |
| | Min - Max | 1.9 - 3.7 | 1.8 - 4.1 | |
| GPX (µU/g Hb)Ŧ | Median | 6.1 | 5.8 | 0.766 |
| | 95% CI | 5.3 / 6.5 | 5.3 / 6.7 | |
| | Min - Max | -0.6 - 8.5 | 2.0 – 12.0 | |

*Comparison using student t-test.
ŦComparisons using Mann-Whitney U-test.

forward. On the one hand, lower activities of enzymes would argue for a depletion of antioxidant capacity, that is, making the subject more vulnerable to oxidative damage, while, on the other hand, higher enzymatic activity might signify a compensatory reaction, that is, adding protection in the face of persistent oxidative stress. The observed interplay between *Giardia* intensity and urinary oxidative biomarkers could follow 1 or several causal scenarios. For example, the presence of varying numbers of protozoal pathogens in the intestinal lumen could result in the transmission of stimuli that would provoke oxidation locally within intestinal enterocytes or, with absorption, in somatic cells within the body.

Alternatively, independent exposures producing states of low-level enhancement of cellular oxidation could alter immune defenses to a degree that permit the colonization by *Giardia*. The rank-order association of the phenomena would logically make the intensity of infestation driving a reactive oxidation into the more plausible scenario. It is axiomatic, however, that an association in no way proves causality. Indeed, a third (unmeasured) factor operating in the environment in a graded manner could simultaneously set the stage for both susceptibility to colonization by *Giardia* and expression of cellular oxidative stress. Insofar as no a priori hypothesis re-

Table 3. Spearman correlations for Giardia intestinalis absorbance and oxidation biomarkers measured in urine and erythrocytes (n=74).

| Biomarker vs *Gi* absorbance | r-value | p-value |
|---|---|---|
| 8-OHdG (ng/mL) | 0.076 | 0.522 |
| F2-ISO (ng/mL) | 0.446 | <0.001* |
| CAT (nmol/seg/g Hb) | 0.260 | 0.025* |
| SOD (U/g Hb) | 0.283 | 0.014* |
| GSR (umol/min/g Hb) | 0.166 | 0.159 |
| GPX (µU/g Hb) | -0.103 | 0.384 |

*Significant correlation

garding the protozoa-oxidation interaction was part of the study design, our finding could be a unique and nonreproducible statistical association in this instance; only prospective investigation for confirmation in this and other settings of *Giardia* endemicity would establish the relation as a predictable phenomenon.

We acknowledge a series of strengths and weaknesses in the study. The major strength of the study was the convergence of data collected in a common group of preschool children that allowed a post hoc examination for an association having both a sample size of sufficient power and the possibility for scaling intensity. Moreover we had 2 fecal samples for diagnosis of each child's giardiasis status; it has been suggested that having ≥ 2 separate samples improves sensitivity[22,29,30].

Insofar as the range of scaling is limited by the nature of the ELISA method, however, as discussed above, we may have been limited in ascertaining the true degree of association.

The failure to have had the foresight to have diluted the fecal samples with the highest absorbance signals to extend the range is a limitation.

In conclusion, interesting and potentially important associations were found between the prevalence of asymptomatic giardiasis and with a proxy for intensity of infection and certain elements in an array of biomarkers related to systemic oxidation in Guatemalan preschool children across 3 settings. Although clear directional causality cannot be established, oxidation may represent a potential mediating factor in growth impairment associated with Giardia colonization of the intestine.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# SECTION D

# THE NATURE OF VARIANCE FOR BIOMARKERS IN PRESCHOOL CHILDREN


Sheny Romero, *El saludo de la mañana*, Quetzaltenango 2012

# Chapter 9

# Variation of Selected Diagnostic Measurements and Biomarkers in Preschool Children: Implications for Design of Field Surveys, Comparative Studies and Intervention Trials

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# IN PREPARATION

## VARIATION OF SELECTED DIANOSTIC MESUAREMENTS AND BIOMARKERS IN PRESCHOOL CHILDREN: IMPLICATIONS FOR DESIGN OF FIELD SURVEYS, COMPARATIVE STUDIES AND INTERVENTION TRIALS

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**Abstract**

*Background:* Sample-size considerations and estimations are critical to assess a field study's power to obtain a stable estimate of prevalence, or to avoid a Type II error of accepting a null hypothesis, when there in fact is difference. As novel biomarkers are introduced into population research, information on variance in the variables and its impact on sample-size adequacy is often missing. *Objective:* To illustrate heterogeneity of variance among the variables in a multidimensional child health study in the Guatemala highlands.

*Methods:* Fifty-three variables to characterize growth, hematology, hydration, oxidation, inflammation, and infection were included in the design, collected from 66-87 Guatemalan preschoolers. Descriptive statistics were enumerated; the conventional formula to establish sample-size required for comparison was applied, using a 20% effect-size assumption.

The conventional formula for sample-size and prevalence assessment was used in the 33 variable with cut-off criteria for abnormality.

*Results:* Among the 53 variables, the relative standard deviation (coefficien of variance) ranged from 0.02-12.42%. Among the 33 categorical variables,the sample size needed to estimate a stable prevalence for a confidence level of 95% ranged from 91-2078. Sample-size for comparison with a power (1-ß) of 0.8 ranged from below 10 per group to up to 93,000. *Conclusion:* Sample-size considerations must be applied to novel variables in all field research initiatives prospectively. Failure to do so risks erroneously false outcome conclusions, publication bias and suppression of follow-up research.

Keywords: *sample-size; variance; prevalence; statistical power; filed research; Guatemala*

**Introduction**

With the advance of diagnostic technology to assess nutrient status field- and population-research in human nutrition has become increasingly sophisticated and thoughtful. Moreover, the number of hypotheses on interactions between nutritional status and non-dietary environmental factors have proliferated[1-6]. Future field research will increasingly combine a mixture of measurements and biomarkers from both, nutrient-status and environmental-exposure domains.

We were cognizant of such a projection in the design of the project "Study on the normative state and inter-and intra-individual variation in growth, hematology, hydration, and markers of oxidation, infection and inflammation in pre-school children with a similar dietary intake"[7].
For convenience in this paper, we have combined inflammation and infection into a common domain. We previously published descriptive and analytic findings employing many of the 53 variables chosen for further, in depth analysis in this article[7-13]. With the exception of anthropometric and hematological areas, few of the variables had previously been measured and reported in preschool children from the Western Highlands of Guatemala. Thus, we entered the project without an a priori understanding of the inter-individual variance for most of the collected variables. The size of a sample has implications for both the description of a stable prevalence of abnormal status[14], and for the analysis of associations to test of biological hypotheses[15]. Therefore, when designing prospective field studies with adequate statistical power to describe prevalence or to test hypotheses, inherent issues in the inter-and intra-individual variation of the variables of interest come into consideration[16; 17].
For measurements of previously un known variances, the experiences of our field study can provide the basis to create a framework in order to assure adequate statistical power in the design of future projects involving the same variables.
We recompiled the distribution of the field-study variables presented previously[7-13] with a particular focus on refining critical sample-sizes that provide sufficient statistical power to meet criteria for stable assessment of prevalence, and to avoid type II errors over inter-group comparisons.
We present here the findings of these analyses.

## Methods

The **Appendix** presents 53 variables in spreadsheet fashion, organized in fiv domains of interest.
The columns provide the name of the variable with measurement units, number of valid values; median value, 25$^{th}$ and 75$^{th}$ percentiles, minimum and maximum values of the distribution, highest-to-lowest range, the arithmetic mean and standard deviation (SD), skewness, kurtosis and the variance (defined as the SD$^2$).
Although we presented the SD of the arithmetic mean for all variables, we indicate by an asterisk, which variables are distributed with sufficient normality to be appropriately represented by the arithmetic mean. These variables are suitable for parametric statistical tests.
Detailes on analytic methods for most variables have been summarized in the previous publications[7-13]. The methods used are outline briefly in the subsequent sections.

### *Growth and anthropometry*

Height and weight are two primary anthropometric variables[11]. There are fiv additional derived indices in this field calculated from these primary values, some of which taking age in months into consideration. These indices include the Z scores of weight-for-age (WAZ), height-for-age (HAZ), weight-for-height (WHZ), body mass index for age (BMIZ-age)[18-19] as well as simple body mass index (BMI).

### *Hematology*

Eleven variables were in the hematological domain were, not heretofore published except for white blood cell count

(wbc). Although grouped with hematology here, these values were published earlier in our paper on inflammatio [8].
The hematological variables are listed in the second tier of Appendix. All were measured using routine automated cell counter technology in a hospital clinical laboratory in Guatemala.

*Hydration*

The 11 hydration values have been presented in previous publication[7,9,13]. Twenty-four hour urine volume was a directly measured. Creatinine in plasma was determined in Spain by the Scientifi Instrumentation Center (CIC) bio-analysis unit using the automatic biochemical analyzer BS200 (Mindray, Shenzhen, China); and urinary creatinine was measured in Guatemala by the traditional Jaffe method.
Osmolality was analyzed by freezing-point depression osmometry. Urinary urea and uric acid were also assayed by biochemical analyzer, and the four cationic electrolytes were measured by inductively-coupled plasma emission.
Inorganic phosphorus was determined by use of a commercial kit.

*Oxidation and anti-oxidant defense*

Eleven variables were included in this domain[12]. The two variables of oxidative, free-radical exposure, 8-hydroxy-deoxyguanisine (8OHdG) and 15-isoprostanes-F2t (F2-Iso) were measured by commercial kits. Anti-oxidant enzyme activity (catalase (CAT); superoxide dismutase (SOD); glutathione reductase (GSR); and glutathione peroxidase (GPX) was measured by reaction spectrophotometry. Concentrations of vitamin and vitamin-like substances (retinol; α-tocopherol, β-carotene, and co-enzymes Q9 and Q10) in the plasma were measured by high-precision liquid chromatography.

*Infection and inflammation*

Among the diagnostic indicators for inflammation were: urinary white blood cells per field, not heretofore published; giardia intensity determined by an ELISA kit (please add detais), using the optical density reading of absorbance as a continuous scale[10]. The inflammator biomarkers were determined ino plasma; saliva; and faeces[8]. The five cytokines (interleukins 1β, 6, 8 and 10 and tumor necrosis factor-alpha (TNF-α) were measured using Luminex® chemoluminescence assays for both plasma and saliva samples. A single marker of intra-intestinal inflammation was the concentration of the calprotectin, derived from activated gut leukocytes.

*Data Handling, and Analysis*

All collected data was entered into an SPSS version 20 database to be analyzed. With relation to the **Appendix**; although many variables in Appendix 1 are no normally distributed in a Gaussian sense, the SD is essential to estimate sample-sizes for comparisons. We indicate with an asterisk, however, which variables are non-normally distributed, for which the median or a transformed mean would be the appropriate expression of the central tendency and non-parametric statistical tests would be applicable for hypothesis testing.

*Estimation of variance*

The first illustration was of the variance within the distributions. Despite the caveat expressed above regarding the application of the arithmetic mean and its SD for non-normal distributions, for comparative purposes the relative standard deviation (coefficient of variable) was generated for each variable. This is the SD/arithmetic mean x 100, and expressed as a percentage.

*Stability of estimates on population prevalence*

The second exercise involved determining the sample size that would provide a stable prevalence estimate with respect to confidence level and determined confide - ce intervals.
We used the formula shown below where we solve for a sample size (ss) using a determined confidence level (Z) and selected confidence intervals (c) in the formula the observed percentage or prevalence of abnormality is expressed a decimal (p).

$$ss = Z\,2 * (p) * (1-p) / c\,2$$

In this exercise, our confidence level Z was consistently 95%, and our c were alternatively 0.05 and 0.01, corresponding to confidence intervals of 95% and 99%, respectively.
In terms of selecting the direction of abnormality, with height-for-age, for example, the prevalence of stunting (low height) was of interest. Regarding BMI for age Z scores, the cut-off for overweight (elevated weight) was chosen.
Similarly, for Hb or red cell count, the downside direction was of interest, whereas for white cell count and red cell distribution width the upside direction was defined a abnormal. We have been able to assign cut-off point for abnormality in only 33 of the 53 total variables (= 62%). We generated the data using a free-access web page sample size calculator based on the formula given above[20].

*Estimation of sample-size for inter-group comparisons*

In this case is the sample size required in each side of the comparison to resolve the null hypothesis of a difference between the means of two distributions. Za is the two-sided constant value for the alpha error. Z (1–β) corresponds to the desired power, e.g., 0.8 or 0.9. The σ represents the observed or estimated SD, squared. The effect size in the denominator (Δ) is a decimal of the resolvable difference of interest between the two distributions.

$$n = 2(Za+Z1-\beta)\sigma^2/\Delta^2$$

In this exercise our alpha error is 0.05, and our 1–β has been entered both at the 0.8 and 0.9 levels, and we used the SD of our own observations.
On this basis we calculated the sample size to resolve an intergroup effect-size of 0.2. We generated the data for all the 53 study variables using a free-access web page sample size calculator based on the given formula[21].
We acknowledge the caveat that not all of our distributions are Gaussian normal but the formula required the standard deviation of the arithmetic mean; in the Appendix we have indicated the variables for which the data are not normally distributed.

