ASSOCIATIONS BETWEEN DEMOGRAPHIC FACTORS AND FECAL CALPROTECTIN IN THE INDIGENOUS SHUAR OF ECUADORIAN AMAZONIA: A WINDOW INTO INFLAMMATORY BOWEL DISEASE (IBD)

by

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Associations Between Demographic Factors and Fecal Calprotectin in the Indigenous Shuar of Ecuadorian Amazonia: A Window into Inflammatory Bowel Disease (IBD)

Approved: _____

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Previous research has shown that autoimmune disorders like inflammatory bowel disease (IBD) are on the rise in wealthy nations such as the United States. However, virtually no research on IBD or its precursors, has been conducted in less wealthy nations. The present study addresses this issue by looking at demographic trends in levels of fecal calprotectin (FC), a biomarker of intestinal inflammation closely related to IBD, in a sample (n=79; 37 males, 42 females) of Shuar forager-horticulturalists from Ecuadorian Amazonia. There was a significant negative relationship between FC and age (p=0.04) (as age increased, FC decreased), not a curvilinear relationship as would be seen with IBD (where FC is high in childhood and late adulthood, and low in the middle of life). T-test results provided no evidence of a sex difference in mean FC levels (t=-1.14, *p*=0.259), and F-test results provide no evidence of sex differences in FC variance (ratio=1.15, p=0.66). These results indicate that Shuar had lower levels of FC in older age, whereas with more industrialized nations there is a curvilinear age pattern with an increase of FC in old age (50+ years old). This suggests that FC levels in this setting are not driven by IBD, but instead by other factors, perhaps parasite burden. Further study in what these other factors are should be done to better understand how they shape IBD pathology in industrializing nations.

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INTRODUCTION

In recent decades, autoimmune diseases have become a major focus of Western biomedicine as wealthy nations are experiencing an unprecedented rise in prevalence and incidence of these diseases (Cooper, Bynum, & Somers, 2009). Since the 1950s, the global rates of type 1 diabetes, asthma, Crohn's disease, and multiple sclerosis have increased by 300% or more (Lerner et al., 2016; Scudellari, 2017). It is impossible for a genetic predisposition to sweep through the worldwide population in this time frame, however there may be some genetic component with regards to susceptible individuals (Lerner et al., 2016). This suggests that environmental factors play a primary role. While the exact mechanisms are not completely understood, some of these proposed environmental factors include shifts in childhood animal exposure, family size, and exposure to immunoregulatory microbes, to name just a few possibilities (Rook, 2012; Scudellari, 2017). Hundreds of studies have confirmed that autoimmune diseases are on the rise in wealthy nations, and risk factors for these diseases are related to wealth and extent of economic development. Normally, in epidemiological research it is a search for new variables that contribute to an outcome; however, evidence suggests it is the *absence* of immunoregulatory interactions with our environment that may play a primary role in the rise in autoimmune diseases.

The Hygiene Hypothesis appeared first in 1989 and came about as a result of a suspicion that reduction in infections (like mumps, measles, and tuberculosis) and an increase in hygienic behavior associated with economic development might be creating a dysregulation in the human immune system, resulting in increased incidence of allergies and asthma (Martinez & Holt, 1999; Strachan, 1989; von Mutius, 2007). These

previously cited studies make up the foundational work done on the Hygiene Hypothesis. One such study in 1999 hypothesized that delayed maturation of immune responses due to improvements in hygiene and decreased microbe (specifically a bacterial endotoxin) exposure put children at higher risk for allergies and asthma (Martinez & Holt, 1999). Another study found that the post industrialized rise in hay fever was related to decreased family size, and higher standards of hygiene, suggesting that allergic diseases were prevented by infection in early childhood from infected siblings or prenatal infections from mother to child (Strachan, 1989). Though it was not until later, however, that a mechanism was proposed to explain the Hygiene Hypothesis.

The molecular mechanism proposed for the Hygiene Hypothesis was the activation of T helper 1 (Th1) cells by bacteria, protozoa, and endotoxin that led to the decrease in T helper 2 (Th2) cells, an indicator of allergic disorder (Scudellari, 2017). Martinez and Holt hypothesized that a bias towards a Th2 immune response in childhood would increase allergen sensitivity as well as allergic reactions, asthma, and immune dysfunction (Martinez & Holt, 1999). This meant that for children living without infections, their Th1 counts would be low and subsequently their Th2 counts would be high. However, it was later discovered that children with high levels of infections (specifically from helminths [parasitic worms]) still had high Th2 counts, so this was not the likely mechanism explaining the cause of the immune dysregulation (Scudellari, 2017). In the years to come, this mechanism and hypothesis was revised and is now commonly referred to as the Old Friends Hypothesis.

