Nutrition, Immunity, Infection and Metabolic Health in Ecuador

Focus on role of zinc in immune function and of anti-oxidants in metabolic syndrome

Fernando Sempértegui Ontaneda

The studies described in the thesis were performed at the Department of Immunology, Central University of Ecuador, Quito, Ecuador in collaboration with the institutes indicated in the various chapters.

The composition of this thesis was performed in close collaboration with the Department of Immunology, Erasmus MC. The printing of this thesis was supported by the Editorial Universitaria, Quito, Ecuador.

ISBN/EAN: 978-94-9181-108-1

Illustrations: Fernando Sempértegui
Cover: Fernando Sempértegui
Lay-out: Erna Moerland-van Eenennaam
Printing: Editorial Universidad Central del Ecuador

Nutrition, Immunity, Infection and Metabolic Health in Ecuador

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Voeding, immuniteit, infectie en metabole gezondheid in Ecuador

De rol van zink in immuunfunctie en van anti-oxidanten in metabool syndroom

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 7 oktober 2015 om 15.30 uur

door

Fernando Efraín Sempértegui Ontaneda

geboren te Loja, Ecuador

Erasmus University Rotterdam

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Al esplendor de las vidas que florecen más allá:

María Fernanda, Paulina, Ana Cristina, Ana Sofía, Jeremías.
Mercedes y Myriam.

To the splendor of the lives that flourish beyond:

María Fernanda, Paulina, Ana Cristina, Ana Sofía, Jeremías.
Mercedes and Myriam.

CONTENTS

Chapter 1

General Introduction

INTRODUCTION

This thesis is a compilation of several articles with as subject nutrition, immunity, infection and metabolic health in Ecuador. Focus is in particular on

1. The association of zinc deficiency with immune functioning and with infections in children and aged (generally over 60 years) under-privileged Ecuadorian urban populations
2. The prevalence of metabolic syndrome and its association with micronutrient deficiencies in aged under-privileged Ecuadorian urban populations.

The investigations are based on studies carried out at both the community level and in hospitals.

The introduction describes of what is known in the literature on micronutrient deficiencies in Ecuadorian children and older subjects with emphasis on zinc deficiency, the incidence of infections in the same Ecuadorian populations, and the prevalence and the epidemiology of the metabolic syndrome (MetS)/Type 2 diabetes in Ecuadorian older subjects.

Micronutrient deficiencies in Ecuador: Focus on zinc special for the immune system

In young children (< 5 years old) there are three anthropometric indices used to evaluate their nutritional status: weight-for-age, height-for-age, and weight-for-height. Sustained low weight-for-age (underweight) in an individual child suggests insufficient caloric availability in the diet, low height-for-age (stunted) suggests a combined protein/caloric chronic deficiency in the diet, and low weight-for-height (wasted) is an indicator of sudden energy deprivation. At community level height-for-age provides a good estimate of the nutritional situation both in terms of food availability and health status. If food availability is constrained children are vulnerable to infections which in turn affect linear growth. Although the prevalence of underweight in Ecuador has been reduced during the last two decades, stunted remains a public health problem, especially in rural areas in the Andean region [1].

Linear growth depends on good provision of food at critical times. Given the accelerated rate of growth the first two years of life deserve more attention [2]. During this period food restriction affects growth and makes children vulnerable to respiratory and diarrheal infections, which worsen the adverse effects of food deficiency on growth [3]. Linear growth not only requires a balanced caloric-protein diet but also some critical micronutrients like iron and zinc. Deficiency of these micronutrients is prevalent across all regions of Ecuador, mainly in Andean areas [3].

Anemia due to iron deficiency is a negative condition for growth and especially for development. Its deleterious effect on the long term has been evidenced by some studies, which have shown that young people who suffered from severe anemia at childhood have low performance of some intellectual abilities, mainly those related to memory and logic-solve problems [4]. Severe anemia in young children has been reduced in Ecuador due to provision of iron supplements at health centers. However, it continues to be a problem in rural villages where the adhesion to supplements is scarce.

Zinc deficiency is prevalent in Ecuador as it is in all Andean countries [5]. The sources of dietary zinc are animal food and cereals. The availability of zinc-rich animal food is limited for poor populations at both urban and rural areas. Although legumes and cereals like wheat and barley provide some zinc, the daily amounts obtained by regular consumption are not enough to meet the daily requirements [5]. Furthermore, in Andean areas the traditional domestic practice of preparing unrefined barley flour, which contains phytic acid, lowers even more the zinc bioavailability due to phytate formation in the intestine [6]. An approach to provide daily supplements of zinc to these populations is pending.

Zinc is distributed in the extracellular and, mostly, in the intracellular compartment of the body [7]. The cellular fraction is included in some enzymes, proteins and transcription factors associated with growth and immune response [8]. Some studies have shown that extracellular zinc diminishes during acute infection, which might indicate that it is used to improve the immune response trough zinc-dependent intracellular mechanisms [9]. I was involved in a multicenter study in children with acute *P. falciparum* malaria which showed that plasma zinc concentration was very low at admission. Furthermore, the change in C-reactive protein (CRP) concentration from admission to 72 hours showed that CRP levels were negatively associated with plasma zinc concentrations. This negative relation between the 2 variables shows that as CRP declined, plasma zinc levels increased [10]. Thus zinc status assessed by plasma zinc concentration should be adjusted for CRP concentration.