## Results

*Intrinsic Variance among the Measures and Biomarkers*

The width across the distribution or between the 25$^{th}$ and 75$^{th}$ centiles is considered indicators of variance, but in a relative sense this is colored by the choice of units. If one reports height in centimeters, there is a 100-fold higher absolute maximum to minimum range than if they are expressed in meters. A similar issue is seen in the expression of Hb concentration in g/dL vs g/L.The relative standard deviation, i.e., the standard deviation divided by the arithmetic mean, commonly called the coefficient of variation (CV) provides a better perspective. There is a caveat, of course, that not all distributions have the norma-

**Table 1.** The relative standard deviation (Coefficient of Variation) and the sample size per group needed for detecting a 20% change in the observed average value based on the observed variance in the present study population assuming a power (1- β) of 0.8 and 0.9 for all 53 variables.

| ID | Variable | Estimated sample size | | Relative Standard deviation |
|---|---|---|---|---|
| | | 0.8 | 0.9 | |
| R | Platelet distribution width -PDW- (%) | 1 | 1 | 0.02 |
| O | Mean corpuscular hemoglobin concentration -MCHC- (g/dL) | 1 | 1 | 0.03 |
| M | Mean corpuscular volume -MCV- (fL) | 1 | 1 | 0.04 |
| N | Mean corpuscular hemoglobin -MCH- (pg) | 2 | 3 | 0.06 |
| F | Body Mass Index -BMI- (kg/m2) | 2 | 3 | 0.07 |
| P | Red cells distribution width -RDW- (%) | 3 | 3 | 0.07 |
| H | Hematocrit -Ht- (%) | 3 | 4 | 0.08 |
| J | Hemoglobin, whole blood -Hb- (mg/dL) | 4 | 5 | 0.09 |
| B | Height (cm) | 5 | 6 | 0.10 |
| MM | Coenzyme Q9 -CO-Q9- (mg/L) | 9 | 17 | 0.09 |
| L | Red blood cell count -RBC- (106/mm3) | 15 | 20 | 0.20 |
| NN | Coenzyme Q10 -CO-Q10- (mg/L) | 16 | 22 | 0.17 |
| FF | Catalase activity -CAT- (nmol/seg/ g Hb) | 16 | 21 | 0.20 |
| HH | Gluthathione Reductase activity -GSR- (umol/min/g Hb) | 18 | 24 | 0.21 |
| A | Weight (kg) | 19 | 25 | 0.21 |
| U | Urinary Uric Acid (mg/dL) | 23 | 31 | 0.24 |
| Q | Platelet count (103/mm3) | 23 | 31 | 0.24 |
| KK | α-tocopherol (mg/L) | 24 | 32 | 0.25 |
| X | Plasma creatinine (mg/dL) | 28 | 38 | 0.27 |
| K | White blood cell count -WBC- (103/mm3) | 36 | 48 | 0.30 |
| JJ | Retinol (mg/L) | 38 | 50 | 0.31 |
| II | Gluthathione Peroxidase activity -GPx- (μU/g Hb) | 40 | 54 | 0.32 |
| UU | Plasma TNF-alpha (pg/mL) | 46 | 61 | 0.34 |
| Z | Potassium (mg/dL) | 58 | 78 | 0.38 |
| T | Urinary Osmolality (mOsm/kg) 8th cycle week (Osmomat) | 67 | 89 | 0.41 |
| GG | Superoxide Dismutase activity -SOD- (U/g Hb) | 67 | 90 | 0.41 |
| Y | Sodium (mg/dL) | 76 | 102 | 0.44 |
| D | Height for age -HAZ- (z-score) | 77 | 103 | 0.45 |
| W | Urinary creatinine (mg/dL) 8th cycle week | 99 | 133 | 0.50 |
| SS | Plasma IL-8 (pg/mL) | 101 | 136 | 0.51 |
| I | Hemoglobin, non-invasive -Hb- (mg/dL) | 110 | 147 | 0.53 |
| BB | Magnesium (mg/dL) | 142 | 190 | 0.60 |
| S | Urine volume (mL/24hour) 8th cycle week | 145 | 194 | 0.61 |
| C | Weight for age -WAZ- (z-score) | 145 | 194 | 0.63 |
| CC | Inorganic Phosphorus (mg/dL) | 173 | 231 | 0.66 |
| QQ | Plasma IL-1B (pg/mL) | 201 | 269 | 0.71 |
| AA | Calcium (mg/dL) | 207 | 277 | 0.73 |
| LL | β-carotene (mg/L) | 221 | 296 | 0.75 |
| EE | F2t-Isoprostanes -F2-Iso- (ng/mL) | 262 | 351 | 0.83 |
| V | Urinary Urea (mg/dL) | 291 | 389 | 0.86 |
| DD | 8-Hydroxy-deoxy-guanosine -8OHdG- (ng/mL) | 375 | 501 | 0.98 |
| RR | Plasma IL-6 (pg/mL) | 425 | 569 | 1.04 |
| XX | Salivary IL-8 (pg/mL) | 444 | 594 | 1.06 |
| TT | Plasma IL-10 (pg/mL) | 472 | 631 | 1.10 |
| ZZ | Salivary TNF-alpha (pg/mL) | 493 | 660 | 1.12 |
| YY | Salivary IL-10 (pg/mL) | 526 | 704 | 1.16 |
| PP | Giaridia intestinalis intensity (OD) | 627 | 839 | 1.29 |
| OO | Urinary white blood cells per field | 629 | 841 | 1.27 |
| AAA | Fecal calprotectin (mg/Kg) | 751 | 1006 | 1.38 |
| G | BMI for age -BAZ- (z-score) | 5087 | 6809 | 3.54 |
| WW | Salivary IL-6 (pg/mL) | 5661 | 7578 | 3.80 |
| VV | Salivary IL-1B (pg/mL) | 6742 | 9026 | 4.13 |
| E | Weight for height -WHZ- (z-score) | 93073 | 124598 | 12.42 |

lity that would validate the application of parametric statistics. Nevertheless, it can represent a common calculation across all data presented in the right-hand column of Table 1. It ranged from 0.02% for the platelet distribution width to 12.4% for weight for height Z score. Thirty-six of the CVs were below 1.0 (= 68%), whereas 32% were equal to or greater than 1.0. The highest CV was found for WHZ (12.4%); this was accompanied by three more CVs in excess of 2%: salivary IL-1β (4.13%); salivary IL-6 (3.80%); and BAZ (3.54%).

*Pattern of Sample Size Requirements for Stable Estimate of Measures and Biomarkers treated as Categorical Variables*

We could establish a reported or conventional diagnostic cut-off criterion for an abnormal value for a total of 33 of the 53 variables (62%).

For some variables in which abnormality is bidirectional, such as anthropometric and hematological variables, the direction most relevant to Guatemalan health was chosen. Quantitatively detailed in Table 2 are the respective cut-off criteria with the directionality of abnormality, elevated or depressed, and the distribution across the variables across the spectrum is illustrated in Figure 1. In both illustrations the data are displayed in ascending order of estimated sample-sizes. The letter-codes relate to those ordered by biological domain in the master variable list in the Appendix. Table 2 shows the sample sizes for both a 95 and 99 confidence level. In general, the number for the higher standard is 174% that of the lower one. The sample-size number ranges from a low of 91 subjects survey for elevated calprotectin to a high of 2078, for low platelet count across the graphed distribution.

The median sample-size value across our effective distribution was 183, between high salivary IL-8 (n = 172) and low blood Hb (n = 194). Less than 100 subjects would be required for the 95 CL for four variables, whereas more than 1000 would be required for the final four quantifiable prevalence variables. Five variables had saturated at either the no prevalence of abnormal (plasma IL-8 and co-enzyme Q10) or 100% as abnormal (plasma IL-10 and TNF-α, and co-enzyme Q9), such that no critical sample-size could be computed.

*Pattern of Sample Size Requirements with standard probability (alpha) and power (1-β) assumptions for Comparisons of the Measures and Biomarkers treated as Continuous Variable*

Table 1 and Figure 2 display sample-size value for two-group comparison in the analogous format in ascending order, as the prior illustrations and with the letter-codes of the Appendix. This time, however, data are shown for all 53 study variables. The alpha probability value is 5% and the power (1-ß) is estimated with both, a 0.8 and a 0.9 power assumption. The respective ratio of the critical sample size n is in the order of 1.34 for the comparison of the the higher vs the lower . The estimated critical sample size per group for the listed variables ranges from as low of n = 1 to a high of 93,073; the latter is for weight-for-height Z score. Ten variables compute to a group-size of 10 or lower. Five variables have estimated critical samples sizes of greater than 1000 per group: These include: fecal calprotectin; BAZ-age, salivary IL-6 and IL-8, and the aforementioned WHZ.

## Discussion

As part of our premise, more and more studies in human research are trying to relate and associate variables from different biological domains and the number of measurements in any given study can proliferate.

The size of the sample enrolled and

**Table 2.** The sample size needed for a stable estimate of prevalence of abnormality of the measured variables based on the observed frequency, presenting in ascending order for confidence levels of 95 and 99%

| MID | Variable | N | Cut-off criterion | Observed prevalence % | Estimated sample size | |
|---|---|---|---|---|---|---|
| | | | | | 95% CL | 99% CL |
| I | Hemoglobin, non-invasive -Hb- (mg/dL) | 67 | <12.1 & <12.6 (<60mo & >60mo) | 25 | 90 | 155 |
| AAA | Fecal calprotectine (mg/Kg) | 87 | <50 | 61 | 91 | 158 |
| PP | *Giaridia intestinalis* intensity (OD) 8th week | 87 | <0.05 | 36 | 94 | 163 |
| D | Height for age -HAZ- (z-score) | 87 | <-2 (stunted) | 66 | 97 | 168 |
| EE | F2t-Isoprostanes -F2-Iso- (ng/mL) | 78 | | 76 | 107 | 186 |
| YY | Salivary IL-10 (pg/mL) | 83 | >3.93 | 75 | 111 | 192 |
| ZZ | Salivary TNF-α (pg/mL) | 83 | >19.0 | 75 | 111 | 192 |
| QQ | Plasma IL-1B (pg/mL) | 82 | >1.25 | 23 | 116 | 201 |
| KK | α-Tocopherol (mg/L) | 81 | <5 | 79 | 122 | 212 |
| C | Weight for age -WAZ- (z-score) | 87 | <-2 (underweight) | 23 | 123 | 213 |
| M | Mean corpuscular volume -MCV- (fL) | 82 | <80 | 17 | 145 | 252 |
| OO | Urinary white blood cells per field | 79 | >5 per field | 15 | 155 | 269 |
| G | BMI for age -BAZ- (z-score) | 87 | >+1 (overweight) | 16 | 162 | 281 |
| XX | Salivary IL-8 (pg/mL) | 83 | >578 | 14 | 172 | 298 |
| H | Hematocrit -Ht- (%) | 82 | <37% | 12 | 194 | 337 |
| J | Hemoglobin, whole blood -Hb- (mg/dL) | 82 | <12.1 & <12.6 (<60mo & >60mo) | 11 | 210 | 363 |
| DD | 8-Hydroxy-deoxy-guanosine -8OHdG- (ng/mL) | 78 | | 90 | 217 | 375 |
| JJ | Retinol (µg/L) | 81 | < 30 | 92 | 275 | 476 |
| K | White blood cell count -WBC- (103/mm3) | 82 | >10.8 | 8 | 279 | 483 |
| WW | Salivary IL-6 (pg/mL) | 83 | >10.46 | 6 | 368 | 637 |
| O | Mean corpuscular hemoglobin concentration -MCHC- (g/dL) | 82 | <32 | 5 | 431 | 747 |
| RR | Plasma IL-6 (pg/mL) | 82 | >1.89 | 96 | 534 | 926 |
| VV | Salivary IL-1B (pg/mL) | 83 | >73.0 | 4 | 539 | 934 |
| T | Urinary Osmolality (mOsm/kg) | 78 | >900 | 3 | 669 | 1,159 |
| L | Red blood cell count -RBC- (106/mm3) | 82 | <4.1 | 2 | 1,046 | 1,813 |
| P | Red cells distribution width -RDW- (%) | 82 | >15% | 2 | 1,046 | 1,813 |
| E | Weight for height -WHZ- (z-score) | 87 | <-2 (wasted) | 2 | 1,111 | 1,925 |
| Q | Platelet count (103/mm3) | 82 | <150 | 1 | 2,078 | 3,600 |
| MM | Coenzyme Q9 -CoQ9- (mg/L) | 81 | <0.41 | 100 | n.a. | n.a. |
| NN | Coenzyme Q10 -CoQ10- (mg/L) | 81 | <0.007 | 0 | n.a. | n.a. |
| SS | Plasma IL-8 (pg/mL) | 82 | >13.8 | 0 | n.a. | n.a. |
| TT | Plasma IL-10 (pg/mL) | 82 | >2.7 | 100 | n.a. | n.a. |
| UU | Plasma TNF-α (pg/mL) | 82 | >2.64 | 100 | n.a. | n.a. |

*based on the 33 variables of 53 total for which a cutoff criterion for abnormal can be cited.

analyzed in an investigation is critical to the interpretations and conclusions to be derived[14-17]. Authors are expected to specify prospectively in their methods sections the justification or the sample sizes recruited in terms of the statistical power (1-ß) that they require to avoid a type II error in addressing the null hypothesis[21-24].

When the expected variance is known on the basis of prior experience, appropriate powering can be accomplished using the formula used here or a variant thereof. When there is no previous experience, authors must project a "feasible" variance to create a sample-size estimate.

When the purpose of a study is primarily -or partially- to define a prevalence of a condition in a population, published statistical tables allow specification based on the confidence level one aspired to, e.g. 10%, 5%, 1%, etc, and on the confidenc intervals[25]. This begs the question, of course, of planning and designing studies, without any prior knowledge of what would be an expected population prevalence. As the lower is the prevalence, the greater is the needed sample, survey planners try to err on the safe side such as defining a

**Figure 1:** Frequency distribution of Sample Sizes for 95% Confidence Level.

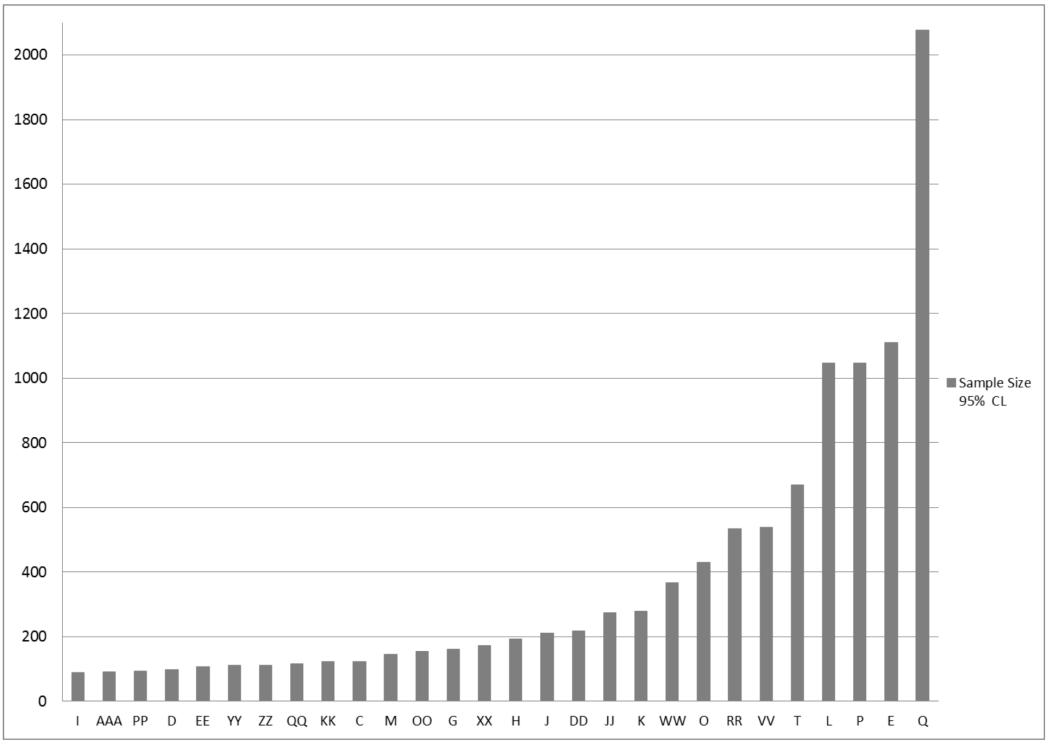
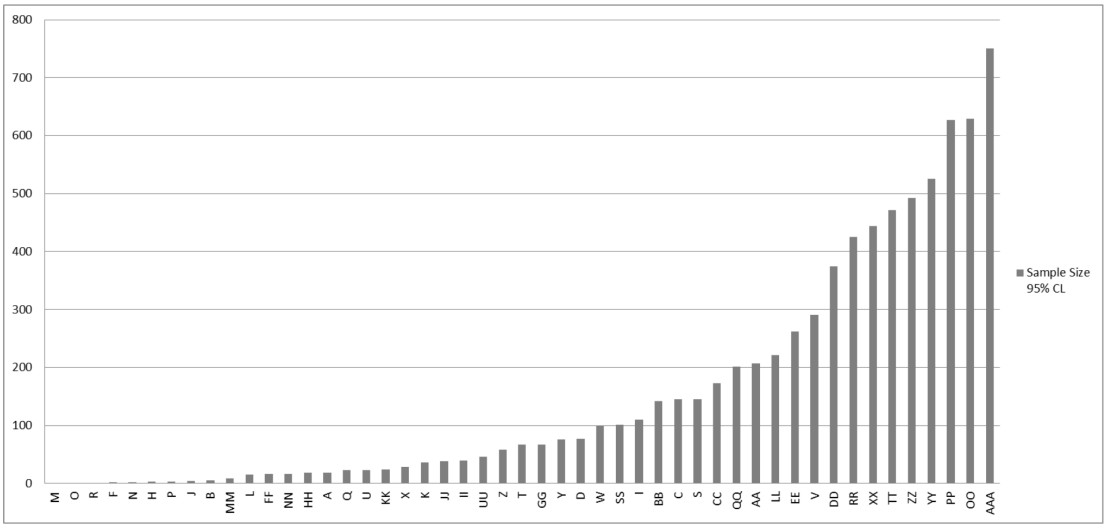



**Figure 2:** Frequency distribution of Sample Sizes for 95% Confidence Level.



sample-size for a 10% frequency when the a priori estimation would be toward a larger prevalence. Such coverage, however, has a negative impact on costs and resources. These types of trade-off considerations arise frequently in the current era of applications of new biomarkers at the population level.