In 2003, Rook proposed a new idea as to why asthma and allergies were on the rise, which he called the Old Friends Hypothesis (Rook et al., 2003). He hypothesized that it was not the reduced infection exposure in children, but instead the pattern of exposure to their microbial environment that was dysregulating their immune systems (Scudellari, 2017; Rook et al., 2003). The 'Old Friends' referred to the immunoregulatory microbes that were present in most past populations and those contemporary populations living outside wealthy countries. This work also expanded the disorders from the Hygiene Hypothesis to include those in the umbrella category of autoimmune disease. Hygiene is still important in this hypothesis; however, Rook hypothesized that radical changes to early life behavior– e.g., decrease in animal cohabitation, increased Cesarean section birth, decreased environmental microbe transmission, and early antibiotic use—has put humans at increased risk for autoimmune disorders such as multiple sclerosis and Type 1 diabetes, among others (Rook, 2012). The Old Friends Hypothesis posits that loss of interaction with microbes and organisms from mud, animals, and feces that were present in earlier eras and in different environmental contexts has disrupted the critical immunoregulating interactions with these factors.

The Old Friends Hypothesis recognizes overarching shifts in the historical pattern of disease burden, sometimes referred to as epidemiolocal transitions. This term refers to the watershed moments in human evolutionary history where mortality and morbidity have been altered significantly due to some sort of societal change, technological improvement, or other factor. The first epidemiological transition occurred about 10,000 years ago with the advent of agriculture and animal husbandry;

this is known as the Neolithic transition and led to a more sedentary lifestyle with permanent settlements (Barrett et al., 1998; Rook, 2010). Living in permanent settlements increased frequency of inter- and intra-personal contact and the accumulation of human waste, which led to increased acute infections and dispersal of macro parasites and gastrointestinal infections (Barrett et al., 1998). Along with increased infections, animal cohabitation led to increased transmission of zoonoses (Barrett et al., 1998). The second epidemiological transition began in North America and Europe in the mid-19th century, roughly coinciding with the latter part of the Industrial Revolution (Barrett et al., 1998). From this transition, mortality due to infectious disease decreased as a result of pasteurization, education, improved public hygiene, and medical science advancements, among other things (Woods, 1991). Changes in environmental exposure to immunoregulatory microbes and microorganisms, especially during development, are most likely contributing to this rise in chronic inflammatory disorders and autoimmune diseases in the 20th century and today (Rook, 2010; Rook, 2012). Among these vital organisms and microbes are soil transmitted helminths (*Trichuris suis)*, bacteria (*H. pylori* or *Salmonella),* and protozoa (*Toxoplasma gondii)* (Rook, 2010). It might seem counterintuitive that living a more sanitized, seemingly healthier childhood and life would lead to more sickness, but current research (Fleming et al., 2017; Shepherd, Wangchuk, & Loukas, 2018; Smallwood et al., 2017) suggests that lost contact with heirloom organisms and microbes could be the cause of this increase of people living with autoimmune diseases.

Some examples of the myriad autoimmune diseases afflicting wealthy nations such as the United States include lupus, celiac disease, inflammatory bowel disease, and

type 1 diabetes. Between 1980 and 1994, the incidence of type 1 diabetes increased by 75% in the US, and this was not related to pollution levels. During the same period, type 1 diabetes prevalence remained relatively unchanged in Low and Middle-Income Countries (Zuk, 2008). Among the many categories of autoimmune diseases increasing worldwide, gastrointestinal autoimmune diseases have seen a particularly rapid rise (Lerner et al., 2016). Globally in the last 30 years, rheumatic, endocrinological, and gastrointestinal autoimmune disease has increased annually by 7.1%, 6.3% and 6.2% respectively (Lerner et al., 2016). A common form of gastrointestinal autoimmune disease is inflammatory bowel disease (IBD). This diagnosis that has no cure, afflicts many, and most likely has environmental triggers related to societal and environmental changes associated with the epidemiological transition (Matsuoka et al., 2018; Molodecky et al., 2012).

Inflammatory Bowel Disease and the Role of the Environment

Inflammatory Bowel Disease (IBD) is the umbrella category comprising ulcerative colitis (UC) and Crohn's disease (CD). The cause of IBD remains unknown but a combination of genetics and environmental factors are thought to contribute to the development of this disease, as will be discussed in detail below. UC is a non-specific inflammatory disease that impacts colonic mucosa near the rectum, resulting in the formation of ulcers and/or erosions (Matsuoka et al., 2018). CD impacts several regions in the digestive tract, most commonly the intestines and perianal region, presenting as fistulas and/or transmural granulomas (Matsuoka et al., 2018). IBD appears to become more prevalent as societies become wealthier, making this a largescale issue and public

health matter that needs further research to identity what environmental factors can shape its onset.

IBD is a global disease with the highest prevalence in wealthy nations, but IBD has also emerged in newly industrializing countries in Asia, the Middle East, Africa, and South America and its incidence is rising dramatically (Molodecky et al., 2012). Since 1990, the incidence of Crohn's disease has increased by 11.1% in Brazil and 4% in Taiwan, while in the same period, the incidence of ulcerative colitis increased by 14.9% in Brazil and 4.8% in Taiwan (Ng et al., 2017). This increase in areas that are experiencing lifestyle change associated with economic development suggests that development of IBD is driven primarily by environmental factors. Although increased reporting and awareness of UC and CD may have contributed to some of the sharp increase over the 20th century, IBD has been reported to be increasing in prevalence in over 200 epidemiologic studies (Kaplan & Ng, 2017). Research has been done to identify exactly what has changed to increase the prevalence and incidence of this disease.

