

RESEARCH ARTICLE

# 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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## Abstract

### Background

Undernutrition and inflammation are related in many ways; for instance, non-hygienic environments are associated with both poor growth and immunostimulation in children.

### Objective

To describe any existing interaction among different inflammation biomarkers measured in the distinct anatomical compartments of whole blood, feces, plasma and saliva.

### Methods

In this descriptive, cross-sectional study, samples of whole blood, feces, plasma and saliva were collected on the 8<sup>th</sup> 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- $\alpha$ . 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.

marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

## 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 significant  $r$  value, whereas 7 of 10 possible associations among plasma cytokines were significant. Associations 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 anatomical compartment separately, IL-6, IL-8 and TNF- $\alpha$  emerged as predictors in plasma ( $r^2 = 0.514$ ) and IL-1B, IL-8 and TNF- $\alpha$  remained as independent predictors in saliva ( $r^2 = 0.762$ ). 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 specific 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-inflammatory 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 life [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

inflammatory 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 inflammation, 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 refferent 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](https://clinicaltrials.gov) as NCT02203890.

## Anthropometric Measurements

We performed anthropometric determinations during the last 7<sup>th</sup> week of each daycare 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 Frankfurt 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 adjustment 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 daycare 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 disposable 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 Cryogenic 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 interleukin-1-beta (IL-1B), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF- $\alpha$ ).

**Fecal samples.** In the 7<sup>th</sup> 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 daycare 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<sup>3</sup> volume.

**Fecal Calprotectin.** Assays were performed in Quetzaltenango, Guatemala. ELISA assays were executed using the CALPRO Calprotectin ELISA Test from CALPRO AS (Lysaker, Norway). 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- $\beta$ , IL-6, IL-8, IL-10 and TNF- $\alpha$ . 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.

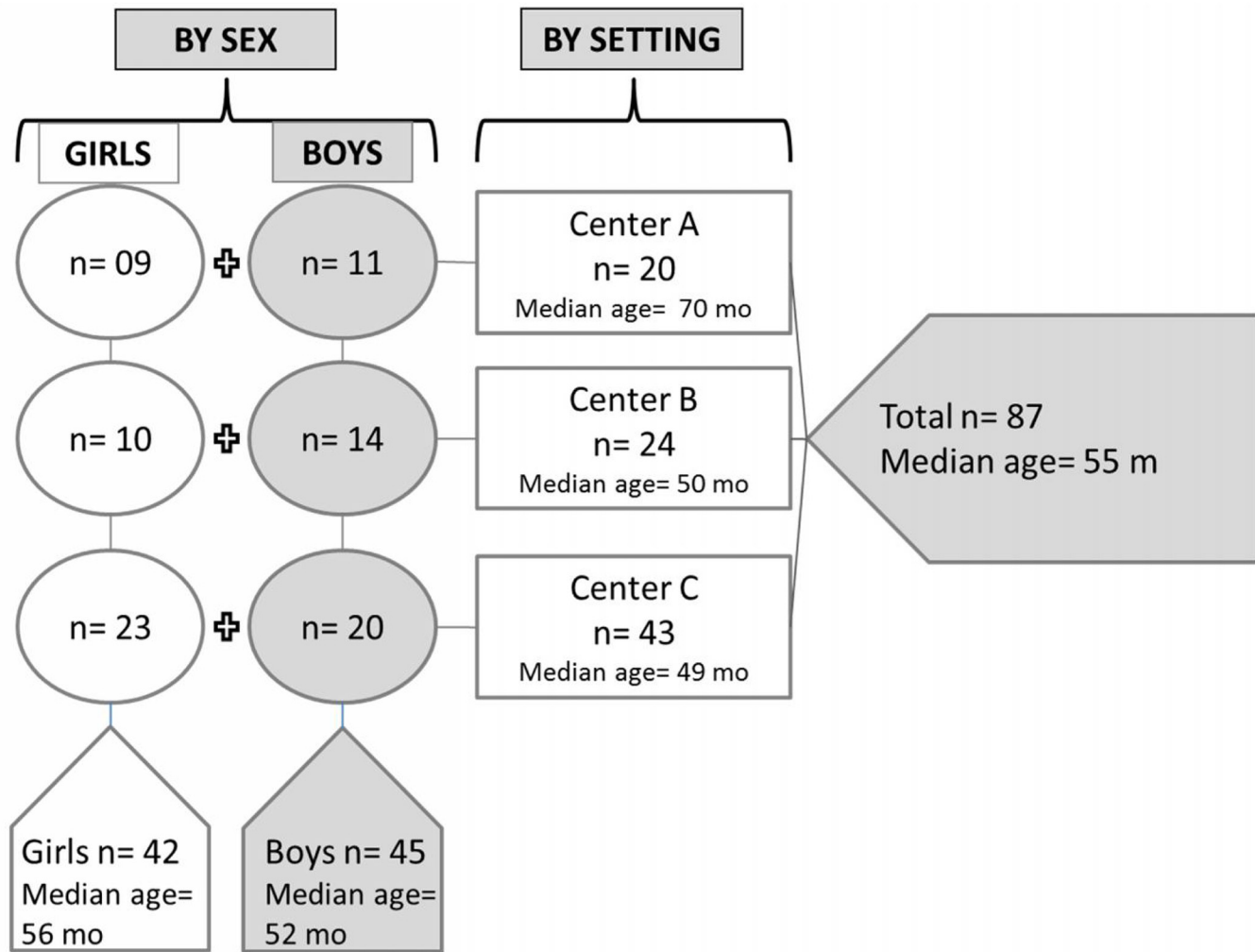
## Data Handling and Statistical Analyses