When the objective of a study is comparative analysis of distributions, the selection of the sample-size is also tied to the variance of a specific -and usually the most important- outcome variable.

For instance, CeSSIAM was involved with an intervention study related to the randomized substitution of a non-coffee beverage in half of a group of 101 coffee-consuming children, mean age 17.3 mo in Guatemala, which resulted in three publications[26-28].

The sample size was based initially on detecting a change in hemoglobin concentration[27]; for this, 80 subjects were considered adequate based on anticipated variance. However, over the course of the three component publications, we reported comparative results on additional outcome variables including: hematocrit; zinc protoporphyrin, plasma ferritin; plasma iron; plasma zinc; plasma copper; weight; weight-gain; height; height-gain; weight-for-age Z score; height-for-age Z-score; weight-for-height Z-score; temperament; and sleep duration. Clearly, the project was not necessarily simultaneously powered to address the null hypothesis of treatment-related differences with respect to all of these additional variables. In a retrospective manner, however, one can always perform a post-hoc assessment of the actual statistical power based on the observed variance in the variables of secondary interest[29]. In practice, however, these calculations are not often presented in the publications. Our present field project was primarily descriptive in its intent.

Literature and institutional experience was available in the domains of anthropometry, hematology and parasitic and protozoan infections[10-11].

We were cognizant of our ignorance of variance in a host of measurements that had never been reported such as urinary osmolality and urine solutes in the hydration domain, urinary biomarkers of oxidative stress and antioxidant enzymes in the oxidation domain, and calprotectin and salivary and plasma cytokines in the infla - mation domain. Our aspiration for sample size had been a convenient, round number of 100 subjects across three centers, but we fell 13 short of recruiting and retaining that number. We were working with novel biomarkers heretofore not applied to preschool-age population samples[7;9-13].

Despite the primarily descriptive nature of the design, we did not constrain ourselves to not reporting subgroup comparisons for which the project had not been powered such as between the sexes or the center sites; in many instances in fact, we were able to find statistically-significant differences despite small subgroup sample sizes[9-10].

In retrospect, however, we recognize that many of our conclusions for no-difference were unlikely to have had the statistical power to arrive at a definitive negative finding. Hence, we were motivated here to create a comprehensive and systematic appraisal of the implications of the inter-individual variance with the hope it would aid future estimation of sample sizes for prevalence assessment in survey activities and prospective comparison studies, either observational or intervention.

In terms of the stability of our values for sample prevalence, 10 variables (30%) ranged between 25 and 75%, which would mean that ~87 observations (70%) would provide the number of subjects for a stable estimate within a 95% confidence-level framework. Assuming no change in background factors, a prospective survey for elevated F2-Iso should include 107 sub-

jects to meet the minimal criteria of confidence. For anemia, this number would be 210 for this setting. Indeed, for these 33 variables, the range of sample-sizes for confident prevalence estimates is vast. As shown in Table 2, the sample to estimate a stable rate of hipohydration (urinary osmolality > 900 mOsm/kg) would be over 1000 subjects.

In many ways, this analysis should be considered a case-study rather than a generic treatise with respect to the sample-size estimates for specific variables.

That a relatively small survey size could derive a stable estimate of stunting rate or giardial colonization is a consequence of nearly half of the subjects being affected. This would not be generalizable to an industrialized nation. On the other hand, the huge sample needed for wasting is also unique to Guatemala, and would be much lower in Africa[30]. Variables that might have a prevalence similar to other areas would be in the hematological domain, where our distributions would differ from peers in most parts of the world.

The plasma cytokines in this study were performed in the same laboratory in Granada as a study in Spanish children by Olza, et al.[31].

This compared three biomarkers, IL-6, IL-8, and TNF-α, in common with our own. Accessing the data reported, there were two groups of 223 schoolchildren each, one of normal weight and the other obese. The respective standard deviations of IL-6 were 8.21 and 13.29 for a pooled standard deviation of 10.75. Applying the formula for the minimum sample-size to discriminate a 20% effect size, the number was 2397. For our children, with a SD of 5.20, it would have required a smaller sample size of n = 425 (Table 1). With respect to the other two common cytokines, IL-8 and TNF- α, the sample-size requirements fulfilling our assumptions were 722 and 139 for the Spanish schoolchildren, and 101 and 46 for the present Guatemalan preschoolers. Consistently, the lower variance and smaller sample requirement are found in Guatemala.

There are a series of variables that, applying the formula, would only require one subject in each of two groups in order to encounter a 20% effect-size difference. These include variables such as mean corpuscular volume, mean corpuscular hemoglobin concentration and platelet distribution width.

Despite the seductive appeal of such variables for study design, one must reflect on the biological nature of these measures as to why they are so tightly distributed. It might be that the intrinsic stability produces resistance in making even the smallest variation in an individual. By the same token, therefore, one would avoid the temptation to apply regression analyses to these variables, as the clustering is so tight on their axis that little association with another distribution of variables would likely be detectable.

## Conclusions

The printed page gives us the luxury of discussing the exigencies of sample-size selection to optimize the statistical certainty for surveys, comparative studies, regression models and interventions. The laborious and expensive nature of field r - search only rarely allows for the mounting of projects that permit optimal sample sizes for all analytical purposes for all variables. Scientific knowledge, however, will only advance as emerging, novel and innovative biomarkers are brought into epidemiological studies at the population level. Our present compilation, therefore, is aimed as a note of caution and guidance for the ubiquitous situations in which a priori estimates of variance in one or another variable are unknown. Such measurements should indeed be made within the confines and limitations of resources of field research, but diagnostic measure-

ments should best be treated as secondary objectives in a preliminary context. These serve as first approximations of the frequency of abnormal values; thus, they provide guidance for formal survey design if the stable prevalence is desired. In reporting the preliminary values, transparency in acknowledging the limits of stability are appropriate in the limitations discussion.

In comparative studies between or among different population groups or in randomized controlled intervention trials (RCTs), the use of outcome variables of unknown variance takes on added significance. First, the publication of a study with a negative result discourages further follow-up[32], and if this is a falsely negative study due to inadequate sample-size, a setback to advancing knowledge can be guaranteed. Second, not publishing a study with a negative outcome also takes its toll. Much is known of the consequences for the scientific literature of the underreporting-bias for studies with a negative outcome, whether falsely or truly negative [33,34]. In the era of systematic review and meta-analysis for evidence-based practice, truly-negative outcomes are vital in shaping the final conclusion on efficacy or e fectiveness.

Finally, on a strictly bioethical basis, any comparative observational or intervention study performed in human subjects should have the solidity of design, including adequate sample-size, to be published irrespective of its outcome conclusions.

In this respect, preliminary descriptive investigation with innovative variables in an observational context, as exemplified here, is invaluable for the planning of observational and intervention research.

Our findings here help to prepare the observation investigator or the RCT researcher to appropriately power studies. In the former domain, for example, would be comparisons across different environmental exposures using urinary biomarkers of free-radical damage. Into the latter instance, for example, would fall inflammation-modification trials using fecal calprotectin or salivary cytokines. Our lessons learned and illustrated here can hopefully provide guidance to research teams embarking on analogous descriptive field studies of broad biological scope to focus on the variance in their own reporting of findings and direct the attention to variance issues in prospective field-study design

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## Appendix: Master tabulation of Descriptive Statistics of the 53 Study Variables

| Letter code | Variable | n valid values | Median | Percentiles 25th | Percentiles 75th | Minimum | Maximum | Range | Arithmetic Mean | Std. Deviation | Skewness | Kurtosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Growth and Anthropometry* | | | | | | | | | | | | |
| A | Weight (kg) | 87 | 14.20 | 12.20 | 17.30 | 8.90 | 23.30 | 14.40 | 14.58 | 3.12 | 0.32 | -0.45 |
| B | Height (cm) | 87 | 95.00 | 90.00 | 104.00 | 74.00 | 115.00 | 41.00 | 95.86 | 10.02 | -0.13 | -0.80 |
| C | Weight for age -WAZ- (z-score) | 87 | -1.37 | -1.98 | -0.80 | -3.41 | 1.60 | 5.01 | -1.41 | 0.88 | 0.20 | 0.76 |
| D | Height for age -HAZ- (z-score) | 87 | -2.39 | -2.98 | -1.62 | -4.64 | 1.36 | 6.00 | -2.33 | 1.04 | 0.43 | 1.14 |
| E | Weight for height -WHZ- (z-score) | 87 | -0.10 | -0.52 | 0.55 | -2.20 | 1.33 | 3.53 | -0.06 | 0.77 | -0.35 | 0.05 |
| F | Body Mass Index -BMI- ($kg/m^2$) | 87 | 15.72 | 15.02 | 16.42 | 12.80 | 17.96 | 5.16 | 15.72 | 1.03 | -0.05 | -0.01 |
| G | BMI for age -BAZ- (z-score) | 87 | 0.25 | -0.19 | 0.69 | -1.97 | 1.59 | 3.56 | 0.20 | 0.72 | -0.56 | 0.72 |
| *Hemogram* | | | | | | | | | | | | |
| H | Hematocrit -Ht- (%) | 82 | 39.45 | 11.30 | 13.85 | 20.10 | 43.60 | 23.50 | 39.12 | 3.07 | -2.97 | 17.23 |
| I | Hemoglobin, non-invasive -Hb- (mg/dL) | 85 | 13.00 | 12.70 | 14.00 | 1.00 | 15.70 | 15.70 | 10.39 | 5.50 | -1.32 | -0.08 |
| J | Hemoglobin, whole blood -Hb- (mg/dL) | 82 | 13.25 | 6.68 | 8.90 | 6.50 | 15.20 | 8.70 | 13.23 | 1.17 | -2.39 | 12.46 |
| K | White blood cell count -WBC- ($10^3/mm^3$) | 82 | 7.60 | 4.55 | 5.00 | 1.80 | 17.60 | 15.80 | 7.95 | 2.39 | 1.40 | 4.35 |
| L | Red blood cell count -RBC- ($10^6/mm^3$) | 82 | 4.74 | 37.78 | 41.10 | 2.40 | 12.40 | 10.00 | 4.84 | 0.94 | 6.26 | 52.38 |
| M | Mean corpuscular volume -MCV- (fL) | 82 | 82.45 | 80.58 | 85.00 | 71.60 | 89.90 | 18.30 | 82.65 | 3.44 | -0.24 | 0.77 |
| N | Mean corpuscular hemoglobin -MCH- (pg) | 82 | 27.85 | 26.80 | 29.13 | 22.20 | 31.90 | 9.70 | 27.95 | 1.73 | -0.10 | 0.56 |
| O | Mean corpuscular hemoglobin concentration -MCHC- (g/dL) | 82 | 33.85 | 32.98 | 34.73 | 31.10 | 36.90 | 5.80 | 33.81 | 1.16 | -0.11 | -0.21 |
| P | Red cells distribution width -RDW- (%) | 82 | 13.00 | 12.50 | 13.53 | 11.70 | 18.70 | 7.00 | 13.13 | 0.94 | 2.76 | 14.52 |
| Q | Platelet count ($10^3/mm^3$) | 82 | 307.00 | 270.50 | 350.75 | 62.00 | 506.00 | 444.00 | 314.73 | 76.02 | 0.15 | 1.08 |
| R | Platelet distribution width -PDW- (%) | 82 | 15.80 | 15.50 | 16.10 | 14.90 | 16.90 | 2.00 | 15.81 | 0.39 | 0.35 | 0.07 |
| *Hydration* | | | | | | | | | | | | |
| S | Urine volume (mL/24hour) 8th cycle week | 78 | 460.00 | 285.00 | 680.00 | 65.00 | 1670.00 | 1605.00 | 505.58 | 306.52 | 1.12 | 1.85 |
| T | Urinary Osmolality (mOsm/kg) 8th cycle week (Osmomat) | 78 | 430.00 | 327.00 | 511.75 | 115.00 | 1102.00 | 987.00 | 448.97 | 184.56 | 1.18 | 2.45 |
| U | Urinary Uric Acid (mg/dL) | 78 | 15.47 | 13.65 | 17.26 | 6.85 | 26.79 | 19.94 | 15.61 | 3.73 | 0.63 | 1.56 |
| V | Urinary Urea (mg/dL) | 66 | 99.97 | 9.31 | 155.59 | 0.02 | 283.04 | 283.02 | 96.78 | 83.19 | 0.46 | -0.88 |
| W | Urinary creatinine (mg/dL) 8th cycle week | 78 | 31.00 | 22.53 | 37.77 | 9.88 | 109.00 | 99.12 | 33.03 | 16.57 | 2.00 | 6.03 |
| X | Plasma creatinine (mg/dL) | 82 | 0.48 | 0.38 | 0.53 | 0.07 | 0.72 | 0.65 | 0.46 | 0.12 | -0.55 | 0.64 |
| Y | Sodium (mg/dL) | 77 | 183.00 | 134.30 | 235.60 | 19.30 | 441.60 | 422.30 | 191.28 | 83.86 | 0.60 | 0.75 |
| Z | Potassium (mg/dL) | 77 | 114.20 | 93.95 | 153.50 | 68.51 | 325.60 | 257.09 | 129.83 | 49.74 | 1.44 | 2.43 |
| AA | Calcium (mg/dL) | 77 | 6.28 | 3.98 | 9.21 | 0.64 | 34.38 | 33.74 | 7.44 | 5.40 | 2.15 | 7.55 |
| BB | Magnesium (mg/dL) | 77 | 5.72 | 3.46 | 9.13 | 0.65 | 15.20 | 14.55 | 6.38 | 3.81 | 0.60 | -0.56 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CC | Inorganic Phosphorus (mg/dL) | 78 | 21.92 | 14.87 | 34.05 | 2.35 | 75.15 | 72.80 | 27.77 | 18.41 | 0.99 | 0.15 |
| *Oxidation and Antioxidant Defense* | | | | | | | | | | | | |
| DD | 8-Hydroxy-deoxy-guanosine -8OHdG- (ng/mL) | 78 | 5.69 | 3.37 | 8.80 | 0.55 | 38.61 | 38.07 | 8.03 | 7.86 | 2.10 | 4.34 |
| EE | F2t-Isoprostanes -F2-Iso- (ng/mL) | 78 | 1.75 | 0.97 | 3.33 | 0.50 | 11.80 | 11.30 | 2.37 | 1.96 | 2.17 | 6.62 |
| FF | Catalase activity -CAT- (nmol/seg/ g Hb) | 82 | 6.68 | 5.70 | 7.69 | 4.31 | 9.91 | 5.60 | 6.76 | 1.34 | 0.33 | -0.41 |
| GG | Superoxide Dismutase activity -SOD- (U/g Hb) | 82 | 1.57 | 1.06 | 2.11 | 0.24 | 2.78 | 2.54 | 1.55 | 0.64 | -0.22 | -0.83 |
| HH | Gluthathione Reductase activity -GSR- (umol/min/g Hb) | 82 | 2.82 | 2.21 | 3.24 | 1.79 | 4.06 | 2.27 | 2.80 | 0.59 | 0.12 | -1.08 |
| II | Gluthathione Peroxidase activity -GPx- (μU/g Hb) | 82 | 6.05 | 4.80 | 7.05 | -0.63 | 11.99 | 12.62 | 5.99 | 1.91 | 0.01 | 2.24 |
| JJ | Retinol (mg/L) | 81 | 8.06 | 6.58 | 9.74 | 3.70 | 18.11 | 14.41 | 8.29 | 2.56 | 0.92 | 1.83 |
| KK | α-tocopherol (mg/L) | 81 | 1.54 | 1.33 | 1.83 | 0.79 | 2.64 | 1.85 | 1.57 | 0.39 | 0.29 | -0.22 |
| LL | β-carotene (mg/L) | 81 | 0.60 | 0.34 | 1.16 | 0.11 | 2.77 | 2.66 | 0.80 | 0.60 | 1.24 | 1.06 |
| MM | Coenzyme $Q_9$ -CO-$Q_9$- (mg/L) | 81 | 0.04 | 0.04 | 0.05 | 0.04 | 0.06 | 0.02 | 0.04 | 0.00 | 1.18 | 1.31 |
| NN | Coenzyme $Q_{10}$ -CO-$Q_{10}$- (mg/L) | 81 | 0.20 | 0.18 | 0.24 | 0.15 | 0.32 | 0.17 | 0.21 | 0.04 | 0.61 | 0.04 |
| *Infection and Inflammation* | | | | | | | | | | | | |
| OO | Urinary white blood cells per field | 79 | 3.00 | 1.00 | 5.00 | 1.00 | 26.00 | 26.00 | 4.29 | 5.44 | 2.70 | 7.60 |
| PP | *Giaridia intestinalis* intensity (OD) | 87 | 0.10 | 0.01 | 2.68 | -0.02 | 2.84 | 2.86 | 0.93 | 1.20 | 0.76 | -1.30 |
| QQ | Plasma IL-1β (pg/mL) | 82 | 0.88 | 0.66 | 1.20 | 0.27 | 5.69 | 5.42 | 1.05 | 0.75 | 3.79 | 19.76 |
| RR | Plasma IL-6 (pg/mL) | 82 | 3.67 | 2.81 | 5.21 | 1.57 | 44.20 | 42.63 | 4.98 | 5.20 | 5.76 | 40.89 |
| SS | Plasma IL-8 (pg/mL) | 82 | 4.48 | 3.15 | 5.99 | 1.46 | 13.41 | 11.95 | 5.06 | 2.56 | 1.30 | 1.64 |
| TT | Plasma IL-10 (pg/mL) | 82 | 52.22 | 39.28 | 75.59 | 16.99 | 608.11 | 591.12 | 69.68 | 76.38 | 5.42 | 33.97 |
| UU | Plasma TNF-α (pg/mL) | 82 | 7.49 | 6.08 | 9.53 | 4.43 | 17.96 | 13.53 | 8.08 | 2.76 | 1.33 | 1.98 |
| VV | Salivary IL-1β (pg/mL) | 83 | 1.70 | 0.63 | 5.09 | 0.33 | 407.08 | 406.75 | 11.99 | 49.53 | 6.95 | 52.20 |
| WW | Salivary IL-6 (pg/mL) | 83 | 1.36 | 0.68 | 2.72 | 0.11 | 151.25 | 151.14 | 4.40 | 16.71 | 8.49 | 75.09 |
| XX | Salivary IL-8 (pg/mL) | 83 | 120.44 | 46.17 | 298.95 | 1.92 | 734.29 | 732.37 | 207.83 | 220.94 | 1.17 | -0.09 |
| YY | Salivary IL-10 (pg/mL) | 83 | 11.02 | 3.92 | 26.20 | 1.24 | 108.76 | 107.52 | 18.53 | 21.46 | 2.08 | 4.56 |
| ZZ | Salivary TNF-α (pg/mL) | 83 | 53.19 | 18.98 | 149.93 | 0.94 | 373.16 | 372.22 | 100.75 | 112.88 | 1.21 | -0.02 |
| AAA | Fecal calprotectin (mg/Kg) | 87 | 57.50 | 30.00 | 100.00 | 10.00 | 950.00 | 940.00 | 98.02 | 135.62 | 4.01 | 20.07 |