Another study found that better living conditions during childhood are associated with increased risk for IBD while childhood respiratory infection and gastroenteritis were protective factors in an urban population in Spain (López-Serrano et al., 2010). Some studies have tried to explain the increase of IBD with lack of exposure to infectious agents, suggesting that patients are developing IBD as a result of not developing conditions such as *Helicobacter pylori* gastritis or intestinal parasite infections (Loftus Jr., 2004). These studies have added to the evidence supporting the Old Friends and Hygiene Hypothesis, as they show how improved living conditions and

decreased childhood infection by bacteria and parasites have likely contributed to the rise in this autoimmune disease. Parasitic infections by helminths in particular have been a major topic of investigation—this includes potential use in therapies for treating the symptoms of IBD. Humans and helminths have co-existed throughout our evolutionary history (Cox, 2002). However, due to widespread use of anthelmintic (helminth targeting) drugs and WHO initiatives in the 1950s, there has been a dramatic reduction in intestinal helminth infections in wealthy nations (Shepherd et al., 2018).

Since immune-mediated diseases like IBD are the result of dysregulated inflammation in the intestines, and intestinal worms produce anti-inflammatory factors to remain disguised from the host's immune system, treating IBD patients with intestinal parasites is being tested in clinical trials. Work on animal models (such as the rag mouse, *Mus musculus*) demonstrate that intestinal helminth infections inhibit development of intestinal inflammation and so began the first human trials with the pig whipworm *(Trichuris suis)* (Helmby, 2009, 2015). *T. suis* eggs administered every few weeks in an oral solution only produced weak reduction in symptoms for Crohn's and ulcerative colitis patients; thus a new, more invasive approach with hookworm (*Necator americanus)* was pursued (Helmby, 2015). Further clinical trials are expected, but early results indicate symptoms improving for Crohn's patients infected with 25-50 hookworm larvae (Helmby, 2015). However, it is a delicate balance between suppressing IBD symptoms and preventing the onset of negative health effects related to high levels of helminth infection like nutritional deficiency and respiratory issues.

These diverse lines of evidence suggest that decreased infections by intestinal helminths are associated with the rising prevalence of IBD, which is important for

populations that have not transitioned to a low infectious/parasitic burden lifestyle as they may have protection from IBD. Despite numerous publications about IBD, there still exists a need for research on the incidence and prevalence of IBD in less wealthy countries going through economic development and lifestyle change. Additionally, fewer studies have examined patterns of intestinal inflammation in poorer nations also experiencing high rates of pathogen exposure.

To address this gap in the understanding of autoimmune disease and market integration, the Shuar Life History Project (SHLHP) has been researching the Shuar as they undergo market integration to understand how this impacts human health, including immune dysregulating disorders. This research concentrates on the Shuar, an indigenous subsistence population that experiences high levels of parasitic infection, common to most low income non-urban settings (Cepon-Robins et al., 2014; Gildner et al., 2016). Additionally, this population and other poor populations in Latin America have high rates of neglected tropical diseases (NTDs) like dengue, schistosomiasis, leishmaniasis, trachoma, leprosy, and lymphatic filariasis (Hotez et al., 2008).

Because the symptoms and experiences of IBD vary widely patient to patient and the onset of symptoms can take many years, diagnosing this disease requires several components. This diagnosis usually requires a colonoscopy, radiological work and/or a test for biomarker levels, among other components (D'Angelo et al., 2017). This biomarker is called fecal calprotectin (FC), and the levels present in a sample can be used to determine levels of intestinal inflammation and investigate IBD to track its global reach.

Fecal Calprotectin and Population Screening

The present study is the first of its kind examining FC in an indigenous population in the process of rapid changes from a forager-horticulturalist lifestyle to a more marked integrated lifestyle. Investigating how this population is experiencing intestinal inflammation can help scientists better understand how undergoing epidemiological transitions and market integration (as many poor nations currently are) influence the emergence of IBD. As the global prevalence and incidence of IBD continue to rise, new populations will be put at risk and identifying the environmental factors responsible will help in protecting future generations. The present study is the beginning of further research that will hopefully find those factors. Thus, this study will begin this process by examining population trends found with FC levels. For wealthy nations, it has been found that FC levels are high in childhood (Fagerberg et al., 2003), low in early adulthood but then towards late adulthood FC levels starts to increase again (Poullis et al., 2004).

In order to study FC in this less wealthy setting, age and sex was examined using population trends of FC from the available literature. Comparative data for the national prevalence or incidence of IBD in Ecuador is unavailable, so a comparison with wealthy nations will be used to provide a general trend of what would be predicted if IBD was driving the intestinal inflammation present. A study done on children aged 1-18 months in Shanghai demonstrated a decline in FC levels from birth, with the highest mean being 375.2 μg/g for age 1-3 months (Li et al., 2015). A UK study found that children aged 1-3.9 years had significantly higher levels of FC than adults, while there was no significant difference between older children and adults (Davidson &

Lock, 2017). Another UK study of older adults from south London found a positive relationship between FC and older age (all participants were over 50 years) in a random population (Poullis et al., 2004). The trends in these studies were found cross-sectionally, just as the present study was composed. This suggests that for random populations from wealthy nations, FC levels are high at birth, then decline into adulthood and increase again into late adulthood.