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  $\leq 0.05$  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  $54 \pm 16$  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](#)).



**Fig 1. Characteristics of the Subjects.** Legend: 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.

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

Setting	N	Height-for-Age (stunting)	Weight-for-Age (underweight) Z-score (% subnormal)	Weight-for-Height (wasting)
Total Sample	87	-2.39 (66%)	-1.37 (23%)	-1.00 (2%)
Semi-Urban (A)	20	-1.61 <sup>a</sup> (35%)	-0.96 <sup>a</sup> (0%)	-0.10 (0%)
Marginal-Urban (B)	24	-2.42 <sup>ab</sup> (63%)	-1.46 <sup>b</sup> (17%)	-0.16 (4%)
Rural-(C)	43	-2.73 <sup>b</sup> (81%)	-1.47 <sup>b</sup> (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

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		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- $\alpha$ (pg/mL)	Salivary IL-10 (pg/mL)	Salivary IL-1B (pg/mL)	Salivary IL-6 (pg/mL)	Salivary IL-8 (pg/mL)	Salivary TNF- $\alpha$ (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- $\alpha$ [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- $\alpha$ [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	

**Fig 2. Spearman Correlation Coefficient Hemi-Matrix for Inter-Relationships of Biomarkers.** Legend: 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.

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### Descriptive statistics of the inflammatory 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- $\alpha$ ; IL-8; IL-6; and IL-1B. For saliva, corresponding values are: IL-8; IL-10; IL-1B; IL-6; and TNF- $\alpha$ . Only IL-8 and IL-1B had higher concentrations in saliva as compared to plasma, whereas IL-10; IL-6 and TNF- $\alpha$  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 y 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 significantly associated at the level of  $p \leq 0.05$ . In the face of multiple comparisons with this number

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

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

x = manufacturer’s suggested cut-off for normal calprotectin

y = published normative post-menopausal females’ for cytokines [Browne et al., 2013], expressed as 25<sup>th</sup> and 75<sup>th</sup> percentiles

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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<sup>-3</sup>. The lowest r values still reaching the criterion for significance were a pair at 0.222 and 0.226 involving paired 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-fit 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 in saliva. Only in the IL-10 association with IL-6 did the curve-forms differ between anatomical compartments: for plasma, the form was cubic and for saliva, it was the power form. In this instance, plasma showed the stronger association.



**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- $\alpha$	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- $\alpha$	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- $\alpha$	0.390	0.378	Sigmoid
Plasma IL-8	Plasma TNF- $\alpha$	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- $\alpha$	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- $\alpha$	0.780	0.872	Cubic
Salivary IL-6	Salivary IL-8	0.657	0.707	Sigmoid
	Salivary TNF- $\alpha$	0.616	0.635	Power
Salivary IL-8	Salivary TNF- $\alpha$	0.871	0.854	Compound