# Chapter 10

# Considerations of Probability in Multiple Comparison Analysis

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**Considerations of Probability in Multiple Comparison Analysis**

Statistical assessments are based upon an assigned probability of a phenomenon's occurring by chance (1,2). For instance, when we assign a critical probability of 5%, we mean that 5 times in 100 it could be happening by chance alone and 95 times could be not by chance. This leads to a problem when one is dealing with multiple comparisons. If we demand a 5% probability with only 5 comparisons and find 1 to be significant, it is four times greater than by chance alone; with the same probability criteria and 20 comparisons, this could clearly occur by chance alone. Our exploratory approach in **Chapters 3, 4, 5** and **6** to determine associations between variables, was to apply the Spearman rank-order correlation to paired-variables, filtering for probability levels at the 5% level or less.

In **Chapter 3**, we examined the association of six urinary variables including volume, osmolality and unadjusted and adjusted concentrations of two oxidative stress biomarkers within a total of 15 cells in a hemi-matrix. In **Chapter 4**, the interactive domains were oxidation variables reflected on themselves. With a total of 11 variables in a hemi-matrix configuration, the number of multiple comparisons was 55. In **Chapter 5**, domains were self-reflected inflammation biomarkers. With a total of 12 variables, the number of cells rose to 66 in the hemi-matrix. Finally, in **Chapter 6**, the oxidation and the inflammation variables constituted a full-matrix of 132 cross-association cells. Each of these represents a situation in which the pitfalls of multiple comparisons could be encounter.

We became sensitized to the issue when the first submission of **Chapter 5** was returned from a journal without review citing a design flaw of "multiple comparison", the same manuscript was promptly accepted in a second submission, that to *PLoS ONE*; however, we have done a self-examination of the four articles involving the multiple Spearman correlations to see how justified or not was the critique of a multiple comparison problem.

In **Table 1**, we used the basic probability criterion of 5% as a screening level for statistical significance across the four papers. This table includes the absolute number of significant correlations and the total cells of the hemi- or full-matrix in each of the four articles, among which the percentage of significant cells ranges from 80%

in **Chapter 3** down to barely 10% in **Chapter 6**. Expressed as fold-excess, this would be 16 times the expected number of significant associations in the former chapter to twice the expected in the latter.

**Table 1: Proportion of associations at statistical significance of less than 5% in the hemi-matrix and full-matrix Spearman correlations across chapters 3, 4, 5 and 6.**

| | **Hydration Oxidation** | **Oxidation Cluster** | **Inflammation Cluster** | **Oxidation-Inflammation** |
|---|---|---|---|---|
| | **Chapter 3** | **Chapter 4** | **Chapter 5** | **Chapter 6** |
| **Type of Matrix** | Hemi-matrix | Hemi-matrix | Hemi-matrix | Full-matrix |
| Total significant | 12 | 28 | 22 | 13 |
| Total cells | 15 | 55 | 66 | 132 |
| Percentage significant | 80% | 51% | 33.3% | 9.8% |
| Expected Percentage | 5% | 5% | 5% | 5% |
| Fold-excess | 16.0 | 10.2 | 6.6 | 2.0 |

To further examine the multiple comparison issue, we graded our associations by the degree of probability into three descending levels: ≤ 0.05 to >0.01; ≤ 0.01 to >0.001; and ≤ 0.001. **Table 2** provides the cumulative number of associations in the various statistical significance ranges in the Spearman correlation hemi- and full-matrix. The importance at each level of probability can be understood better, however, with the expressions in **Table 3**, which provides a per mil standard of reference.

**Table 2: Cumulative number of associations in the various statistical significance range in the hemi-matrix and full-matrix Spearman correlations in Chapters 3, 4, 5 and 6.**

| | Hydration Oxidation | Oxidation Cluster | Inflammation Cluster | Oxidation Inflammation |
|---|---|---|---|---|
| | **Chapter 3** | **Chapter 4** | **Chapter 5** | **Chapter 6** |
| **Type of Matrix** | Hemi-matrix | Hemi-matrix | Hemi-matrix | Full-matrix |
| **≤ 0.05 to >0.01** | -- | 9 | 3 | 6 |
| **≤ 0.01 to >0.001** | 1 | 5 | 2 | 6 |
| **≤ 0.001** | 11 | 14 | 17 | 1 |
| **Total significant** | 12 | 28 | 22 | 13 |

We have transformed the data to be expressed on a per mil basis. If we have 1000 comparisons, a 5% probability would reflect a total of 50 significant findings by chance; 40 of these would be expected to fall within the range of ≤ 0.05 to >0.01 by chance, and 10 would be below. Of these 10, nine would be expected to be in the range of ≤ 0.01 to >0.001 and the final probability would be one in 1000 (≤ 0.001). Transforming the absolute values in **Table 2** to a per mil expression in **Table 3,** we find curiously no associations within the ≤ 0.05 to >0.01 range in **Chapter 6**. For **Chapter 4** on oxidation variables interactions, we find four times the expected associations in this lowest range of significance. Therefore, at least three of every four associations would not be expected by chance alone. However, in **Chapter 5** on inflammation biomarkers interactions and in **Chapter 6** on oxidation-inflammation interactions, almost all of the 45 cross-correlations at the first significance level could occur by chance alone.

Moving to the second level of probability, ≤ 0.01 to >0.001, all associations are well above the reference nine per thousand that would be expected; this ranges from the 10-fold excess in **Chapter 4** to 3.3-fold excess in **Chapter 5**. Returning to

**Table 2**, with the exception of **Chapter 6**, the vast majority of significant associations fall at the one-in-a-thousand or less level. In a per mil expression, the association at this level are 733 times higher than chance alone in **Chapter 3**, and above 250 times for the remaining two chapters. There is essentially no reasonable possibility that any of those associations are "chance findings".

**Table 3: Number of associations in the various statistical significance range in the hemi-matrix and full-matrix Spearman correlations in Chapters 1, 4, 5 and 6, expressed as per mil.**

| | Reference Standard (per mil) | Hydration Oxidation | Oxidation Cluster | Inflammation Cluster | Oxidation-Inflammation |
|---|---|---|---|---|---|
| | | **Chapter 1** | **Chapter 4** | **Chapter 5** | **Chapter 6** |
| **Type of matrix** | **--** | Hemi-matrix | Hemi-matrix | Hemi-matrix | Full-matrix |
| **≤ 0.05 to >0.01** | 40 | -- | 164 | 45 | 45 |
| **≤ 0.01 to >0.001** | 9 | 67 | 90 | 30 | 45 |
| **≤ 0.001** | 1 | 733 | 254 | 258 | 7 |

Spearman rank-order correlation enforces a linear assumption about the relationship between the X- and Y-axis variables. In biology nature, however, not all interactions would follow a mutually-linear gradient. In all of the four chapters, we further examined the statistically significant associations under the microscope of the goodness-of-fit modeling (3). This model seeks to find the alternative, non-linear associations across paired variables with a closer fit to curves such as cubic, quadratic, exponential, sigmoid, etc. If the strength of association, as indicated by the correlation coefficient (r), improves with the goodness-of-fit model as compared to the Spearman $r_s$ value, this adds credibility to the validity as a true association. Across the ≤ 0.05 to >0.01 probability range, one or another non-linear assumption showed a much stronger association than the rank-order correlation.

In summary, the approach of Spearman correlations between paired variables is a useful exploratory tool for initial examination of associations. Many

investigators, however, have fallen into the trap of multiple comparisons. Our experience with manuscript submissions sensitized the authorship to this possibility. Our present examination highlights the fact that most of the findings are supported with probability of chance association of less than one in a thousand and many of the others have an inherently non-linear configuration to their interaction. Thus, we have minimized the confounding of our conclusions by randomly-occurring associations.

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# VI. DISCUSSION

Jeniece Alvey, *Hoy  no quiero jugar*, Quetzaltenango 2012

The main objective of this study was to describe the variance of a series of markers related to nutrition and health in preschool children from the Western Highlands of Guatemala. The major strength in the methodology of this study was the common dietary offering of 4 from 5 meals per day, creating a variance that is independent of the diet and could be e.g. explained by environmental and genetic exposure. This dissertation attempted to inform about possible interaction within and across different research domains, such as growth, hematology, hydration, oxidation, inflammation and infection. In the case of the inflammation domain, interactions were also analyzed across different anatomical compartments (saliva and plasma).

Studies in children will always involve ethical and methodological concerns that should be considered in order to plan future investigations in nutrition and health (68). Because of the vulnerability of this age group, one of those concerns is the invasiveness of sample collection. Another concern would be to take a minimum amount of samples (blood, tissue, etc.), if invasive collection is imperative.

**Hydration status and its interactions**

Because very few studies have focused on the hydration subject, reporting beverage consumption and comparing it to recommendations of water intake (69-71), one of the main interests in this dissertation was to collect and present data on hydration status in Guatemala, as determined by urine osmolality.

Urine osmolality, is a non-invasive marker of hydration status well investigated in different countries (72-76). Especially in Germany, several reports have been published regarding the steady-state of hydration (73,77-78) and the completeness of 24-h samples (79) as part of the DONALD study. **Chapter 1** compares hydration status of our study subjects, who were in the narrow age range from 2-6 years of age, with data from children and juveniles in developed countries who were up to 18 years old (72-76). Our results show that Guatemalan children are as well hydrated as the children from a 1$^{st}$ world country.

One cannot rely on plasma osmolality when it comes to assess human hydration, as it is tightly and homeostatically regulated within a narrow range of 280-295 mOsm/kg of plasma (80). It is common clinical practice to use a surrogate estimate for plasma osmolality. The corresponding formula includes the circulating

concentrations of Na, K, glucose and urea nitrogen. Therefore, in **Chapter 2**, we investigated how dependent Uosm is on the concentration of the urinary solutes. By using the backwards elimination multiple regression modeling in our population, we showed that 95% of the variance in Uosm is explained by the concentrations of Na, K, Mg, Pi and urea.

An interesting finding was the inverse correlation of urinary Uosm with urea. This is explained as follows: in order to retain water within the body, and hence to excrete more concentrated urine, urea is more aggressively reabsorbed in the kidney, using the counter-current exchange at the terminal inner medullary collecting duct in the loop of Henle (82). Hence, less urea in the urine generates higher osmolality and vice versa.