Another important finding is that among subsistence populations (including the Shuar) it has been found that helminth burden is higher in sub-adults and subsequently decreases with age (Gildner et al., 2016). Children in these low income, high parasite burdened populations are exposed to high levels of parasitic infections at early ages. Parasite levels decrease over time in populations similar to the Shuar, which have a high parasite burden (Blackwell et al., 2011). The peak-shift model predicts that populations with high exposure to parasite infection develop immunity earlier and transmissions rates decline over time as partial immunity is acquired by these groups (Blackwell et al., 2011). If intestinal inflammation is primarily driven by parasite burden, FC levels should exhibit a similar pattern of age-related decline. However, if FC levels of the Shuar are driven by IBD-related processes, FC levels would be expected to increase with age as demonstrated for wealthy populations (Poullis et al., 2004; Joshi et al., 2010). Looking at what differences between age groups might (or might not) exist in the sample population will lend a view into if the intestinal inflammation detected in this group is due to IBD related processes or something else like parasite infection. I produced a flowchart outlining the factors that play a role in the increase of risk of inflammatory bowel disease in wealthy nations (see figure 1). This flowchart is a

general overview and is not the entire list of factors that could be contributing to the rise in IBD, however helps demonstrate the issue and how the factors relate.

Sex Difference and Immunoglobulin E

The available variables for the samples in the present study are age, FC levels, location of community, and sex. Since nearly all participants were from the same community, sex (along with age) was analyzed to understand if intestinal inflammation present was due to IBD-related processes or something else. The available literature is unclear on if IBD prevalence and mean FC values are affected by sex (Poullis et al., 2004; Liu et al, 2012; Yang et al., 2001; Zelinkova & van der Woude, 2014). Because of this, we turned to another biomarker to develop predictions about sex differences in fecal calprotectin.

Immunoglobulin E (IgE) is a biomarker related to parasite burden. In populations of wealthy nations, IgE is found in very low levels as there are low levels of parasitic infection (Blackwell et al., 2011). High levels of IgE indicate a resistance that is acquired with age after repeated parasite infection (Cepon-Robins et al., 2018). An unpublished cross-sectional Shuar study has found a negative association between FC and IgE, demonstrating that IgE increases with age-acquired helminth immunity while FC declines (Cepon-Robins et al., 2018). Ultimately this finding is important because it demonstrates that FC and IgE might be inversely related and justifies why testing sex difference would lend insight into the presence of IBD related intestinal inflammation in this population.

It has been found that men have higher IgE levels than women in studies on subsistence populations like the Shuar possibly due to cultural factors related to

helminth exposure (De Paula Couto et al., 2014; Blackwell et al., 2011). The possible mechanism responsible for this sex difference in IgE could be due to women being more likely to be infected with *A. lumbricoides*, which may be related to cultural practice and societal differences present (Blackwell et al., 2011). If FC follows a similar sex trend as demonstrated by IgE, this would indicate a likely association between intestinal inflammation and parasitic infection, rather than IBD related pathology. Looking at the sex trend, along with age, will help in understanding if intestinal inflammation detected in this population is due to parasitic infection or IBD.

HYPOTHESES

Hypothesis 1: Levels of FC among Shuar will exhibit a different age-related pattern than wealthy populations due to differences in lifestyle and infectious disease exposure; meaning a high, low, high pattern with age will not be present.

If intestinal inflammation in less wealthy settings exhibits the same age trend as FC levels in wealthy settings, then intestinal inflammation is likely driven by IBD-related processes. If intestinal inflammation is primarily driven by parasite burden or some other pathology present in the Shuar that is not IBD, FC levels should exhibit a pattern different from the high, low, high (curvilinear) pattern in wealthy populations (Poullis et al., 2004; Fagerberg et al., 2003).

Hypothesis 2: Following a pattern similar to sex differences in IgE, Shuar men will exhibit higher FC levels than women as women are more likely to be infected with A. lumbricoides.

If men exhibit higher FC levels than women, then FC sex differences follow a similar trend to IgE meaning intestinal inflammation present could be due to parasitic infection rather than IBD related pathology. Since women are more likely to be infected with *A. lumbricoides*, a species soil transmitted helminth, men may have higher IgE levels possibly due to additional resistance to this specific helminth (Blackwell et al., 2011). If there is no sex difference detected, FC levels and IgE may not be closely related and/or high levels of intestinal inflammation could be due to IBD or a combination of IBD and parasitic infection.

MATERIALS AND METHODS

Study Population

As the second largest indigenous population in Ecuador, there are somewhere between 40,000 to 100,000 Shuar living in Ecuador today, mostly in the southeastern region of the country (Urlacher et al., 2016). The Shuar are largely a natural fertility population with an average age at menarche of 13 years, average age of first parturition of 17.5, and 8.4 average live births per woman (Madimenos et al., 2012). They rely largely on human-powered subsistence activities such as fishing, hunting, foraging, and horticulture (Liebert et al., 2013). Previous research indicates that among the groups living in this region, 44-65% are infected with at least one soil transmitted helminth (STH) species (Gildner et al., 2016). There may be lifelong consequences for high levels of parasite infections during childhood such as cognitive impairment and growth stunting (Gildner et al., 2016). Shuar communities are undergoing rapid and uneven market integration, with increasing rates of participation in wage labor, sale of agricultural and pastoral products, and consmumption of market goods (Urlacher et al., 2016; Liebert et al., 2013).