The association between zinc status and linear growth in young children has been reported in some studies, including a meta-analysis [11]. Therefore, it has been suggested that at community level the prevalence of stunting is an indicator of chronic zinc deficiency. Precisely, due to the prevalence of stunting, the Andean region has been characterized as one of moderate to severe chronic zinc deficiency areas in the world [5]. The high concentration of zinc at bones and its regulative role on insulin-like growth factor I (IGF-I) in osteoblastic cells suggests a direct effect of this mineral on linear growth [12]. Furthermore, the presence of "zinc finger" DNA motifs adds plausibility to this conjecture [13].

Prevalence of infectious diseases in zinc-deficient populations in both children and elderly could be associated with impaired immune responses. Precisely our studies at community level have been aimed at providing evidence on this potential association in Ecuadorian populations. In fact, the effect of zinc supplementation on the incidence of respiratory infections was our first intervention trial and it is reported in Chapter 4. Furthermore, the effect of Zn provision as an adjunct to the standard treatment on the clinical evolution of severe pneumonia has been evaluated by our team in hospitalized children, and the study is reported in Chapter 5. It is likely that micronutrient, including dietary zinc deficiency also affects Ecuadorian older subjects, and this has been the impetus for our studies in a poor elderly population in Quito city as described in Chapter 6.

Incidence of acute infections in Ecuador: diarrhea, respiratory infections in children and elderly

Respiratory infections, mainly pneumonia, remain the leading cause of mortality in young children in Ecuador [14]. When we started our studies on zinc and infection in children in 1992, acute diarrhea was a leading cause of mortality in children, but improvement of safe drinking water access, especially in urban areas, as well as the oral rehydration therapy has lowered both the morbidity and mortality associated with diarrhea infection. However, in some rural areas diarrhea incidence is still high compared to that in urban areas. In the epidemiological context of the nineties, we chose to study the putative relationship of zinc deficiency and respiratory infections in malnourished children, especially in those with low height-for-age Z score.

The diagnosis of pneumonia in Ecuador is always based on symptoms and signs. Microbiological identification of pathogens is lacking even in hospitals. Then, standard treatment of pneumonia includes antibiotics. Our initial studies of pneumonia followed the symptomatic diagnosis. Recently, we carried out the first study in Ecuador aimed at identifying the effect of type of pathogen, bacteria and viruses, on the clinical evolution of severe pneumonia in hospitalized children who were given zinc or placebo plus the standard treatment [15]. We found that most pneumonia episodes are associated with viral infections.

Accurate data on infections in Ecuadorian older and poor subjects are lacking. However, our preliminary study, the first in Ecuador in an elderly population, showed that older subjects suffer from a high incidence of respiratory infections. In fact, a 6 months recall survey reported that 54% of subjects, who live in urban slums, had at least one episode of respiratory infection. Of these, 47% sought care at a hospital or from a physician [16]. In another one-month recall study carried out by me and my team in a larger sample, participants recalled having had colds or influenza-like syndromes (62.8%) and cough (61.0%) [17].

According to data of the year 2012 from the National Institute on Census and Statistics (INEC), 7400 subjects older than 65 years old were hospitalized in the country due to respiratory infections. From them, 3256 had diagnoses of pneumonia, and 1672 chronic obstructive pulmonary disease (COPD) [18]. In 2010, mortality in older adults (aged 65 and over) was 35.6 per 1,000 populations, with 50.9% of the deaths occurring in men. The leading causes of death included pneumonia, hypertension, diabetes, cardiac insufficiency, and acute myocardial infarction [19].

Since the high incidence of infections in older Ecuadorian subjects could be related to immune senescence as well as zinc deficiency, we carried out a cross-sectional larger study in a sample of elderly living in urban slums in order to obtain more accurate information on the incidence of respiratory infections as well as the zinc, and other micronutrient status and the cellular immune response through a Delayed Type Hypersensitivity (DTH) skin test and an ex vivo production of Th1-dependent cytokines IL-2, and IFN-γ. Data are presented in chapter 7 .

It has been found that chronic gastric infection by *H. pylori* leads to increased reactive oxygen species (ROS) and reactive nitrogen species (NOS) production [20]. We carried out a study, reported in Chapter 9, in adults suffering from clinical gastritis associated with *H. pylori* infection [21], aimed at determining whether infiltration of the gastric mucosa by mononuclear and polymorphonuclear cells (PMNs) was associated with lower zinc tissue concentrations to try to relate active gastritis to zinc deficiency . The study is described in chapter 9.

Metabolic syndrome and diabetes in Ecuador and a potential role of micronutrient, including zinc deficiency

On 2002 we carried out a preliminary study, aimed at describing the nutritional status of Ecuadorian older subjects (n=142). That study, was the first one carried out in this population. The collected data suggested a high prevalence of the metabolic syndrome (MetS) [22].

Although the etiology of the MetS has not been fully elucidated, available evidence suggests that it is the result of a complex interaction between genetic, metabolic and environmental factors [23]. Nutritional factors are the most prominent environmental influences, including obesity, dietary glycemic index (GI) [24], fruit and vegetable intake [25], total and type of fat intake [26, 27], antioxidant nutrients [28], B vitamins and dairy products [29]. In fact, our preliminary study in elderly Ecuadorians, who lived in poor peri-urban communities, showed a high prevalence of elevated waist circumference and low HDL cholesterol concentration, both components of MetS [16]. Energy intake was mainly dependent on high carbohydrate consumption (76.7% of total energy). Furthermore, a high prevalence of several micronutrient deficiencies was found [16]. These findings

suggested that the increasing elderly population of Ecuador is at risk for MetS, although no specific information was available for them.