Reproducibility has important implications for the design of multicenter studies, in this case, about hydration. We performed comparisons related to temperature of sample storage, and on the correspondence between two osmometers of different brands. No statistically significant differences were found when comparing $0^{o}C$ and $-80^{o}C$ storage. Regarding the use of different osmometers, it turned out that use of the same equipment to determine urine osmolality is necessary, even in multicenter studies.

When applying the Remer (79) criteria for completeness of 24-h urine samples based on creatinine, only 57% of our sample met those criteria. However, a recent study in adults compared the timing over the day of spot urine sampling with the osmolality of the pooled sample. Casual samples collected in the afternoon best reflected the average across-day osmolality (83). Even if incomplete, the more urine was collected and pooled, the better it represented the hydration status given in Uosm. Granted that all urine samples were more than a casual spot sample, our experience shows that valuable information about urinary biomarkers can be derived from prolonged, but not necessarily completed, collections.

The observations made in **Chapter 3** combined the analyses of hydration status through Uosm and their interactions with two urinary indicators of oxidative stress, at the systemic level: the F2-Iso, derived from the oxidation of fatty acids in the lipid membrane and circulating lipoproteins (84) and 8-OHdG, a degradation product of the nucleoside guanine derived from DNA (85). Even though, these two

indicators reflect free radical exposure at different intracellular sites, our results revealed a strong correlation between the hydration status and the excretion of both oxidative stress biomarkers.

On the hydration side, López-Plaza *et al.* (86) demonstrated recently that healthy adults with adequate hydration status as assessed with bioelectrical impedance rather than urinary Uosm, presented lower concentrations of pro-inflammatory cytokines. Such observations confirm the general principle that a mild variation in hydration status can be associated with considerable changes in a functional biomarker.

**Interactions within and across biomarkers of oxidation and inflammation**

With regards to the domains of oxidation and inflammation, we were expecting heterogeneity across sample, despite the narrow age-range and the common institutional menu offering.

A list of factors compiled by Berk *et al*. (87), links the causality of the environmental stimuli to inflammation. These factors include psychosocial stressors, poor diet, physical inactivity, obesity, smoking, altered gut permeability, atopy, dental cares, sleep and vitamin D deficiency. We believe the listed factors may serve as a basis for oxidation, as well. We can assume that all possible factors except for obesity and sleep disturbance would be applicable in our setting, due to poor quality of water, rudimentary sanitation (88), indoor oven smoke (89) and diverse parasites (90).

**Chapter 4**, analyzes two biomarkers of systemic oxidation (F2-Iso and 8-OHdG), four antioxidant enzymes (CAT, SOD, GSR and GPX) and five antioxidant nutrients (retinol, α-tocopherol, β-carotene and coenzyme Q9 and Q10). As expected, the concentrations of biomarkers of oxidative stress were above the reference values cited on the assay kits, or above values from other reference populations. Moreover, we found low circulating levels of alpha-tocopherol. In 64 of 81 subjects (79%) they were below the normal range of 5 to 20 mg/dL. This finding has implications for public health in Guatemala because Vitamin E deficiency is associated with an increased risk of chronic diseases, such as coronary heart disease, type II diabetes, and some types of cancer (91-93).

In our data set, urinary excretion of oxidative biomarkers associates directly with the activity of antioxidant enzymes and inversely with the vitamin concentration; 51% of the paired comparisons in the correlation hemi-matrix were statistically significant. Our most striking findings are the apparent patterns of harmonious interactions among the elements of the oxidation-antioxidation systems within an environment with a variety of mild to moderate oxidative stress in daily life.

In **Chapter 5**, we generated descriptive information and interactions across 12 biomarkers of inflammation from the whole blood (WBC), plasmatic, salivary (IL-1β, IL-6, IL-8, IL-10 and TNF-α), and fecal (calprotectin) anatomical compartments. As shown for oxidative stress markers, these indicators showed high values when compared commonly used reference levels (94), due to the above-mentioned environmental and psychosocial conditions.

In this case, 33% of the hemi-matrix correlations had significant r values. Strong correlations were found within each anatomical compartment, whereas modest to none correlations were found between different compartments. This is consistent with several studies reporting lack of such correlations (95-97). Calprotectin, the fecal biomarker of inflammation, did not correlate with other biomarkers.

IL-10 was the only anti-inflammatory cytokine that was evaluated in our study, and it showed a wide series of correlations. Therefore, we decided to run two backwards elimination multiple regression models for IL-10, one in the salivary and the other in the plasmatic anatomical compartment. In each model three variables emerged as independent predictors for the IL-10 concentration variance, two of these predictors were IL-8 and TNF-α, in both anatomical compartments. IL-1β, and IL-6 emerged as the third predictor, in the salivary and in the plasmatic compartment, respectively.

Due to the interesting findings in the oxidation and inflammation domains, individually described in the corresponding articles, we decided to explore possible inter-domain associations in **Chapter 6**. The same methods as in the two previous chapters were used in this exploration, but instead of constructing a hemi-matrix, the Spearman rank-order correlations were presented in a full-matrix. Results of this

combined analysis showed significant associations only in 10% of the 132 possible paired correlations.

The biomarkers that showed more paired-correlations among the matrix were β-carotene and SOD, on the oxidation side, and salivary IL-8 and IL-10 and plasmatic TNF-α, on the inflammation side. Again, we ran the backwards elimination multiple regression model for the former two biomarkers with their respective significantly associated pairs in the inflammation domain. Curiously, plasmatic TNF-α and salivary IL-8 remained as independent predictors for the variance of these two biomarkers of oxidation, after running 4 elimination models.

Both, oxidative and inflammatory stress biomarkers, showed wide variation -but relative high values- in low-income and deprived preschoolers of predominantly Mayan ascent sharing a highly uniform dietary offering.

**Intestinal Health**

Emerging technology allows non-invasive diagnostic assessment of intestinal inflammation. Calprotectin, a fecal marker of intra-intestinal inflammation, is widely available and the most studied of diagnostic markers. We attempted to link this variable to other markers that could be associated with intestinal inflammation, e.g. growth, intestinal infection, level of intestinal *giardia* and helminthes infestation, etc.

Values of calprotectin decline with age (98). The age variation made direct comparisons among sites difficult to interpret, although the median value of 55 mg/kg of feces reported in **Chapter 7** is close to data reported in Norwegian five-year-old children (99). Our distribution, as expected, is shifted to the right, due to calprotectin values up to 950 mg/kg.

In comparison with other countries, no matter whether highly or less developed, our central-tendency values are the highest reported so far (57-59,99-102). According to the diagnostic criterion, 5 of 10, or 6 of 10 subjects had a high calprotectin stool concentration, and 1 in 10 show concentrations corresponding to clinically active inflammatory bowel disease. Because of the narrow range of age included in this data set compared with other studies, and because of the age dependency of calprotectin, further research needs to be performed with a wider age range.

Although no specific associations were found between fecal calprotectin concentration and the diagnostic or intensity of parasite infestations, the higher concentration of fecal calprotectin in Guatemalan children, suggests a severe inflammatory state in the intestine.

An additional aspect of intestinal health is presented in **Chapter 8**. Here we tested the use of O.D. in the ELISA readings from the *Giardia* fecal antibody assay. Here, 53% of our sample was diagnosed as positive for *Giardia* antigen with the ELISA assay, showing a significantly higher percentage of positive cases in the marginal-urban center (B).

A previous study at CeSSIAM, showed an inverse correlation between *Giardia* infestation and height; and reported that the reaction intensity for absorbance reaches its maximum level at an O.D. of 3.0. As a conclusion of this study, it was recommended an expansion of the range through a dilution of high-response samples to further improve the rank-ordering (34). Nevertheless, using the entire range that the ProSpecT-Giardia-EZ microplate allows, we found a meaningful biological association between *Giardia* absorbance and oxidation biomarkers. This finding shows the potential validity of the former as an indicator of the *Giardia* infestation intensity.

Knowing there is a predisposition to transmission of *Giardia* in Guatemalan children attending daycare centers (10), we decided to test the hypothesis of an association of asymptomatic giardiasis and oxidative stress. This was possible due to data of fecal assays for *G intestinalis* and a battery of biomarkers for both oxidation and antioxidation systems for urine, plasma, and erythrocytes collected from 74 preschool children.

Our results showed significant higher concentrations of F2-Iso and GSR in children with a positive test for giardiasis. With respect to intensity, significant positive associations were found between O.D. absorbance values and urinary F2-Iso, CAT, and SOD.

In our study population, we found so far unrecognized associations between RBC antioxidant enzymes and urine oxidative stress biomarkers with the prevalence and intensity of asymptomatic giardiasis. A greater excretion of urinary biomarkers

signifies a greater systemic oxidation. For enzyme activity the interpretation is not so straightforward. On the one hand, lower activities of enzymes may suggest a depletion of antioxidant capacity; i.e. the subject is more vulnerable to oxidative damage. On the other hand, higher enzymatic activity may signal a compensatory reaction; i.e. adding protection in the face of persistent oxidative damage. Clear directional causality cannot be established with the obtained results.

A unique feature of highland preschoolers is their high degree of linear growth retardation. Stunting varied from 35% in center A to 81% in center C, which is consistent with the position of Guatemala as the nation with the highest under-five stunting rate in the Western Hemisphere (54%) (103). We are aware of the fact that growth retardation reaches its maximum at 2 years of age (104), while preschoolers enrolled in this dissertation were 2-7 years old. Although they received good nutrition and care at the moment of sample collection, our results suggest that the particularities of home environment, psychosocial factors, genetics and prenatal factors take their toll on these children's failure in growth, nutrition and health status previous to their enrollment at the daycare centers.

**The nature of variance**

More and more studies in human nutrition research are trying to link variables from different biological domains. The size of samples enrolled to analyze a specific variable is very important for the interpretations of the results and the conclusions drawn (105,106).

When the expected variance is known from previous experience, proper statistical power of a study can be accomplished by enrolling an adequate number of candidates. But without previous experience authors must estimate sample-sizes projecting a feasible variance.

Fifty-three of the 61 variables measured in this dissertation were selected to illustrate the heterogeneity in this respect across our sample in **Chapter 9**. Thirty-three variables had a cut-off criterion of abnormality that allowed us to use a conventional formula to calculate sample size. Among the 53 variables relative standard deviation (coefficient of variance) ranged from 90 to 2073. Accordingly,

sample-size for comparison with a power (1-ß) of 0.8 ranged from below 10 per group up to 93,000.

Some of the resultant sample size estimates are the consequence of the high number of Guatemalan children being affected by different conditions e.g. stunting and giardiasis. For that reason, most of our results regarding variance and sample sizes could not be generalized to other nations. However, preliminary descriptive investigation with innovative variables in a new observational context, as exemplified here, is of great value for the planning of observational and intervention research, in similar contexts to the setting of this study.

**Strengths and limitations of the study**

A major strength of this set of studies is that it addresses the age-group of preschool children living in a context of poor hygienic environment. The common and controlled dietary offering of foods and beverages to children during the time spent at a daycare center should theoretically narrow the sample-wide variance, this could be regarded as an strength or a limitation.

This set of studies uses modern assessment equipment and micro-techniques for analysis in samples collected in a group of young children at the community level. The possibility to relate a large variety of biomarkers within and across different study domains collected from different anatomical compartments is an important strength.

We were ambitious enough to include 24-h urine collections in the study and were quite successful in obtaining complete collections. We recognize, however, that we did not obtain full adherence in this effort due to the young age of the subjects.

A weakness regarding some of the biomarkers analyzed in this dissertation project is the cross-sectional, point-in-time characteristic of the dissertation. This allowed us to analyze distribution but not to understand day-to-day variation, especially in variables that present higher values as compared to corresponding references.

For some of the study analyses, our sample number was modest and results could lack statistical significance when comparing groups. An intrinsic limitation is that statistically significant associations do not imply causality because of the absenceof longitudinal or intervention design.

# VII. CONCLUSIONS

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**Practical and methodological advances**

1. This dissertation revealed new public health insights by applying innovative and novel biomarkers in an epidemiological field investigation in children of preschool age. For example, fecal calprotectin as a marker of enteric inflammation and use of Giardia antigen in an ELISA assay to indicate prevalence and intensity of asymptomatic giardiasis infestation were applied for the first time in Guatemala.

2. Useful and valuable diagnostic information has been gained by use of collection techniques and measurements that are innocuous and non-invasive. Anthropometric measurements, skin contact probes and salivary, urinary and fecal sampling were explored in this study.

3. Complete quantitative urine sample collection over 24 h is tedious and cumbersome in preschool children. Our study showed that total urine output does not need to be collected to provide useful information on human metabolism. Reproducibility studies reveal that frozen- or long-term-storage has no effect on the consistency of osmolality measurement. Results are not sufficiently reproducible across different osmometry equipment, however, to allow pooling of values obtained with different devices in a common analysis.

**New biological discovery**

4. Despite a common dietary offering for 80% of the average daily calorie intake in a given group of preschoolers attending a day-care center system, wide variation in different biomarkers was documented. Thus, uniformity of the diet is no guarantee of uniformity in the nutritional, physiological, oxidative or inflammatory status across a group.

5. Household and community environments generated more oxidative stress in our preschooler population than expected. However, apparent patterns of harmonious interactions among the elements of the oxidation-antioxidation systems were observed. Additionally, the biomarkers of systemic oxidation showed associations with the intensity of *Giardia intestinalis* infestation in our study

population. Although causality cannot be established by use of our study design, oxidation may represent a potential mediating factor in the growth impairment associated with *Giardia* colonization.

6. Biomarkers of inflammation measured in different anatomical compartments were elevated above the reference levels, suggesting a response to the microbial and antigenic challenge of the poorly-hygienic situation in which preschoolers from the Western Highlands of Guatemala live.

7. There are abundant factors to explain the oxidant and immune alteration in our study population, e.g. altered gut permeability, scarce dental care, vitamin D deficiency and indoor smoke contamination. Inter-domain analysis of oxidation and inflammation did not yield results that were as robust or harmonic as the ones found in intra-domain analyses. However, interesting interactions of anti-oxidation biomarkers, such as superoxide dismutase and β-carotene, with those of inflammation, such as salivary IL-8 and plasmatic TNF-α, were found.

**Public health relevance for Guatemala**

8. The hydration status of children in our study, as assessed by urinary osmolality, can be interpreted as adequate, even as superior to contemporary findings in other areas of the world. The most remarkable features overall in the finding is the inter-individual variation in excretion of solutes, and the confirmation of the dominant roles of urea and the principal electrolytes, sodium and potassium, in urine osmolality. These are determined, in turn, by the level of protein in diet (urea), the contribution of an intracellular fraction of the diet (potassium), and the extent of processed foods and discretional salt use (sodium). Moreover, our results suggest that a superior hydration status associates with quantitative reductions in urinary excretion of markers of cellular oxidation.

9. Low circulating levels of alpha-tocopherol (< 5 mg/dL) in 79% of our study population hints towards a previously unrecognized micronutrient deficiency with respect to vitamin E in the country.

**Remaining work to be done**

10. In order to confirm our findings regarding interactions between different biomarkers, and to corroborate the direction of causality of those interactions, intervention trials and cohort studies should be performed for different variables of interest.