The Shuar Health and Life History Project (SHLHP; http://www.bonesandbehavior.org/shuar/) is a collaborative research effort that focuses on the indigenous Shuar of Ecuador and involves scientists from several US universities, Ecuadorian health providers, and Shuar colleagues. The project examines markers of immune function and inflammation such as IgE to better understand the regulation of inflammation. The results from this project have important implications

for research on inflammation and diseases of aging worldwide. Researchers from the SHLHP have expanded the view of the extent of health change with market integration and have advanced human biology methods necessary for such research. Research results are regularly presented to Ministry of Health colleagues and the Shuar Federation Health directorate as well as to the participant communities in order to assist the development and dissemination of public health policy and practices.

Study Participants

In 2016, SHLHP researchers collected data for this study from Shuar living in the neotropical rainforests of the Cross-Cútucú region of Morona Santiago Province, southeastern Ecuador (Figure 2). The Shuar in the Cross-Cutucú are less market integrated, where they still rely mostly on traditional practices for daily subsistence (Gildner et al., 2016). Not included in this study are the Shuar in the Upano Valley that are more market-integrated, where traditional horticulture is supplemented with agro-pastoral production for sale at markets (Gildner et al., 2016).

There were a total of 79 participants in the present study, including 37 males and 42 females aged from 0 to 75 years. The mean participant age was 22 years. Age was determined for Shuar children with health clinic and school records that provided birth dates accurate to the month. For Shuar adults, government identification was cross-checked with genealogical information from SHLHP (Blackwell et al., 2011; Gildner et al., 2016). Permission to conduct this study was obtained with informed verbal consent provided by participants, and for participants under the age of 15 (the local consent age) child and parental verbal consent was obtained (Gildner et al., 2016). The study and procedures for consent were approved by the University of Oregon Institutional Review

Board and were endorsed by the *Federación Interprovincial de Centros Shaur* (FICSH) and local leaders.

Fecal Calprotectin Levels

Calprotectin is a 36 kDa neutrophil-derived protein that can be measured in fecal samples to detect intestinal inflammation and diagnose IBD (D'Angelo et al., 2017). In clinical diagnosis, cutoff values of FC levels are used to identify intestinal inflammation. Samples with an FC level <50 μg/g are negative, FC level between 50 - 100 μg/g are weakly positive for intestinal inflammation, and those with an FC level > 100 μg/g are strongly positive for intestinal inflammation (D'Angelo et al., 2017). Fecal calprotectin level concentrations are just one component of the multidisciplinary diagnostic tests required for diagnosing IBD; this study is not finding the prevalence of IBD in this population rather measuring the level of intestinal inflammation present.

Fecal samples collected in Ecuador were frozen immediately and then shipped via courier service to the Global Health Biomarker Lab at the University of Oregon, where they were stored at -28°C until analysis. The Buhlmann CALEX® Cap Device was used to extract exactly 10 μL of fecal material from each sample (lot number 0442N, kit number B-CALEX-C200-U). Buhlmann fCAL ELISA was used to measure calprotectin levels (lot number 46120.X.U, kit number EK-CAL-U). FC level is reported as μg FC per gram of feces (μg/g). Samples with FC levels above the limit of detection were re-run at a 1:10 dilution. High and low controls were within the expected range and R^2 for the PL standard curve was >0.95 for every plate run. Each fecal sample was labeled with a participant identification number (PID), which can be used in a database to search for participant info (age, sex, location of household, height, weight,

etc.). However, these additional data were not available for a large enough portion of subjects, limiting our analysis to age and sex, which was available for all samples.

Statistics

Statistical analyses were run in R-Studio version 1.1442. As with most biomarker data, the raw values were not normally distributed. FC levels were normalized by natural log-transformation for analysis (logFC). A sex difference was tested for in the means and variances of logFC using an independent samples t-test and an F-test. An ordinary least squares multiple regression model regressing age and sex on logFC was also done. To test whether there was a curvilinear relationship between age and logFC, an additional model including age squared in the model was specified, in addition to the covariates in the previous model.

RESULTS

Descriptive Statistics

Using the clinical cutoff values for IBD (D'Angelo et al., 2017), 50 individuals were negative for intestinal inflammation (FC level <50 μg/g), 21 individuals were weakly positive for intestinal inflammation (FC level between 50 - 100 μg/g), and 8 individuals were positive for intestinal inflammation (FC level > 100 μg/g). From this it was determined 37% of the population was positive for intestinal inflammation (combined weak and strong categories) while the majority of the population did not have any kind of intestinal inflammation.