Given the morbidity and mortality data we had in the year 2003, and the potential risk factors we found in our preliminary study in older subjects, we carried out the epidemiologic study on MetS in a larger population (n= 352) as described in Chapter 8, to better understand the relationship between MetS and nutritional status, mainly the micronutrient status. Since MetS is associated with inflammatory markers, and zinc is an antioxidant through its role in the superoxide dismutase enzyme as well as in lowering the production of NO. due to the inactivation of inducible nitric oxide synthase (iNOS) in endothelial cells [30], our study included the evaluation of the zinc status and that of other antioxidants like vitamins C and E.

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Chapter 2

Experimental aims of this Thesis

EXPERIMENTAL AIMS OF THIS THESIS

More specifically the following aims for the studies presented in this thesis were:

1. To know the prevalence of micronutrient, and in particular zinc deficiency in younger and older Ecuadorian populations.

2. To perform limited studies on the immunological consequences of micronutrient and in particular zinc deficiency.
   For that purpose we determined in the older subjects as described in chapters 6 and 7 the following immune tests:
   a. DTH skin tests with some recall antigens
   b. Ex vivo IL-2 production in cultured mononuclear cells
   c. Ex vivo INF-γ production in cultured mononuclear cells

3. To study the prevalence of respiratory infections in both children and older subjects and its association with zinc deficiency.

4. To study the effects of zinc supplementation on the prevention of respiratory symptoms in young children, and determine the lowest efficacious zinc dose to prevent acute infections in young children.

5. To study the effects of zinc supplementation as an adjunct to the standard treatment of severe pneumonia in hospitalized children.

6. To study the prevalence of the MetS in an older Ecuadorian population and to determine its association with micronutrient deficiencies.

7. To determine whether infiltration of the gastric mucosa by mononuclear and polymorphonuclear cells (PMNs) in *H. pylori* infected adults was associated with lower zinc tissue concentrations to try to relate active gastritis to zinc deficiency.

Chapter 3

Dose-response trial of prophylactic zinc supplements, with or without copper, in young Ecuadorian children at risk of zinc deficiency

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American Journal of Clinical Nutrition 2008; 87; 723-733

Dose-response trial of prophylactic zinc supplements, with or without copper, in young Ecuadorian children at risk of zinc deficiency[1–3]

Sara E Wuehler, Fernando Sempértegui, and Kenneth H Brown

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ABSTRACT

Background: Multiple studies have shown the benefits of zinc supplementation among young children in high-risk populations. However, the optimal dose and safe upper level of zinc have not been determined.

Objectives: The objectives of this study were to measure the effects of different doses of supplemental zinc on the plasma zinc concentration, morbidity, and growth of young children; to detect any adverse effects of 10 mg supplemental Zn on markers of copper or iron status; and to determine whether any adverse effects are alleviated by providing copper with zinc.

Design: This randomized, double-masked, community-based intervention trial was conducted in 631 Ecuadorian children who were 12–30 mo old at baseline and who had initial length-for-age *z* scores <-1.3. Children received 1 of 5 daily supplements for 6 mo: 3, 7, or 10 mg Zn as zinc sulfate, 10 mg Zn + 0.5 mg Cu as copper sulfate, or placebo.

Results: The change in plasma zinc concentration from baseline was positively related to the zinc dose ($P < 0.001$). Zinc supplementation, including doses as low as 3 mg/d, reduced the incidence of diarrhea by 21–42% ($P < 0.01$). There were no other significant group-wise differences.

Conclusions: Zinc supplementation with a dose as low as 3 mg/d increased plasma zinc concentrations and reduced diarrhea incidence in the study population. There were no observed adverse effects of 10 mg Zn/d on indicators of copper or iron status. The current tolerable upper level of zinc recommended by the Institute of Medicine should be reassessed for young children. *Am J Clin Nutr* 2008;87:723–33.

KEY WORDS Zinc, copper, dose response, tolerable upper level, diarrhea, lipoprotein concentration, iron, growth response

INTRODUCTION

The beneficial effects of zinc supplementation include reductions in the incidence and prevalence of diarrhea (1, 2), the incidence of pneumonia (1, 2), and the rates of mortality (3–8) among young children in low-income countries. Moreover, zinc supplementation increases the growth of stunted children (9). However, the dose of supplemental zinc required to achieve these beneficial outcomes, while avoiding potential adverse effects of excessive zinc intake, remains unknown. Previously, the recommended dietary allowance of zinc for preschool children was set at 10 mg/d by national and international organizations (10, 11), but the United States Food and Nutrition Board–Institute of Medicine, the International Zinc Nutrition Consultative Group (IZiNCG), and the World Health Organization (WHO) now recommend a lower recommended dietary allowance of 3 mg Zn/d for 1–3-y-old children (12–14). These expert groups also recommended tolerable upper levels (ULs) of zinc intake ranging from 7 to 23 mg/d to avoid the possible adverse effects of excessive intakes of zinc on copper and iron status and lipoprotein concentrations, which are the first expected signs of excessive zinc intake.