11. Poor linear growth, a disturbing public health problem in Guatemala, is the consequence of a series of conditions that Guatemalan children are exposed to. The ones that are already described in the country (lack of food, lack of particular nutrients, etc.), and some others unexplored hitherto, are described across this study. Most of these conditions can be tackled by the nation leaders with public actions, e.g. by an appropriate investment in clean water and adequate sanitation systems that are complemented with basic health and nutrition education.

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# IX. ANNEXES

Sheny Romero, *Jugando en la niebla*, Quetzaltenango 2012

## **Annex 1.** 40-Day Rotating Menu from the SOSEP System

*Week 1*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 hard-boiled egg<br>1 tbsp Chirmol<br>1 piece of French Bread or 1 Tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup beans<br>1 tsp of cream<br>1 baked banana<br>1 piece of French Bread or 1 Tortilla | 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 torta de huevo<br>1 tbsp of Chirmol<br>1 French Bread or 1 Tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup beans<br>1 tbsp of cheese<br>1 French Bread or 1 Tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 torta de huevo w tomato and onion<br>1 French Bread or Tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 4 oz Chicken with Chipilín and Vegetables (Recipe 41)<br>1/2 cup white rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>1 glass Orange and Carrot Juice (Recipe 60) | 3 oz Beef Steak (Recipe 33)<br>1/2 cup Mashed Potatoes (Recipe 5)<br>1/2 cup Lettuce w Cucumbers (Recipe 53)<br>1 tortilla or 1 tamalito<br>1 cup Horchata (Recipe 67) | 4 oz Chicken in Pepián (Recipe 42)<br>1/2 cup Rice with Vegetables (Recipe 1)<br>1 tortilla or 1 tamalito<br>1 cup Tropical Juice (Recipe 66) | 3 oz Breaded Liver (Recipe 35)<br>1/2 cup Mashed Potatoes (Recipe 5)<br>1/2 cup Beet Salad (Recipe 57)<br>1 tortilla or 1 tamalito<br>1 cup Orange and Carrot Juice (Recipe 60) | 3 oz Meatballs w Sauce (Recipe 36)<br>1/2 cup Spaghetti w Margarine (Recipe 6)<br>1/2 cup steamed Güicoyitos (Recipe 20)<br>1 tortilla or 1 tamalito<br>1 cup Rosa de Jamaica Juice (Recipe 64) |
| SNACK (3:30pm) | | | | |
| 1 glass Pineapple Juice (Recipe 59)<br>1 piece bread w beans | 1 6-oz cup Bienestarina (Recipe 71) | 1 cup Tropical Juice (Recipe 66)<br>1 piece bread w Frijoles Colados | 1 6-oz cup Bienestarina (Recipe 71) | 1 cup Rosa de Jamaica Juice (Recipe 64)<br>1 Pancake w Honey |

*Week 2*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1/2 cup Corn Flakes (or other breakfast cereal)<br>1 6-oz glass Milk (Recipe 75)<br>1 banana | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup frijol colado<br>1 tbsp cheese<br>1 piece french bread or 1 tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 torta de huevo<br>1 french bread or 1 tortilla | 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1/2 cup frijol colado<br>1 tbsp cheese<br>1 french bread or 1 tortilla | 1 6-oz cup Avena<br>1 scrambled egg w tomato<br>1 french Bread or tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 1 Beef Patty (Recipe 28)<br>1/2 cup Spaghetti w Sauce (Recipe 10)<br>1/2 cup Cucumber Salad w Mayo (Recipe 55)<br>1 tortilla or 1 tamalito<br>1 cup Starfruit and Pineapple Juice (Recipe 65) | 1 serving Chow Mein (Recipe 12)<br>1/2 cup Pea Soup (Recipe 18)<br>1 tortilla or 1 tamalito<br>1 cup Horchata (Recipe 67) | 3 oz Beef Stew (Recipe 32)<br>1/2 cup mashed potatoes (Recipe 5)<br>1/2 cup of Carrot, Lettuce, and Tomato Salad (Recipe 56)<br>1 tortilla or 1 tamalito<br>1 cup Pineapple Juice (Recipe 59) | 1 Chilaquila w Güisquil and Cheese (Recipe 25)<br>1/2 cup White Rice (Recipe 2)<br>1/2 cup Beet Salad (Recipe 57)<br>1 tortilla or 1 tamalito<br>1 cup Tamarind Juice (Recipe 62) | 3 oz Carne Asada w 1 tbsp Chirmol (Recipe 38)<br>2 tbsp Guacamole<br>1/2 cup White Rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>1 cup Carrot and Orange Juice (Recipe 60) |
| SNACK (3:30pm) | | | | |
| 1 cup Starfruit and Pineapple Juice (Recipe 65)<br>1 piece bread w frijoles colados | 1 6-oz cup Bienestarina (Recipe 71) | 1 cup Pineapple Juice (Recipe 59)<br>Plantain w Beans (Recipe 76) | 1 6-oz cup Incaparina Gruel (Recipe 72) | 1/2 cup Dulce de Leche (Recipe 80)<br>1 cup Horchata (Recipe 67) |

*Week 3*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 hard-boiled egg<br>1 tbsp Chirmol<br>1 french Bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup beans<br>1 tsp cream<br>1 cooked banana<br>1 french bread or 1 tortilla | 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 torta de huevo<br>1 tbsp Chirmol<br>1 french Bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup beans<br>1 tbsp of cheese<br>1 french bread or 1 tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 torta de huevo w tomato and onion<br>1 french bread or 1 tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 4 oz Chicken Broth w Vegetables (Recipe 43)<br>1/2 cup Noodles (for Soup) (Recipe 11)<br>1 tortilla or 1 tamalito<br>1 cup Blackberry Juice (Recipe 63) | 3 oz Fish Fillet (Recipe 51)<br>1 Steamed Potato (Recipe 4)<br>1/2 cup Carrot, Lettuce, Tomato Salad (Recipe 56)<br>1 tortilla or 1 tamalito<br>1 cup Lemonade (Recipe 61) | 4 oz Barbecue Chicken (Recipe 44)<br>1/2 cup Vegetable Soup (Recipe 15)<br>1/2 cup Spaghetti w Margarine (Recipe 8)<br>1 tortilla or 1 tamalito<br>1 cup of Orange and Watermelon Juice (Recipe 68) | 1 Doblada w Cheese and Loroco (Recipe 48)<br>1/2 cup Rice w Vegetables (Recipe 1)<br>1/2 cup Beet Salad (Recipe 57)<br>1 cup Tamarind Juice (Recipe 62) | 3 oz Pancakes w Protemás (Recipe 34)<br>1/2 cup Mashed Potatoes (Recipe 5)<br>1 tortilla or 1 tamalito<br>1/2 cup Combined Broccoli (Recipe 58)<br>1 cup Orange and Watermelon Juice (Recipe 68) |
| SNACK (3:30pm) | | | | |
| 1 cup Blackberry Juice (Recipe 63)<br>1 Pancake w Honey (Recipe 77) | 1 6-oz cup Incaparina Gruel (Recipe 72) | 1 cup Orange Juice<br>1/2 cup Dulce de Leche (Recipe 80) | 1 6-oz cup Avena (Recipe 73) | 1 cup Orange and Watermelon Juice (Recipe 68)<br>1 bread w beans |

*Week 4*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1/2 cup Corn Flakes or other breakfast cereal<br>1 6-oz cup Milk (Recipe 75)<br>1 banana | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup frijol colado<br>1 tbsp cheese<br>1 French Bread or 1 Tortilla | 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 torta de huevo<br>1 tbsp Chirmol<br>1 french bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup beans<br>1 tbsp of cheese<br>1 french bread or 1 tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 torta de huevo w tomato and onion<br>1 french bread or 1 tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 1/2 cup Salpicón (Recipe 29)<br>1/2 cup Spinach Soup (Recipe 17)<br>1/2 cup White Rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>1 cup Mango Juice (Recipe 70) | 1 serving Scrambled Eggs w Broccoli or Güisquil (Recipe 23 or 24)<br>1/2 cup Carrot Soup (Recipe 16)<br>1/2 cup Rice w Vegetables (Recipe 1)<br>1 tortilla or 1 tamalito<br>1 cup Carrot and Orange Juice (Recipe 60) | 1 serving Arroz Chino w Chicken (Recipe 3)<br>1/2 cup Carrot Soup (Recipe 16)<br>1 tortilla or 1 tamalito<br>1 cup Blackberry Juice (Recipe 63) | 1/2 cup Carne Picada w Vegetables (Recipe 39)<br>1/2 cup White Rice (Recipe 2)<br>1/2 cup Cucumber, Tomato, and Onion Salad (Recipe 54)<br>1 cup Tamarind Juice (Recipe 62)<br>1 tortilla or 1 tamalito | 1 cup Chicken Soup (Recipe 13)<br>1 tortilla or 1 tamalito<br>1 cup Pineapple Juice (Recipe 59) |
| SNACK (3:30pm) | | | | |
| 1 cup Lemonade<br>1/2 cup Bananas w Cream (Recipe 81) | 1 6-oz cup Avena (Recipe 73) | 1 cup Blackberry Juice (Recipe 63)<br>1 Rellenito de Plátano (Recipe 76) | 1 6-oz cup Bienestarina (Recipe 71) | 1 cup Pineapple Juice (Recipe 59)<br>1/2 cup Mole de Plátano (Recipe 78) |

*Week 5*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1/2 cup Corn Flakes or other breakfast cereal<br>1 6-oz cup Milk (Recipe 75)<br>1 banana | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup Frijol Colado<br>1 tbsp cheese<br>1 french bread or 1 tortilla | 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 torta de huevo<br>1 french bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup Frijol Colado<br>1 tbsp crème<br>1 french bread or 1 tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 hard-boiled egg<br>1 tbsp Chirmol<br>1 french bread or 1 tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 1/2 cup Beef and Vegetable Soup (Recipe 30)<br>1/2 cup Noodles (for Soup) (Recipe 11)<br>1 tortilla or 1 tamalito<br>1 cup Lemonade (Recipe 61) | 4 oz Pollo Asado (Recipe 45)<br>1/2 cup Carrot Soup (Recipe 16)<br>1/2 cup Mashed Potatoes (Recipe 5)<br>1 tortilla or 1 tamalito<br>1 cup Carrot and Orange Juice (Recipe 60) | 3 oz Carne Molda Con Verduras (Recipe 40)<br>1/2 cup Spaghetti w Cream (Recipe 8)<br>1/2 cup salad<br>1 tortilla or 1 tamalito<br>1 cup Blackberry Juice (Recipe 63) | 1 cup Green Beans w Egg (Recipe 21)<br>1/2 cup Spaghetti w Margarine (Recipe 6)<br>1/2 cup Vegetable Soup (Recipe 15)<br>1 tortilla or 1 tamalito<br>1 cup Lemonade (Recipe 61) | 1 serving Spaghetti w Bolognese Sauce (Recipe 9)<br>1/2 cup Spinach Soup (Recipe 17)<br>1 tortilla or 1 tamalito<br>1 cup Lemonade (Recipe 61) |
| SNACK (3:30pm) | | | | |
| 1 6-oz cup plantain porridge | 1 6-oz cup Bienestarina (Recipe 71) | 1/2 cup Jello w Fruit (Recipe 79) or 1 serving of fruit | 1 6-oz cup Avena (Recipe 73) | 1 Fruit Ice |

*Week 6*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 hard-boiled egg<br>1 tbsp Chirmol<br>1 french bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup beans<br>1 tsp cream<br>1 cooked plantain<br>1 french bread or 1 tortilla | 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 torta de huevo<br>1 tbsp Chirmol<br>1 french bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup beans<br>1 tbsp cheese<br>1 french bread or 1 tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 scrambled egg w tomato and onion<br>1 french bread or 1 tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 3 oz Breaded Beef (Recipe 37)<br>1/2 cup Mashed Potatoes (Recipe 5)<br>1 tortilla or 1 tamalito<br>1/2 cup Cucumber, Tomato, and Onion Salad (Recipe 54)<br>1 cup Tamarind Juice (Recipe 62) | 3 oz Carne Asada (Recipe 38)<br>1 tbsp chirmol<br>1/2 cup White Rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>2 tbsp guacamole<br>1 cup orange juice | 1/2 cup Chop Suey (Recipe 14)<br>1/2 cup White Rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>1 cup Carrot and Orange Juice (Recipe 60) | 4 oz Stewed Chicken (Recipe 46)<br>1/2 cup White Rice (Recipe 2)<br>1/2 cup Lettuce and Cucumber Salad (Recipe 53)<br>1 tortilla or 1 tamalito<br>1 cup Rosa de Jamaica Juice (Recipe 64) | 1 serving Arroz Chino con Lomito (Recipe 3)<br>1/2 cup Carrot Soup (Recipe 16)<br>1 tortilla or 1 tamalito<br>1 cup Blackberry Juice (Recipe 63) |
| SNACK (3:30pm) | | | | |
| 1 cup Tamarind Juice (Recipe 62)<br>1 pancake w honey | 1 6-oz cup Avena (Recipe 73) | 1/2 cup Rice Pudding (Recipe 82)<br>1 cup Horchata (Recipe 67) | 1 6-oz cup of Incaparina Gruel (Recipe 72) | 1 cup Carrot and Orange Juice (Recipe 60)<br>1/2 cup Bananas w Cream (Recipe 81) |

*Week 7*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1/2 cup Corn Flakes or other breakfast cereal<br>1 6-oz cup Milk (Recipe 75)<br>1 banana | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup frijol colado<br>1 tbsp cheese<br>1 french bread or 1 tortilla | 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 torta de huevo<br>1 tbsp Chirmol<br>1 french bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup frijol colado<br>1 tbsp cream<br>1 french bread or 1 tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 hard-boiled egg<br>1 tbsp Chirmol<br>1 french bread or 1 tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 1 cup Beef Broth (Recipe 31)<br>1/2 cup Noodles (for Soup) (Recipe 11)<br>1 tortilla or 1 tamalito<br>1 cup Lemonade (Recipe 61) | 1 Mixta (Recipe 50)<br>1/2 cup Pea Soup (Recipe 18)<br>1 cup Horchata (Recipe 67) | 1 Doblada of Beef (Recipe 49)<br>1/2 cup Mashed Potatoes (Recipe 5)<br>2 tbsp Guacamole<br>1 cup Mango Juice (Recipe 70) | 1/2 cup Salpicon (Recipe 29)<br>1/2 cup Spinach Soup (Recipe 17)<br>1/2 cup White Rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>1 cup Mango Juice (Recipe 70) | 4 oz Pollo Asado (Recipe 45)<br>1/2 cup Carrot Soup (Recipe 16)<br>1/2 cup Mashed Potatoes (Recipe 5)<br>1 tortilla or 1 tamalito<br>1 cup Carrot and Orange Juice (Recipe 60) |
| SNACK (3:30pm) | | | | |
| 1 cup Lemonade (Recipe 61)<br>1/4 fried plantain<br>1/2 cup Frijol Colado | 1 6-oz cup Incaparina Gruel (Recipe 72) | 1 cup Mango Juice (Recipe 70)<br>1 bread w beans and cheese | 1 6-oz cup Avena (Recipe 73) | 1 Stuffed Plantain (Recipe 76)<br>1 cup Horchata (Recipe 67) |