Sex Difference

T-test results provided no evidence of a sex difference in mean logFC levels (t=-1.14, p=0.259), and F-test results provide no evidence of sex differences in logFC variance (ratio=1.15, p=0.66). Table 1 shows women and men divided into three age groups (0-14 years, 15-39 years, and 40+ years) and shows no significant difference between sex, age group, or FC (raw or log transformed). Figure 4 shows a violin plot of the distribution of log transformed FC and sex.

Age Trend

In the first regression model, there was no association between age and log FC (β=-0.01, SE=0.009, p=0.23), or sex and logFC (β=0.38, SE=0.32, p=0.024). However, in the third model adding a term for age squared, older age was associated with reduced logFC (β=-0.05 SE=0.03, p=0.04), and the coefficient for age squared approached significance, though the effect size was small (β=0.0006, SE=0.0003, p=0.09). The

graph of age and FC levels can be seen in Figure 3. This was produced using R-studio in a regression model with an age variable while controlling for sex.

DISCUSSION

This study examined the relationships among FC levels, age, and sex in a population with high parasite burden but also rapid market integration, in order to gain a new perspective on IBD in economically developing nations. 79 participants were included in the study, with a mean age of 22 years and an age range of 0-75 years old. It was found that there was no high, low, high pattern between age and FC as there is with populations in wealthy nations. No sex difference was detected in this population in FC, which is consistent with the IBD related literature; however, the violin plot shows a difference in distribution indicating more females above and below the mean than clustered by the mean, whereas for men the curve had a normal bell shape (see Figure 4).

Hypothesis 1 predicted the Shuar would exhibit a different FC pattern than wealthy populations over time. Our results support this hypothesis, as there was a significant negative relationship between log FC and age squared ($p=0.04$), not a high, low, high relationship as would be represented by IBD. Children had much higher levels of intestinal inflammation than adults (see Table 2) and FC levels were lower in older age groups. Participants in adulthood and beyond did not have higher values than those individuals younger than them, this suggests a different pattern from wealthy populations experiencing high prevalence of IBD. Further research with a larger sample and with comparisons between the Upano Valley and Cross-Cutucú are key to understanding how market integration may be increasing IBD in this population, and if groups that are simultaneously market integrated but maintain helminth exposure still have this age-related decline.

Hypothesis 2 predicted men would have higher levels of FC, following the same trend of IgE. This hypothesis was not supported, as men did not have a significantly higher FC mean or variance than woman; in fact, no significant difference was detected. While this does not support the idea that FC would follow the same age trend as IgE because both are related to intestinal inflammation, the violin plot suggests a different distribution for women than men (refer to figure 4). Further research should be done as to why men would have a more bell-shaped distribution than women of FC levels, possibly in comparison with IgE level violin plot distributions.

Different Pattern of Age and FC

While the association between age and FC is generally curvilinear in Western populations, the present study found a different age trend with some evidence of a linear decline in logFC with age among the Shuar. This means that the first hypothesis was supported, though stating it is a clear linear decline is inaccurate. I would speculate that this age patterning has to do with intestinal inflammation being regulated by early and intense parasite infection resulting in FC levels being low early and staying low as immunity is acquired and inflammation is suppressed by anti-inflammatory substances secreted by STHs. In the data, the three participants with the highest FC levels (797.6, 832.79 and 896.72 μg/g) were 4,1, and 21 years old respectively with the closest 4th highest value being 255 μg/g as seen in Table 2. This is consistent with the trend of younger, more parasite infected participants having higher FC than adults with immunity to these parasites. Previous studies among the Shuar have reported age-related declines in parasite burden (Blackwell et al., 2011). Thus, our findings are consistent with the argument that FC levels among the Shuar may be primarily driven

by parasite exposure, rather than idiopathic intestinal inflammation as with wealthy nations. This study shows that intestinal inflammation in a rapidly market transitioning society is nuanced and is not as clearly related to age and lifestyle changes as in wealthy populations.

No apparent sex differences in fecal calprotectin levels

While there were no apparent sex differences present with fecal calprotectin values, Figure 4 demonstrates a difference in the shape of distribution; reasons for why this dual peak was not present in men can be further explored. It has been found that men may exhibit reduced immune function due to the hormone testosterone (Gildner et al., 2016), which could alter their immune response to helminth infection. Additionally, it has been shown that giving birth is a risk factor for developing autoimmune disorders in wealthy population, however work specifically with natural fertility populations and this risk has not been done (Khashan et al., 2011). Age groups from Table 1 were determined based on the Shuar being a natural fertility population. No significant difference was detected among age groups for women, and within the same age group between sexes.

Limitations

The present study suffers from several limitations. First sample size is relatively small and non-representative and because subjects were divided into groups for the analysis, non-representative outliers may have influenced the analysis. More samples would have allowed for more robust statistical analysis and additional subgroups could have been tested. Second, age and sex were the only co-variables available for all

participants; additional data like self-reported symptoms related to IBD would have led to stronger analysis. Finally, this study was a cross sectional study, and because of this it is limited in the ability to identify casual relationships. A longitudinal study could document causation better but requires many years of collection and this was the first year this biomarker was assessed in fecal samples.