Previously reported supplementation trials in young children that lasted ≥2 mo evaluated a single daily dose of zinc ranging from 5 to 20 mg, and most provided ≥9 mg Zn (2, 9); 51% of 1–3-y-old children in the United States consume more dietary zinc than the recommended UL (15). However, possible adverse effects of this level of intake have not been systematically explored. Thus, additional information is needed with respect to the UL for this age group.

The objectives of the present study were to determine the lowest daily dose of supplemental zinc that effectively increased the plasma zinc concentrations, reduced the morbidity, and increased the growth of young Ecuadorian children presumed to be at increased risk of zinc deficiency, on the basis of their relatively low length-for-age *z* score (LAZ), more than did placebo; to determine whether there are adverse effects of 10 mg Zn/d on markers of copper and iron status and lipoprotein concentrations; and to determine whether any observed adverse effects could be prevented by providing 0.5 mg Cu/d in combination with 10 mg Zn/d. The first objective was assessed among children who were given 1 of 5 supplements containing 3, 7, or 10 mg Zn/d, 10 mg Zn + 0.5 mg Cu/d, or placebo; the second and third objectives were assessed among a subset of the children who consumed 10 mg Zn/d, 10 mg Zn + 0.5 mg Cu, or placebo. To maximize the

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[2] Supported by the US Department of Agriculture, the US Agency for International Development Micronutrient Program, the United Nations Children's Fund (UNICEF), Bristol-Meyers/Squibb Freedom to Discover grant, and Grupo Farma del Ecuador.

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Received August 13, 2007.
Accepted for publication October 1, 2007.

likelihood of detecting functional responses to zinc supplementation (13, 16), we selected children with an initial LAZ <-1.3 as compared with 1978 reference data from the WHO and the National Center for Health Statistics (NCHS)(17).

SUBJECTS AND METHODS

Study design and sites

The present study was designed as a community-based, randomized, double-masked intervention trial. It was conducted in 3 sites in Ecuador that were selected in consultation with the Ecuadorian Ministry of Health: El Carmen, a small town in the coastal plains, and the communities surrounding it; Latacunga, a medium-sized town in the Andean highlands, and several surrounding rural communities; and 2 shantytowns in the hills adjacent to the capital city of Quito, also in the Andean highlands. Community surveys, enrollment, and follow-up were conducted between November 2001 and April 2005.

Subjects

Children 9–29 mo old were identified by house-to-house surveys and during well-child checkups at local health centers. To avoid the loss of potentially eligible children due to changes in LAZ during the screening period, the screening LAZ cutoff was set at <-1.25 as compared with the WHO/NCHS international reference data (17). All of these children were given iron sulfate supplements to provide $\approx$2.5 mg Fe · kg body wt^{-1} · d^{-1} (18) for 1 mo, unless they had recently received iron supplementation from the local health center. The intent was to treat any existing iron deficiency before the study to avoid possible interactions between iron and zinc supplements during the study (19–21). After 1 mo of iron supplementation, potentially eligible children were invited to participate in the pretrial screening measurement of blood hemoglobin concentrations conducted with the use of the HemoCue Analyzer (HemoCue AB, Ángelholm, Sweden). If the child's hemoglobin was $\geq$10.5 g/dL [adjusted for altitude (22)], he or she was invited to attend a further screening appointment to determine eligibility for the 6-mo intervention trial. Those children with lower hemoglobin concentrations were provided an additional month of iron supplementation, after which they were reassessed for eligibility for the full study. If anemia persisted beyond 3 mo of iron treatment, the child was excluded from the trial and referred for more detailed medical assessment and care. Final eligibility criteria for the intervention trial included LAZ <-1.3 for children 12–20 mo old and <-1.5 for children 21–29 mo old, assessed by comparison with the WHO/NCHS international reference data (17); hemoglobin $\geq$ 10.5 g/dL, adjusted for altitude (22); and the absence of chronic disease or congenital defects that restrict normal growth. The cutoff of -1.5 LAZ was selected because of meta-analyses indicating that growth response to zinc occurs among populations with LAZ <-1.5 (9). This cutoff was relaxed among children 12–20 mo old, because their LAZ may have still been declining over that age range.

A flow chart shows the numbers of children involved in each step of the initial survey, prescreening examinations, and intervention trial and indicates the reasons why children did not continue at each stage (**Figure 1**). Ultimately, 631 children were included in the trial.