*Week 8*

| MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY |
|---|---|---|---|---|
| BREAKFAST (8am) | | | | |
| 1 6-oz cup Incaparina Gruel (Recipe 72)<br>1 torta de huevo<br>1 tbsp Chirmol<br>1 french bread or 1 tortilla | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup frijol colado<br>1 tbsp cheese<br>1 french bread or 1 tortilla | 1/2 cup Corn Flakes or other breakfast cereal<br>1 6-oz cup Milk (Recipe 75)<br>1 banana | 1 6-oz cup Bienestarina (Recipe 71)<br>1/2 cup frijol colado<br>1 tbsp cream<br>1 French Bread or 1 Tortilla | 1 6-oz cup Avena (Recipe 73)<br>1 scrambled egg w tomato<br>1 French Bread or 1 Tortilla |
| SNACK (10am) | | | | |
| 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit | 1 serving of fruit |
| LUNCH (12:30pm) | | | | |
| 1/2 cup Spaghetti w Sausage (Recipe 7)<br>1/2 cup Spinach Soup (Recipe 17)<br>1 tortilla or 1 tamalito<br>1 cup Strawberry Juice (Recipe 69) | 4 oz Creamed Chicken (Recipe 47)<br>1/2 cup White Rice (Recipe 2)<br>1/2 cup Spinach Soup (Recipe 17)<br>1 tortilla or 1 tamalito<br>1 cup Lemonade (Recipe 61) | 1/2 cup Beans w Spinach or Chipilin (Recipe 26 or 27)<br>1/2 cup White Rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>1 cup Pineapple Juice (Recipe 59) | 4 oz Pollo Asado (Recipe 45)<br>1/2 cup Carrot Soup (Recipe 16)<br>1/2 cup White Rice (Recipe 2)<br>1 tortilla or 1 tamalito<br>1 cup Orange and Watermelon Juice (Recipe 68) | 3 oz Fish Fillet (Recipe 51)<br>1 Steamed Potato (Recipe 4)<br>1/2 cup Carrot, Spinach, and Tomato Salad (Recipe 56)<br>1 tortilla or 1 tamalito<br>1 cup Lemonade (Recipe 61) |
| SNACK (3:30pm) | | | | |
| 1/2 cup Gelatin (Recipe 79) or 1 serving of fruit | 1 6-oz cup Avena (Recipe 73) | 1 serving Mole de Plantain (Recipe 78)<br>1 cup Horchata (Recipe 67) | 1 6-oz cup Bienestarina (Recipe 71) | 1 cup Lemonade (Recipe 61)<br>1 serving Bananas w Cream (Recipe 81) |

**Annex 2.** Energy and nutrient adequacy of the nutrient offering of the 40-day SOSEP menu[1]

| | Mean daily nutrient intake of SOSEP menu | Daily requirement based on UN-RNI system (4-6 y) | Percentage of UN-RNI requirement (%) | Daily requirement based on DRI-RDA system (4-8 y) | Percentage of RDA (%) |
|---|---|---|---|---|---|
| Energy (kcal) | **1200** | 1300[a] | 92 | 1366[d] | 88 |
| Protein (g) | **54.5** | 16.7[b] | 326 | 19.0[e] | 287 |
| Carbohydrates (g) | **167** | -- | -- | 130[e] | 128 |
| Total sugar (g) | **87.0** | -- | -- | -- | -- |
| Total fiber (g) | **15.0** | -- | -- | 25.0[e] | 60 |
| Fat (g) | **37.5** | -- | -- | -- | -- |
| Saturated fatty acid (g) | **12.2** | -- | -- | -- | -- |
| MUFA (g) | **13.5** | -- | -- | -- | -- |
| PUFA (g) | **7.7** | -- | -- | -- | -- |
| Vitamin A (RAE) | **1355** | 450[c] | 301 | 400[f] | 339 |
| Vitamin D (mg) | **4.1** | 5.0[c] | 82 | 15.0[f] | 27 |
| Vitamin E (IU) | **5.3** | 5.0[c] | 106 | 7.0[f] | 76 |
| Vitamin K (mg) | **98.4** | | -- | 55.0[f] | 179 |
| Vitamin C (mg) | **58.1** | 30.0[c] | 194 | 25.0[f] | 232 |
| Thiamine (mg) | **1.0** | 0.6[c] | 167 | 0.6[f] | 167 |
| Riboflavin (mg) | **1.3** | 0.6[c] | 217 | 0.6[f] | 217 |
| Niacin (mg) | **13.4** | 8.0[c] | 168 | 8.0[f] | 168 |
| Vitamin $B_6$ (mg) | **1.2** | 0.6[c] | 200 | 0.6[f] | 200 |
| Pantothenic Acid (mg) | **3.1** | 3.0[c] | 103 | 3.0[f] | 103 |
| Folate DFE (mg) | **330** | 200[c] | 165 | 200[f] | 165 |
| Vitamin $B_{12}$ (µg) | **4.3** | 1.2[c] | 358 | 1.2[f] | 358 |
| Calcium (mg) | **573** | 600[c] | 96 | 1000[f] | 57 |
| Phosphorus (mg) | **810** | -- | -- | 500[f] | 162 |
| Magnesium (mg) | **215** | 76[c] | 283 | 250[f] | 86 |
| Iron (mg) | **11.5** | 6.3[c,g] | 183 | 10.0[f] | 115 |
| Zinc (mg) | **8.6** | 4.8[c,h] | 179 | 5.0[f] | 172 |
| Copper (mg) | **1.2** | -- | -- | 4.4[f] | 27 |
| Manganese (mg) | **1.7** | -- | -- | 1.5[f] | 113 |
| Selenium (mg) | **59.9** | 22.0[c] | 272 | 30.0[f] | 200 |
| Sodium (mg) | **1696** | -- | -- | 1200[f] | 141 |
| Potassium (mg) | **1738** | -- | -- | 3800[f] | 219 |

DFE= Dietary Folate Equivalents; DRI= Dietary Reference Intakes; RAE= retinol activity equivalent; RDA= Recommended Dietary Allowances; RNI=Recommended nutrient intakes; SOSEP="Secretaría de Obras Sociales de la Esposa del Presidente"; UN=United Nations.

[a] The average of the energy requirement for a 4-5 year old boy (1332 kcal) and girl (1265 kcal). The formula used is 77kg for boys and 74kg for girls. The body weight at mid-point of age interval of the 2006 WHO growth standards was used, this is 17.3 kg for boys and 16.9 kg for girls;
[b] The average safe level of protein intake for a 4-6 year old boy (17.1 g) and girl (16.2 g). The assumed body weight is 17.3 kg for boys and 16.9 kg for girls;
[c] Recommended nutrient intakes (RNI) for children aged 4 – 6 year old;
[d] The average of the estimated energy requirement for a 4-5 year old boy (1408 kcal) and girl (1325 kcal). The assumed body weight is 17.3 kg for boys and 16.9 kg for girls;
[e] Recommended intake for children aged 4 – 8 year old;
[f] Recommended Dietary Allowances (RDA) for children aged 4 – 8 year old;
[g] Assuming 10% bioavailability;
[h] Assuming moderate bioavailability.

[1] From the original article: Vossenaar M, Hernández L, Montenegro-Bethancourt G, Soto-Méndez MJ, *et al*. The Nutritional Contribution of Foods and Beverages Provided by Government-Sponsored Day-care Centers in Guatemala. Food Nut Bull. In press.

**Annex 3:** Ethical Approval from the CESSIAM's Ethics Committee

DOCTOR
B
E L
T R A
N E N A
CLINICAS

Guatemala 22 de mayo del 2012

Doctor
Noel W. Solomons
Ceciam

Estimado Dr. Solomons:

Atentamente me permito informarle de mi criterio sobre los trabajos de Investigación de Liza Hernández y colaboradores sobre **"Contribution of leading food sources, meals and presentations to dietary intake in governmet sponsored day-care centers en Guatemala" (SOSEP)**, así mismo el trabajo de alimentación de María José Soto Méndez sobre el **"ESTUDIO DEL ESTADO Y LAS VARIACIONES INTER-SUJETO E INTRA-SUJETO EN LA COMPOSICION CORPORAL, HIDRATACIÓN, MARCADORES DE OXIDACIÓN, INFECCIÓN E INFLAMACIÓN EN NIÑOS PRE-ESCOLARES CON UNA DIETA PARECIDA".**

Es mi criterio, que ambos estudios por la condición y metodología propuesta no ofrece ningún riesgo y guardan la dignidad de las personas, de los niños, objetivos de los trabajos.

Atentamente,

Doctor Fernando Beltranena Valladares

Edificio Plaza Dorada, 4to Nivel, 6ta. Ave. 3-47, Zona 9 Tel.: 2334-0930/2361-2446

# **Annex 4:** Consent form

**CENTRO DE ESTUDIOS EN SENSORIOPATIAS, SENECTUD E IMPEDIMENTOS Y ALTERACIONES METABOLICAS -CESSIAM**
**17 Av. 16-89 (Interior) zona 11**

**Investigador Principal: María José Soto Méndez, MSc.**

**CONSENTIMIENTO INFORMADO**

**ESTUDIO DEL ESTADO Y LAS VARIACIONES INTER-SUJETO E INTRA-SUJETO EN CRECIMIENTO, HEMATOLOGÍA, HIDRATACIÓN, MARCADORES DE OXIDACIÓN E INFLAMACIÓN EN NIÑOS PRE-ESCOLARES CON UNA DIETA PARECIDA.**
**Una investigación utilizando predominantemente, técnicas no-invasivas de recolección de datos biológicos.**

**PARTICIPACIÓN EN ESTUDIO Y TOMA DE MUESTRAS BIOLÓGICAS EN NIÑOS**

Lo hemos invitado el día de hoy para explicarle de qué se trata este estudio y para invitarle a que su hijo(a) participe en el estudio.

**DESCRIPCION DEL ESTUDIO Y RAZON DEL ESTUDIO**

El propósito de este estudio es identificar la situación de su niño(a) respecto a peso, estatura, infecciones, parásitos, hemoglobina y algunos otros componentes que ayudan a conocer mejor el estado de una persona, como la oxidación o envejecimiento del cuerpo, la inflamación y la hidratación o cantidad de agua en el cuerpo.

Su niño(a) está en una edad importante de crecimiento y desarrollo; además está asistiendo a un Hogar Comunitario de la Secretaria de Obras Sociales de la Esposa del Presidente (SOSEP), donde comparte un mismo lugar, la misma alimentación y la misma educación que otros niños(as) de su edad; por esta razón el título del estudio menciona la frase “variaciones inter-sujeto” que significa que queremos saber si existen diferencias entre los resultados de su niño(a) y los de sus compañeritos de la misma edad y que asisten a la misma institución. También se encuentra la frase “variaciones intra-sujeto” que significa que queremos averiguar, en algunos casos, los cambios de un día a otro en su niño(a).

El estudio se realizará en 4 diferentes hogares comunitarios. Su niño(a) está asistiendo a uno de ellos, por lo cual deseamos que su hijo(a) participe en este estudio con nosotros.

Su hijo (a) no tiene que participar en el estudio si usted no lo desea, y puede salirse cuando quiera.

**PROCEDIMIENTO A SEGUIR:**

Durante ocho semanas se estará viendo que a su niño (a) se le entregue su comida tal y como lo pide el menú de Hogares Comunitarios de SOSEP. Al iniciar la semana número seis se empezará la recolección de varias muestras para determinar el estado general de su niño (a).

**Exámenes:** Las muestras que se pedirá a su niño (a) son las siguientes: muestra de heces (2 veces), muestra de orina de 24 horas, o sea de todo un día y una noche (3 veces), muestra de orina común (1 vez), muestra de saliva (1 vez), muestra de 5 mililitros de sangre, que equivalen a 1 cucharadita pequeña (1 vez). Con estas muestras se les realizará exámenes para saber lo siguiente: Peso, estatura, nivel de hemoglobina (en sangre y con un aparato que lo mide por medio de una luz en la piel llamado Pronto 7), envejecimiento del cuerpo (en sangre, en orina y en heces), inflamación (en

saliva, en sangre y en heces), si existe infección (en orina y en sangre), si existen parásitos (en heces),
.

**MOLESTIAS Y RIESGOS:**

- Las muestras de orina, saliva y heces; además del aparato de transmisión por piel no generarán ningún dolor o molestia a su niño (a).

- Durante la toma de la muestra de sangre, su niño(a) podrá sentir un poco de dolor y que se forme un morete en el brazo. La persona que encargada de tomar la muestra tiene mucha experiencia para evitar que deje problemas.

**BENEFICIOS:**

- No recibirá ninguna atención médica especial su niño(a) por estar en el estudio, deberá seguir asistiendo regularmente a los servicios de salud.

- Si en los resultados de la toma de muestra su niño(a) sale con baja hemoglobina se le informará para que aunado a otros exámenes la entidad de salud que lo atiende determine si es por falta de hierro. Si en los resultados de parásitos en heces su niño(a) sale con parásitos, se le proporcionará el tratamiento correspondiente y adecuado para su edad.

**COSTOS:**

- Todos los costos serán cubiertos por el estudio.

- No habrá costos para su familia por la muestra.

**RETIRO DEL ESTUDIO**

- Su hijo(a) puede abandonar el estudio en cualquier momento sin problema.

- Si su médico cree que el estudio representa algún peligro o daño a la salud de su niño(a), por alguna condición o padecimiento, podrá decidir que se retire del estudio.

**EXCLUSIONES:**

La única cosa por la que no podría ayudarnos en este estudio, es que su niño deje de asistir a Hogares Comunitarios de SOSEP.

**CONFIDENCIALIDAD:**

La información obtenida en el estudio será colocada en forma de números, el nombre de su hijo(a) solo será conocido por los investigadores en contacto con él/ella. Los resultados serán publicados eventualmente pero con números, nunca aparecerán los nombres. Tampoco se le harán otras pruebas de laboratorio que usted no nos autorice en este documento.

**CONTACTOS:**

Si usted tiene alguna pregunta sobre los procedimientos o cambios de citas realizadas con nosotros puede comunicarse a Licda. María José Soto al teléfono 24733942 ó 59270017, de 8:00 de la mañana a 3:30 de la tarde, de lunes a viernes. Si usted tiene dudas sobre cualquier aspecto de derechos humanos de los participantes en este estudio, por favor comuníquese con el Dr. Fernando Beltranena (Director del Comité de Derechos Humanos) al teléfono 23310928, de 8:00 de la mañana a 5:00 de la tarde, de lunes a viernes. La dirección de nuestra oficina donde también puede contactarnos está en el membrete de este documento.

**PARTICIPACIÓN VOLUNTARIA:**

Los investigadores estamos en libertad de dar por terminada la participación de su hijo(a) si no cumple con la asistencia a Hogares Comunitarios o si nos es imposible tomar muestras de sangre adecuadas.

**Consentimiento:**

Yo_____he conocido los objetivos de este estudio, entiendo que es un estudio de investigación, entiendo sus riesgos y beneficios, así como la confidencialidad que me ofrecen. Sé que el estudio es gratuito y que no debo pagar un solo centavo por participar. Voluntariamente quiero que mi hijo (a) participe en el estudio y entiendo que en el momento que yo quiera puedo retirar a mi hijo(a) del estudio sin represalias de ningún tipo.