CONCLUSIONS

As postulated by the Old Friends Hypothesis, the demographic pattern of intestinal inflammation in the Shuar, an economically developing population, was different from that observed in wealthier developed populations. The present study examined the relationships among FC levels, age and sex in a population with high parasite burden but also rapid market integration to gain insight into IBD in less wealthy nations. There association between age and FC was not curvilinear --high, low, high-- as there it is with populations in wealthy nations with high prevelnce of IBD. There was some evidence of a linear decline in logFC with age in the Shaur. There was no evidence of sex differences in mean levels or variance of logFC, which does not support the hypothesis that FC would be higher in men as with IgE. While there was no apparent difference, looking at the violin plot created (Figure 4) shows a distinct pattern of distribution of FC levels that is not as clearly present in men.

A study demonstrated that smoking and obesity risk increases with age as does FC level; lifestyle differences along with earlier high parasite burden may keep FC levels low in mid to late adulthood for the Shuar (Poullis et al., 2004). The different age patterns may be due to populations of the Shuar and the industrialized nations being in different stages of the epidemiological transition. Future directions for this research include a larger scale study that is longitudinal and includes members of the Shuar and non-Shuar *Colonos* in the same region to investigate how market integration impacts fecal calprotectin levels.

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List of Figures, Tables and Graphs

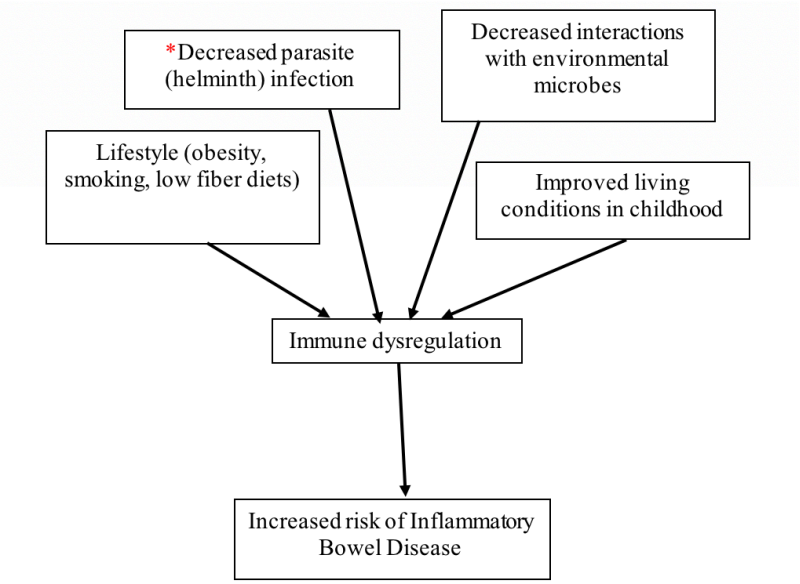

Figure 1: Flowchart demonstrating the relationship between immune regulating factors included by the present study.

The red asterisk indicates the factor most investigated in this study, however it is important to note that there are many other factors that can lead to increased risk of inflammatory bowel disease. This is just a general outline of the ideas involved.

Figure 2: Map of Ecuador and the region of study.

Map of Ecuador with blue shape indicating Shuar territory where the Cross-Cutucú regions and Upano River regions are located.

		n	Untransformed FC Mean (µg/g)	lnFC mean
Women				
	0-14 years	19	96.11 (178.8)	3.539 (1.597)
	15-39 years	14	49.86 (63.74)	3.297 (1.303)
	40-70 years	8	33.4 (34.43)	2.883 (1.334)
Men				
	0-14 years	16	73.64 (196.9)	3.025 (1.494)
	15-39 years	12	103.1 (253)	2.912 (1.963)
	40-70 years	8	33.70 (30.96)	2.945 (1.315)

Table 1: Sex, number of individuals, untransformed fecal calprotectin values and natural log-transformed fecal calprotectin values.

Table showing sex and FC (untransformed and ln transformed). Standard deviation is in parentheses. Age groupings were decided based on groupings during which this population is young, in child bearing age, and old. There is no significant difference between woman of childbearing age with woman of other ages or among men in the same age groups (15-39 years).

Age (years)	Calprotectin level (µg/g),	sex
24	896.72	male
1	832.79	male
4	797.60	female
31	255.50	female

Table 2: The three highest fecal calprotectin values in the sample population along with sex and age noted.

This table shows the three highest fecal calprotectin values collected in the sample were from children or young adults.