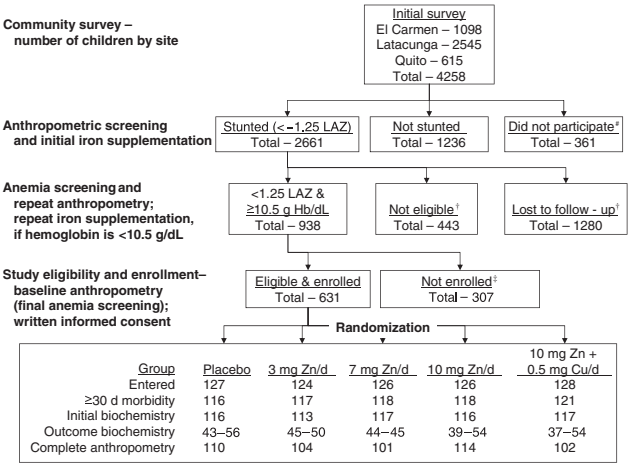

FIGURE 1. Flow sheet of participants in screening survey and 6-mo intervention trial in Ecuadorian children 12–36 mo old. Entry criteria into the full study were defined as length-for-age *z* score (LAZ), which was assessed by comparison with international reference data from the World Health Organization and the National Center for Health Statistics (16)—ie, <-1.3 for children 12–20 mo old and <-1.5 for children 21–29 mo old—and hemoglobin $\geq$ 10.5 g/dL after adjustment for altitude (22). *These children did not report to the neighborhood screening site for anthropometric screening, and no reasons for nonattendance were provided. †Reasons for ineligibility: LAZ >-1.25, 74%; over age, 26%; reasons for loss to follow-up: moved from area or not at home at follow-up, 92%; refused hemoglobin assessment, 8%. ‡Reasons for nonenrollment: refused study design or written consent or did not attend the final eligibility screening (no reason given), 52%; LAZ not <-1.3 or -1.5 according to age group at time of enrollment, 41%; refused blood draw, 7%.

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Written informed consent was obtained from the parents of each child before his or her enrollment in the study; if a child's parents were unable to read, write, or both, oral informed consent was obtained. Permission to conduct this study and approval of informed consent documentation were obtained from the National Institutes of Health–approved Human Subjects Committees of the University of California, Davis, and the Corporación Ecuatoriana de Biotecnología (Quito, Ecuador).

Interventions

Group assignment and supplements

On entering the study, participants were stratified by age (12–20 or 21–30 mo old) and sex and then randomly assigned to a study group. Children in each of the study groups consumed 1 of 5 daily supplements containing 3, 7, or 10 mg Zn as zinc sulfate, 10 mg Zn + 0.5 mg Cu as copper sulfate, or placebo per 5 mL of flavored syrup. The randomization lists for each of these strata were generated independently by using a fixed block randomization procedure.

Supplements were prepared by the Pharmacology Laboratory at the Universidad Central del Ecuador. Mineral concentrations were confirmed by independent laboratory analyses before distribution. Bottles of the assigned supplement were left in each child's home and exchanged twice monthly. Caregivers were encouraged to provide the supplement once daily upon waking the child or between morning meals. Fieldworkers visited the children's homes 3–5 times/wk to record supplement consumption as full or partial and to ascertain whether consumption was observed by the fieldworker or reported by the caregiver. Data were also collected on the consumption of any other nutritional supplements.

Socioeconomic status indicators

The family's socioeconomic status (SES) was assessed by interview and observations of housing quality at the time of entry into the study. SES data included possessions (eg, stove, oven, refrigerator, television, radio, animals, bicycle, automobile, or family-owned shop or roving sales cart), housing characteristics (eg, building materials, type of water source, hygienic services, cooking facilities, availability of electricity, and home ownership), and parental characteristics (educational level and occupation).

Treatments for morbidity

Fieldworkers provided oral rehydration solution for diarrhea and acetaminophen for fever. Children were referred to the study doctor for symptoms of fever (≥38 °C axillary or ear canal temperature), elevated respiratory rate (≥40 respirations/min), diarrhea (≥3 semi-liquid or watery stools/24 h), chronic diarrhea (≥14 consecutive days of diarrhea), or blood in the stool or whenever a referral was deemed necessary by the fieldworker or was requested by the caregiver. The doctor's evaluation, treatment, and, when necessary, referral were provided gratis, either in the child's home or at a centralized clinic, according to previously defined illness categories and treatment algorithms.

Outcomes

Biochemistry

The children's venous blood was drawn in the morning, after either an overnight fast or a delay of ≥2 h since the last reported meal. This procedure was completed at a central location at months 0, 3, and 6 of the intervention. Biochemical markers were measured in plasma, serum, whole blood, and washed red blood cells (RBCs). Zinc status was assessed among all 5 study groups by using the plasma zinc concentration. Copper status was assessed by analyzing the plasma copper concentration, serum ceruloplasmin activity, and activity of erythrocyte zinc-copper superoxide dismutase (SOD)/mg hemoglobin. Iron status was assessed with whole blood hemoglobin and serum ferritin concentrations. Serum total and HDL-cholesterol concentrations were measured to assess lipoprotein responses to the intervention. Serum C-reactive protein (CRP) concentrations were analyzed to control for possible effects of an acute phase response on plasma zinc and copper concentrations and serum ceruloplasmin and ferritin concentrations.

To permit baseline comparisons, initial analyses of plasma zinc and copper concentrations, whole blood hemoglobin, and serum CRP concentrations were conducted on all samples available from participating children. However, final values were assessed only on a randomly selected subset of paired samples, as described later in reference to sample size estimations. Other markers of copper and iron status and lipoprotein concentrations were analyzed only in a subset of the 3 study groups for which possible adverse events were assessed—namely, the placebo, 10 mg Zn/d, and 10 mg Zn + 0.5 mg Cu/d study groups.

Blood was drawn by pediatric phlebotomists into Sarstedt monovettes (Sarstedt AG & Co, Nümbrecht, Germany). Blood for plasma was obtained from trace element–free tubes containing lithium heparin anticoagulant (no. 01.1604.400, with needles, and no. 01.1602.01; Sarstedt AG) and serum from serum separator tubes (no. 01.1602.01; Sarstedt). RBC pellets were obtained from the lithium heparin–treated blood after 3 saline solution washes. Blood samples for plasma were centrifuged within 30 min, and those for serum were centrifuged within 45–60 min. All other blood processing occurred within 2 h of sample collection. All processed samples were stored in coolers until they were transferred to a −20 °C freezer within 6 h of collection. All other sample transportation was on dry ice. Baseline and follow-up analyses for each biochemical marker of a particular child were completed within the same analytic run.