Nombre del Participante: _____

Nombre del Padre/Madre o Encargado del Participante_____

_____

No. de Cédula o DPI_____ Fecha_____

Dirección_____

Firma o huella del (a) Padre/Madre o Encargado del Participante

_____

Nombre del Investigador_____

Firma del Investigador _____

Nombre del Testigo_____

No. de Cédula o DPI _____

Firma o huella del (a) testigo _____

# **Annex 5**: Clinical Trials Registry Form

ClinicalTrials.gov

A service of the U.S. National Institutes of Health

Trial record **1 of 1** for: NCT02203890

**Normative State and Variation in Growth, Hematology, Hydration, Oxidation, Infection, Inflammation, Guatemalan Children**

**This study has been completed.**

**Sponsor:** María José Soto

**Collaborator:** Universidad de Granada

**Information provided by (Responsible Party):** María José Soto, Center for Studies of Sensory Impairment, Aging and Metabolism

**ClinicalTrials.gov Identifier:** NCT02203890

First received: July 25, 2014

Last updated: July 28, 2014

Last verified: July 2014

| **Tracking Information** | |
|---|---|
| **First Received Date** ICMJE | July 25, 2014 |
| **Last Updated Date** | July 28, 2014 |
| **Start Date** ICMJE | June 2012 |
| **Primary Completion Date** | November 2012 (final data collection date for primary outcome measure) |
| **Current Primary Outcome Measures** ICMJE **(submitted: July 28, 2014)** | • Weight [ Time Frame: 7th week of observation ] [ Designated as safety issue: No ]<br>Growth, measured trough weight (kg)<br>• Hemoglobin [ Time Frame: 8th week of observation ] [ Designated as safety issue: No ]<br>Hemoglobin (mg/dl) measured in Biochemistry Laboratory in La Democracia Hospital, Quetzaltenango, Guatemala.<br>• Urine Osmolality-1 [ Time Frame: 6th week of observation ] [ Designated as safety issue: No ]<br>Hydration Status= measured in 24-h urine through urine volume and osmolality in osmometer Model Löser 815. Results in mOsm/kg.<br>• Height [ Time Frame: 7th week of observation ] [ Designated as safety issue: No ]<br>Growth, measured trough height (cm).<br>• Urine Osmolality-2 [ Time Frame: 7th week of observation ] [ Designated as safety issue: No ]<br>Hydration Status= measured in 24-h urine through urine volume and osmolality Osmometer Model Löser 815. Results in mOsm/kg.<br>• Urine Osmolality-3 [ Time Frame: 8th week of observation ] [ Designated as safety issue: No ]<br>Hydration Status= measured in 24-h urine through urine volume and osmolality in two different osmometers (Löser 815 and Osmomat 030). Results in mOsm/kg. |

- Urine Urea [ Time Frame: 8th week of oservation ] [ Designated as safety issue: No ]

Urine solutes measured by the Scientific Instrumentation Center from the Granada University, Spain: urea (mg/l)

- Urine Uric Acid [ Time Frame: 8th week of oservation ] [ Designated as safety issue: No ]

Urine solutes measured by the Scientific Instrumentation Center from the Granada University, Spain: uric acid (mg/l)

- Urine Sodium [ Time Frame: 8th week of oservation ] [ Designated as safety issue: No ]

Urine solutes measured by the Scientific Instrumentation Center from the Granada University, Spain: sodium (mg/l)

- Urine Potassium [ Time Frame: 8th week of oservation ] [ Designated as safety issue: No ]

Urine solutes measured by the Scientific Instrumentation Center from the Granada University, Spain: potassium (mg/l)

- Urine Calcium [ Time Frame: 8th week of oservation ] [ Designated as safety issue: No ]

Urine solutes measured by the Scientific Instrumentation Center from the Granada University, Spain: calcium (mg/l)

- Urine Magnesium [ Time Frame: 8th week of oservation ] [ Designated as safety issue: No ]

Urine solutes measured by the Scientific Instrumentation Center from the Granada University, Spain: magnesium (mg/l)

- Urine Phosphorus [ Time Frame: 8th week of oservation ] [ Designated as safety issue: No ]

Urine solutes measured by the Scientific Instrumentation Center from the Granada University, Spain: inorganic phosphorus (mg/l)

- Urine 15-f2t-Isoprostanes [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Urine Oxidation biomarkers:

15-f2t-Isoprostanes (ng/mL) Measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada using ELISA assay kit (Oxford Biomedical Research, Inc., Catalog # EA84.102606, Michigan, USA)

- Urine 8-Hydroxydeoxyguanosine [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Urine Oxidation biomarkers:

8-Hydroxydeoxyguanosine (ng/mL) Measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada using ELISA assay kits (JaICA, Nikken SEIL Co., Ltd, Catalog# IM-KOGHS 040914E, Shizouka, Japan).

- Catalase Activity [ Time Frame: 8th week of observation ] [ Designated as safety issue: No ]

Activity of antioxidant enzymes in red blood cells; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using ELISA system:

Catalase Activity (nmol/seg/g Hb)

- Superoxide Dismutase Activity [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Activity of antioxidant enzymes in red blood cells; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using ELISA system Superoxide Dismutase Activity (U/g Hb)

- Gluthathione Reductase [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Activity of antioxidant enzymes in red blood cells; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using ELISA system Gluthathione Reductase Activity (umol/min/g Hb)

- Gluthathione Peroxidase [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Activity of antioxidant enzymes in red blood cells; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using ELISA system Gluthathione Peroxidase Activity (uU/g Hb)

- Retinol [ Time Frame: 8th week of observation ] [ Designated as safety issue: No ]

Content of antioxidant compounds in plasma; Scientific Instrumentation Center, University of Granada.

Retinol (mg/dl)

- Alpha-tocopherol [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Content of antioxidant compounds in plasma; Scientific Instrumentation Center, University of Granada.

Alpha-tocopherol (mg/l)

- Beta-Carotenes [ Time Frame: 8th week of observation ] [ Designated as safety issue: No ]

Content of antioxidant compounds in plasma; Scientific Instrumentation Center, University of Granada.

Beta-carotenes (mg/l)

- Coenzyme Q9 [ Time Frame: 8th week of observation ] [ Designated as safety issue: No ]

Content of antioxidant compounds in plasma; Scientific Instrumentation Center, University of Granada.

Coenzyme Q9 (mg/l)

- Coenzyme Q10 [ Time Frame: 8th week of observation ] [ Designated as safety issue: No ]

Content of antioxidant compounds in plasma; Scientific Instrumentation Center, University of Granada.

Coenzyme Q10 (mg/l)

- Plasma Interleukin-1B [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Plasma Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system:
Plasma IL-1β (pg/ml)

- Plasma Interleukin-6 [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Plasma Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system:
Plasma IL-6 (pg/ml)

- Plasma Interleukin-8 [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Plasma Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system:
Plasma IL-8 (pg/ml)

- Plasma Interleukin-10 [ Time Frame: 8th week of observation ]

[ Designated as safety issue: No ]

Plasma Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system:
Plasma IL-10 (pg/ml)

- Plasma Tumor Necrosis Factor-Alpha [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Plasma Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system:
Plasma Tumor Necrosis Factor-Alpha (pg/ml)

- Saliva Interleukin-1B [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Saliva Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system: Saliva IL-1β (pg/ml)

- Saliva Interleukin-6 [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Saliva Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system: Saliva IL-6 (pg/ml)

- Saliva Interleukin-8 [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Saliva Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system: Saliva IL-8 (pg/ml)

- Saliva Interleukin-10 [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Saliva Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system Saliva IL-10 (pg/ml)

- Saliva Tumor Necrosis Factor-Alpha [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Saliva Interleukines; measured at the Institute of Nutrition and Food Technology, Center of Biomedical Research, University of Granada, using the Luminex system:
Saliva Tumor Necrosis Factor-Alpha (pg/ml)

- Fecal Calprotectin [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Fecal Calprotectin (mg/kg) measured using the ELISA assay kit from Calpro-Lab (Catalog # CAL0100).

- Intestinal Parasitosis [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Intestinal Parasitosis, measured by Biochemistry Laboratory, La Democracia Hospital, Quetzaltenango, Guatemala. Parasite cysts presence in feces.

- Giardiasis Infection Presence-1 [ Time Frame: 7th week of observation ]
  [ Designated as safety issue: No ]

Analyses done by ELISA assay kit (ProSpecT Giardia EZ, catalog #R2458596), absorbance (O.D)

- Giardiasis Infection Presence-2 [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: No ]

Analyses done by ELISA assay kit (ProSpecT Giardia EZ, catalog #R2458596), absorbance (O.D)

- White Urine Cell Count [ Time Frame: 8th week of observation ]
  [ Designated as safety issue: Yes ]

White urine cell count (per field), to be determined in Biochemistry Laboratory in La Democracia Hospital, Quetzaltenango Guatemala.

| | |
|---|---|
| | • White Blood Cell Count [ Time Frame: 8th week of observation ]<br>[ Designated as safety issue: Yes ]<br><br>White blood cell count (cells/ul), to be determined in Biochemistry Laboratory in La Democracia Hospital, Quetzaltenango Guatemala. |
| **Original Primary Outcome Measures** ICMJE | *Same as current* |
| **Change History** | Complete list of historical versions of study NCT02203890 on ClinicalTrials.gov Archive Site |
| **Current Secondary Outcome Measures** ICMJE | *Not Provided* |
| **Original Secondary Outcome Measures** ICMJE | *Not Provided* |
| **Current Other Outcome Measures** ICMJE | *Not Provided* |
| **Original Other Outcome Measures** ICMJE | *Not Provided* |
| | |
| **Descriptive Information** | |
| **Brief Title** ICMJE | Normative State and Variation in Growth, Hematology, Hydration, Oxidation, Infection, Inflammation, Guatemalan Children |
| **Official Title** ICMJE | Study on the Normative State and Inter- and Intra-individual Variation in Growth, Hematology, Hydration and Markers of Oxidation, Infection and Inflammation in Preschool Children With a Similar Dietary Intake |
| **Brief Summary** | This study will provide the researchers with an opportunity for the investigation of the biomarkers and different human biology component variable interactions in children; information that can generate new correlations between markers not known at the moment.<br>Regarding their analysis, variation could have multiple connotations. A categorical that refers to a specific state, established with a diagnostic standard. And a numerical, referring to a range or the absolute value limits in a continuous spectrum; this research will allow us to express variability in the distance amplitude of the obtained values level, as well as classifications or categories previously established in the possible cases.<br>OBJECTIVES PRINCIPAL OBJECTIVE<br>To estimate the inter and intra-individual variety in a selected series or biological variables determinant of growth, body composition, infection, oxidation, inflammation and hydration, in preschool children sharing the same institutional-based diet.<br>SPECIFIC OBJECTIVES<br>• To determine the inter and intra-individual variation of growth in preschool children, with a similar diet, who attend Secretariat of Beneficial Works of the First Lady system daycare centers and to compare between semi-urban and rural.<br><br>• To describe the inter-individual variation of the red blood cells |

| | |
|---|---|
| | circulating mass in preschool children, with a similar diet, who attend day care centers and to compare between semi-urban and rural.<br>• To determine the inter-individual variation of systemic and intraintestinal oxidation in preschool children, with a similar diet, who attend daycare centers and to compare between semi-urban and rural.<br>• To describe the inter-individual variation of systemic and intraintestinal inflammation in preschool children, with a similar diet, who attend daycare centers and to compare between semi-urban and rural.<br>• To determine the inter and/or intra-individual variation of urinary tract infection and gastrointestinal parasite infestation in preschool children, with a similar diet, who attend daycare centers and to compare between semi-urban and rural.<br>• To describe the inter and intra-individual variation of the hydration status in preschool children, with a similar diet, who attend daycare centers and to compare between semi-urban and rural.<br>• To explore intra-individual associations in variables of the same research component or different component variables. |
| **Detailed Description** | Please note protocol is also available in Spanish. |
| **Study Type** [ICMJE] | Observational |
| **Study Design** [ICMJE] | Observational Model: Cohort<br>Time Perspective: Cross-Sectional |
| **Target Follow-Up Duration** | *Not Provided* |
| **Biospecimen** | Retention: Samples Without DNA<br>Description:<br>Urine, feces, saliva and blood |
| **Sampling Method** | Non-Probability Sample |
| **Study Population** | Apparently healthy children who attend 3 daycare centers (one semi-urban, one urban-marginal and one rural) from the Secretariat of Beneficial Works of the First Lady (SOSEP) system in the Western Highlands in Quetzaltenango, Guatemala. |
| **Condition** [ICMJE] | Healthy |
| **Intervention** [ICMJE] | *Not Provided* |
| **Study Group/Cohort (s)** | Apparently Healthy<br>Apparently healthy preschool children who attend 3 daycare centers of the Secretariat of Beneficials Works of the First Lady in Guatemalan Western Highlands |
| **Publications *** | Soto-Méndez M, Campos-Oliva R, Aguilera C, Solomons N, Schümann K, Gil A. Urinary osmolality of preschool children with a largely common weekday meal |

| | |
|---|---|
| | offering, from the western highlands of Guatemala. Spanish J Community Nutr. 20:13-9, 2014. |

*** Includes publications given by the data provider as well as publications identified by ClinicalTrials.gov Identifier (NCT Number) in Medline.**

| **Recruitment Information** | |
|---|---|
| **Recruitment Status** [ICMJE] | Completed |
| **Enrollment** [ICMJE] | 91 |
| **Completion Date** | November 2012 |
| **Primary Completion Date** | November 2012 (final data collection date for primary outcome measure) |
| **Eligibility Criteria** [ICMJE] | Inclusion Criteria:<br>• To be attending one of the SOSEP systems assigned daycare centers<br>• To maintain an attendance of 80% during the 8 weeks of the fieldwork<br>• Aged 23 to 84 months<br>• Being apparently healthy and with no dietary restrictions related to the foods offered with the SOSEP menu.<br><br>Exclusion Criteria:<br>• Children whose parents of caregivers did not sign a consent form<br>• Children who refused to participate in the study<br>• Children who refused to adhere to the collections routines. |
| **Gender** | Both |
| **Ages** | 23 Months to 84 Months |
| **Accepts Healthy Volunteers** | Yes |
| **Contacts** [ICMJE] | *Contact information is only displayed when the study is recruiting subjects* |
| **Listed Location Countries** [ICMJE] | Guatemala |
| **Removed Location Countries** | |

| **Administrative Information** | |
|---|---|
| **NCT Number** [ICMJE] | NCT02203890 |

| | |
|---|---|
| **Other Study ID Numbers** [ICMJE] | CeSSIAM_02 |
| **Has Data Monitoring Committee** | No |
| **Responsible Party** | María José Soto, Center for Studies of Sensory Impairment, Aging and Metabolism |
| **Study Sponsor** [ICMJE] | María José Soto |
| **Collaborators** [ICMJE] | Universidad de Granada |
| **Investigators** [ICMJE] | Principal Investigator: Maria J. Soto-Méndez, MSc — Center for Studies of Sensory Impairment, Aging and Metabolism |
| **Information Provided By** | Center for Studies of Sensory Impairment, Aging and Metabolism |
| **Verification Date** | July 2014 |

[ICMJE] **Data element required by the International Committee of Medical Journal Editors and the World Health Organization ICTRP**