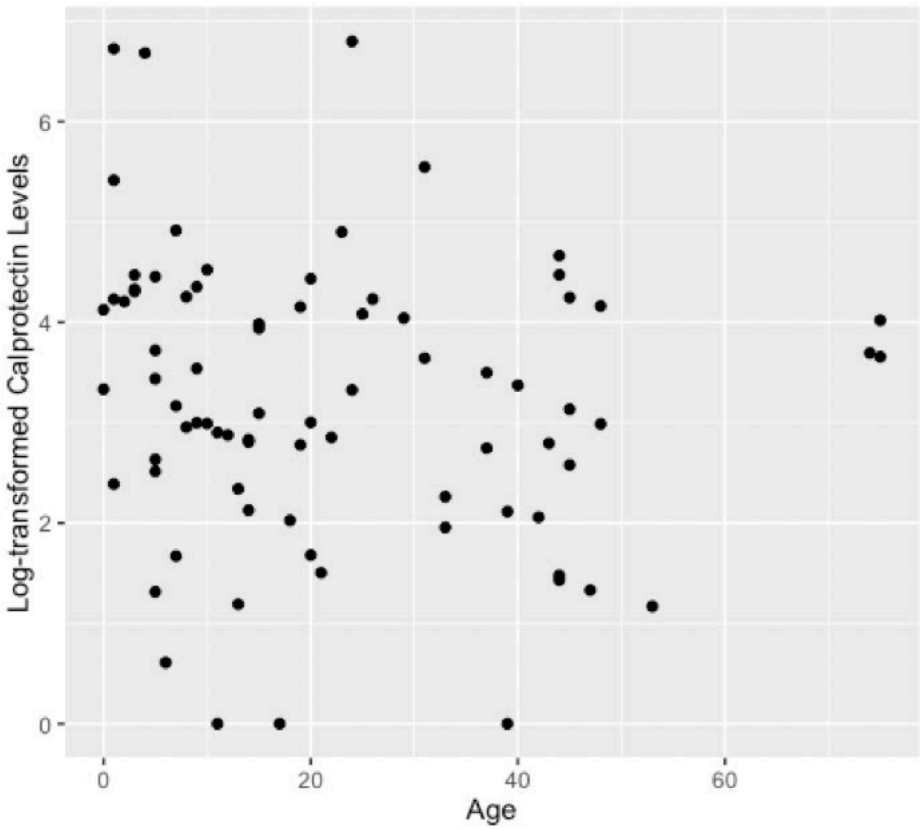

Figure 3: Scatterplot of natural log-transformed fecal calprotectin values and age.

This graph shows the graphical distribution of natural log-transformed fecal calprotectin levels. A significant negative relationship is present (p=0.04).

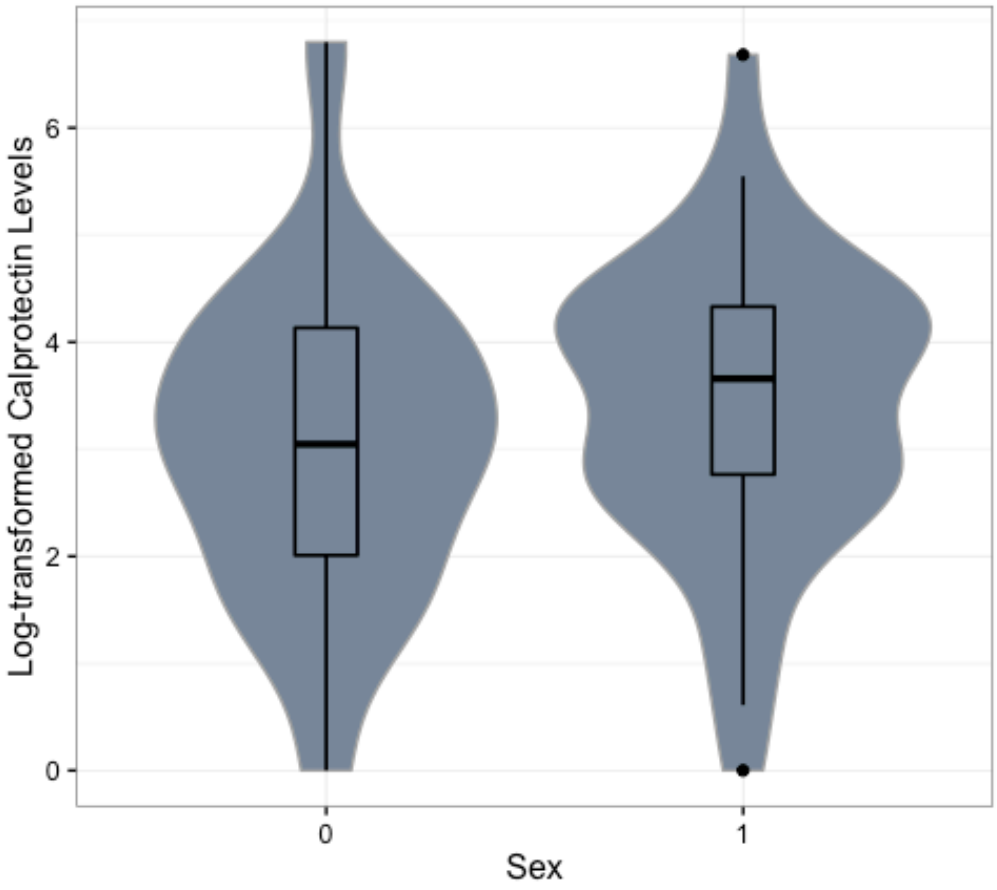

Figure 4: Violin plot of sex and natural log-transformed fecal calprotectin values.

This figure is a visual representation of the male (0) and female (1) distributions of natural log-transformed calprotectin levels. There is a distinct difference in the shape of the male and female plots; that of the men has a normalized bell-shape curve, while women have a dual peak shape, with not as many values around the mean.

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