Plasma zinc and copper concentrations were measured by using an inductively coupled plasma–optical emission spectrometer (ICP-OES; Varian Australia Pty Ltd, Palo Alto, CA); with a zinc standard (#S4400-1000681; CPI International, Santa Rosa, CA) and a bovine liver standard (#SRM 1577b; National Institute of Standards and Technology, Boulder, CO). Serum CRP concentrations were analyzed by using radial immunodiffusion (GT044.3; The Binding Site Limited, Birmingham, United Kingdom). Serum ceruloplasmin activity was measured in response to *o*-dianisidine dihydrochloride (23, 24) and in relation to human ceruloplasmin control (#C4519; Sigma-Aldrich Corporation, St Louis, MO).

Erythrocyte SOD activity was measured according to the method of Peskin and Winterbourn (25) in relation to RBC hemoglobin concentration (Drabkin's solution and standards no. 0320–650; Stanbio Laboratory, Boerne, TX) and standardized to human erythrocyte SOD control (#S9636; Sigma-Aldrich Corporation). A fixed volume of the stored, triple-washed RBC pellet was lysed in cold double-deionized water. After being mixed by vortex and after centrifugation (2500 × *g*, 10 min, 4 °C), a fixed volume of this lysate was mixed with a 37.5%

chloroform–62.5% ethanol solution, mixed by vortex, and centrifuged (2500 × *g*, 10 min, 4 °C) to differentiate zinc-copper SOD from manganese or iron SOD. This aqueous supernatant was stored at −80 °C until analysis, as described above. RBC and whole blood hemoglobin concentrations were measured by using Drabkin's solution. Serum ferritin concentration was measured by using immunoradiometric assay (Coat-A-Count Ferritin IRMA no. IKFE and standards; Diagnostic Products Corporation, Los Angeles, CA). Serum total and HDL-cholesterol concentrations were analyzed colorimetrically (manual kits no. CH201 and CH2673 and standards; Randox Laboratories Ltd, Crumlin, United Kingdom).

Morbidity questionnaire

Morbidity events were recorded during home visits 3–5 times/wk by using a systematic, symptom-based questionnaire and observation of the child. Elicited information included the child's general health status, appetite, number and consistency of stools, and symptoms of cough, fever, nasal discharge, vomiting, and earache or discharge from the ear during each day since the previous visit. Body temperature was measured when fever, cough, or diarrhea was reported and once monthly on a nonillness day. Respiratory rates were measured for 1 min in duplicate by the fieldworker when fever, cough, or diarrhea was reported. The doctor repeated these assessments when the child was referred for illness. Diarrhea was defined as ≥3 semi-liquid or liquid stools in a 24-h period. Criteria for diagnosis of acute upper respiratory infection were concurrent presence of cough and purulent nasal discharge, and those for acute lower respiratory infection were concurrent presence of cough and respiratory rate ≥ 40 rpm. For the purposes of this report, incidence refers to the number of new episodes (separated by ≥1 nonillness day) per 100 d of observation, and the duration of diarrhea is the number of days that each episode lasted.

Anthropometry

When the children reported to the central location at the 0-, 3-, and 6-mo time-points of the intervention, their length and weight were measured. Weight was measured in duplicate to the nearest 0.1 kg by using a digital scale with tare function (model 882; Seca, Ontario, CA) while the child was wearing minimal clothing and was either standing alone or being held in the caregiver's arms. Length was measured in duplicate or triplicate to the nearest 1 mm (26) on an infantometer (model 418; Seca) by each of 2 trained anthropometrists who passed frequent standardization procedures. Length measurements were repeated when the averaged measurements differed by >3 mm between the 2 participating anthropometrists. The 2 closest measurements were averaged. For statistical analysis, weight and length measures were converted to nutritional status indexes of LAZ, weight-for-age (WAZ), and weight-for-length (WLZ) *z* scores as compared with the 2006 WHO child growth standards (27).

Sample size estimation and statistical analyses

The sample size was designed to detect a difference among groups of 0.5 SD units in anthropometry at 80% power, which resulted in a planned sample size of 97 children per group (plus 25 children to account for possible attrition), or 122 children per intervention group. To detect a group-wise difference of 0.7 SD units with 80% power in change in the biochemical indicators, a sample size of 50/group was selected for analyses that included all 5 study groups, and a sample size of 40/group was selected for analysis of just 3 study groups. For these latter analyses, a subset of paired specimens was randomly selected from the 472 subjects who had sufficient quantities of both baseline and final samples of blood to allow the conduct of all required analyses. Although study compliance markers were assessed, primary analyses were completed on an intention-to-treat basis.