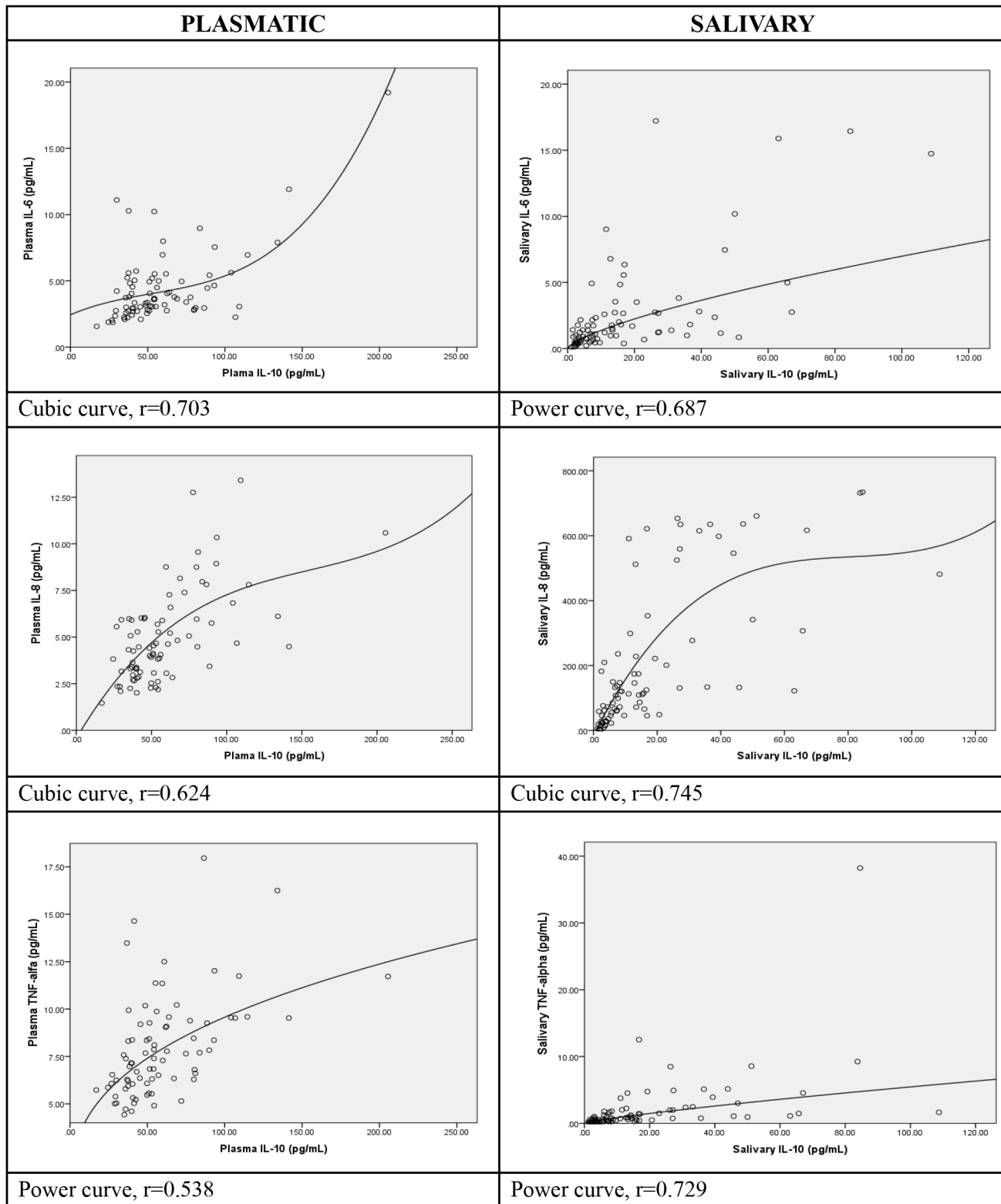
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### Associations by Backwards-Elimination Multiple-Regression modeling

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-inflammatory) cytokines in the series. Restricted to the domain of only the plasma cytokines, the regression modeling produced the parsimonious model with an  $r^2$  value of 0.514, with IL-6, IL-8 and TNF- $\alpha$  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- $\alpha$ , IL-6, IL-8; and salivary IL-1B. On the salivary IL-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- $\alpha$  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



**Fig 3. Selected Associations between Variables with the Superimposition of the Goodness-of-Fit Curve Form.** Legend: Six examples of binary associations between inflammatory biomarker variables with application of the Goodness-of-Fit regressions 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 ( $r$ ).

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**Table 4. Coefficients of the multiple regression model for the dependent variables plasmatic and salivary IL-10 as independent variables pro-inflammatory cytokines, measured in the same body element (IL1B, IL-6, 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
<b>Dependent variable: Plasmatic IL-10*</b>								
2	(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
	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
<b>Dependent Variable: Salivary IL-10**</b>								
2	(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
	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

\*r<sup>2</sup> = 0.514; r = 0.717 (n = 80)

\*\*r<sup>2</sup> = 0.762; r = 0.873 (n = 79)

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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, but followed the pattern of decreased stature. Wasting was virtually non-existent.

### Comparative analysis of biomarker distributions

Leukocytosis is an elevation of the WBC above 10,500/mm<sup>3</sup>. 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 inflammation [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 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- $\alpha$ . In general, with some exceptions, 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 compartment. 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- $\alpha$  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 postmenopausal 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-Bostrán 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- $\gamma$ , 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- $\alpha$  [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 coefficient 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 response 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- $\alpha$ . 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- $\alpha$ , 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- $\alpha$  and INF- $\gamma$  [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 additional 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- $\alpha$  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

**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.

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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.**  
(DOC)

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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 work: MJSM MERA.

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