Statistical analyses were completed by using SAS for WINDOWS software (version 9.1; SAS Institute Inc, Cary, NC). Logarithmic transformations were required to normalize distributions for concentrations of zinc, ferritin, ceruloplasmin, erythrocyte SOD, and HDL and total cholesterol and for the presence of purulent nasal discharge. The incidence of diarrhea and the presence of low appetite, cough, fever, and vomiting were normalized by square-root transformations. Suitable transformations were not found for full or partial supplement consumption or for rates of earache or discharge, elevated respiratory rate, and acute upper or lower respiratory infection, and thus these variables were analyzed by using nonparametric statistics. Factor analyses were conducted to aggregate SES characteristics into 3 main SES factors, which generally corresponded to material possessions, housing quality, and parental characteristics. Possession factors were given a value of 1 if present and of 0 if not present. Parental and housing characteristics were graded from worst to best as 0 to a maximum value, depending on the possible number of categories. Each of these graded values was transformed into a fraction between 0 and 1 by dividing the actual value into the maximal value for each characteristic. The value of each SES factor is a summation of these transformed values and the 0–1 values of the SES characteristics included in each respective SES factor. Thus, the maximal values for material possessions, housing quality, and parental characteristics are 7, 6, and 4, respectively.

Group-wise differences at baseline were compared by using analysis of variance (ANOVA) for continuous variables and a chi-square test for categorical variables. All group-wise postintervention changes were compared by using analysis of covariance (ANCOVA) for continuous variables after control for relevant baseline variables. Baseline variables used as covariates included site, age, sex, SES factors, baseline values of the respective dependent variables, elevation of the respective CRP (in the case of biochemical outcomes) (yes or no), and baseline anthropometric *z* scores. Possible interactions between selected covariates and treatment group were tested by ANCOVA, and all significant covariates were retained. Because no suitable transformation was found, supplement consumption was analyzed by using the Kruskall-Wallis test, with no covariates, and the nonnormally distributed morbidity variables mentioned above were analyzed by using ANCOVA based on ranks. Morbidity analyses were weighted by the number of days of observation and were restricted to children for whom ≥30 d of data were available (>80% of all children). However, including all available data did not affect the results. When overall results were statistically significant, group means were compared with the use of the Tukey-Kramer test after control for significant covariates. To test for nonlinearity of the response of plasma zinc concentrations to supplemental zinc, the supplemental zinc dose was included as a continuous variable, supplemental copper was included as a categorical variable, and both linear and higher-order polynomial terms were tested in the model.

RESULTS

Enrollment, attrition, and baseline characteristics

Of the 938 surveyed children who were eligible for the full intervention study, 631 were enrolled (Figure 1). Of these 631 children, 531 (84%) completed the 6-mo intervention with at least the final anthropometry assessment. Of the 100 enrolled children who were lost to follow-up during the intervention period, 55 moved out of the study area, 11 refused to continue supplement consumption, 25 refused blood draws, and 9 withdrew consent without a specified reason. There were no significant differences in rates of attrition by treatment group (**Table 1**) nor any significant differences between the baseline characteristics of the children who left the study prematurely and those of children who completed the full 6-mo intervention.

No significant group-wise differences were found at baseline by age, sex, SES markers, anthropometric measurements, or biochemical status (Table 1, and **Tables 2**, **3**, and **4**). Fifty-three percent of children were male, and the mean ± SD age at entrance into the full intervention trial was 20.9 ± 5.4 mo. On average, mothers and fathers had completed 4.3 ± 1.9 and 4.7 ± 1.9 y of education, respectively. Electricity and propane cooking stoves were available in most households (99%), but there was greater diversity in other material possessions and home quality indicators, such as building materials, water source, and latrine facilities. The overall geometric mean plasma zinc concentration at baseline was 70.8 μg/dL (95% CI: 69.6, 72.1 μg/dL), and 31.8% of children had values < 65 μg/dL. The mean plasma copper concentration was 136.4 ± 26.5 μg/dL; <1% of children had values < 80 μg/dL. Mean baseline whole blood hemoglobin was 11.7 ± 1.1 g/dL after adjustment for altitude (22), and the geometric mean serum ferritin concentration among the 3 groups measured was 27.7 ng/mL (95% CI: 24.7, 31.0 ng/mL). Although children were treated for iron deficiency anemia before beginning the study and although they had hemoglobin concentrations ≥ 10.5 g/dL by HemoCue analyses, when status was confirmed by using venous blood samples, 18.0% of children began the study with adjusted hemoglobin concentrations < 11.0 g/dL, and 12.8% had ferritin values < 11.2 ng/mL. The baseline geometric mean serum HDL-cholesterol concentration of the children was 40.1 mg/dL (95% CI: 37.3, 43.0 mg/dL), and their total cholesterol concentration was 106.9 mg/dL (95% CI: 102.4, 111.6 mg/dL). The mean baseline LAZ was −2.3 ± 0.6, and 60% of children were stunted (<−2 LAZ). The mean baseline WAZ was −1.2 ± 0.8, and 15% of children had WAZ <−2. The mean baseline WLZ was −0.1 ± 0.9, and <3% of children had WLZ <−2.

Several differences were found in the children's baseline characteristics by study site. Children in El Carmen began the study with the lowest values for WAZ and WLZ, plasma zinc and copper concentrations, and all 3 SES factors. Children in the Latacunga area tended to be the oldest, and children in the Quito area tended to be the youngest and most stunted. However, children were assigned to treatment groups within each study site, and thus, despite these site-specific differences, there were no significant differences in any initial biochemical or anthropometric indicators by treatment group (Tables 1–4).

Adherence to supplement consumption

Supplement consumption was observed by fieldworkers on 34% of the possible days of administration. The entire supplement was consumed on 95% of the observed days and was reported to be consumed on 91% of the remaining days. According to both observed and reported data, the entire supplement dose was consumed on 91.5% of observed days and at least half of the dose was consumed on another 3% of observed days. The 7 children with

TABLE 1
Baseline characteristics and supplement consumption by intervention group, in Ecuadorian children 12–36 mo old with low initial length-for-age during a 6-mo intervention[1]

	Intervention group					
Variable	Placebo	3 mg Zn/d	7 mg Zn/d	10 mg Zn/d	10 mg Zn + 0.5 mg Cu/d	P^2
Subjects (n)	127	124	126	126	128	
Age (mo)	21.2 ± 5.3[3]	20.9 ± 5.1	20.8 ± 5.2	20.6 ± 5.6	21.2 ± 5.5	0.90[4]
Male (%)	52.8	53.2	53.2	53.2	53.1	1.0[5]
SES factor						
Possession[6]	3.3 ± 1.5	3.3 ± 1.5	3.3 ± 1.7	3.4 ± 1.6	3.3 ± 1.4	0.95[4]
Parental[7]	1.5 ± 0.6	1.5 ± 0.6	1.4 ± 0.6	1.4 ± 0.6	1.5 ± 0.5	0.97[4]
Housing[8]	4.1 ± 1.1	4.1 ± 1.0	4.1 ± 1.1	4.1 ± 1.1	4.1 ± 1.0	0.99[4]
Full supplement consumption (% of d)	92.4 ± 10.8	92.3 ± 12.7	90.9 ± 14.8	92.3 ± 12.5	89.5 ± 15.0	0.52[9]
Partial supplement consumption (% of d)	2.9 ± 6.6	2.2 ± 9.3	3.9 ± 12.4	2.7 ± 10.4	4.9 ± 12.2	0.15[9]
Attrition before 6 mo (% of children)	9.5	12.9	13.5	7.1	13.3	0.40[5]

[1] SES, socioeconomic status.
[2] P values are group-wise.
[3] $\bar{x}$ ± SD (all such values).
[4] ANOVA.
[5] Chi-square test.
[6] Includes ownership of household appliances, animals, and vehicles.
[7] Includes the parents' years of education and vocation.
[8] Includes the materials from which the home was constructed, water source, and type of latrine or toilet facilities and whether the house was rented, owned, or borrowed.
[9] Kruskall-Wallis test.

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TABLE 2
Baseline biochemical indicators, by intervention group, of zinc and iron status and changes from baseline in Ecuadorian children 12–36 mo old with low initial length-for-age during a 6-mo intervention[1]

	Intervention group					
Variable	Placebo	3 mg Zn/d	7 mg Zn/d	10 mg Zn/d	10 mg Zn + 0.5 mg Cu/d	*P*
Maximum number of subjects						
Baseline (*n*)	116	113	117	116	117	
Change from baseline (*n*)[2]	56	50	52	54	54	
Initial plasma zinc concentration (μg/dL)[3]	69.2 (66.5, 72.0)	69.8 (67.1, 72.7)	70.8 (68.0, 73.6)	70.5 (67.8, 73.4)	73.8 (71.0, 76.7)	0.18[4]
Children with initial plasma zinc concentration <65 μg/dL (%)	39.7	31.9	29.1	31.0	27.4	0.31[5]
Change in plasma zinc concentration (μg/dL)[3]	0.2[a] (−5.0, 5.8)	10.5[a,b] (4.1, 17.4)	16.4[b] (9.5, 23.9)	20.8[b] (14.2, 27.9)	16.6[b] (10.0, 23.8)	<0.01[6]
Initial serum CRP >10 mg/L (%)	11.0	15.9	13.8	23.2	14.7	0.13[5]
Initial Hb concentration (g/dL)[7]	12.8 ± 1.4	12.5 ± 1.5	12.7 ± 1.4	12.6 ± 1.3	12.8 ± 1.4	0.64[4]
Change in Hb concentration (g/dL)[8]	−0.68 (−1.05, −0.31)	−0.31 (−0.69, 0.07)	−0.43 (−0.84, −0.02)	−0.39 (−0.75, −0.04)	−0.48 (−0.86, −0.09)	0.71[6]
Initial serum ferritin concentration (ng/mL)[3]	28.1 (23.2, 34.1)	—	—	27.2 (22.3, 33.2)	27.6 (22.7, 33.6)	0.97[4]
Change in serum ferritin concentration (ng/mL)[3]	−8.2 (−11.2, −4.8)	—	—	−5.7 (−9.1, −1.6)	−6.4 (−9.7, −2.5)	0.57[6]

[1] CRP, C-reactive protein; Hb, hemoglobin. Values in a row with different superscript letters were significantly different, $P < 0.01$; the difference between the placebo group and the 3 mg Zn/d group was not significant, $P = 0.08$.
[2] Actual samples analyzed and remaining in final analyses ranged from 44 to 56/group. Smaller sample sizes are due to inadequate sample volumes.
[3] Geometric $\bar{x}$ (95% CI); change in group means was based on the percentage change multiplied by the baseline overall geometric mean. Values were normalized by log transformation for analyses.
[4] Group means were compared by using ANOVA; means in the table were not adjusted for covariates.
[5] Group populations were compared by using a chi-square test.
[6] ANCOVA; means in the table we adjusted for covariates.
[7] Unadjusted $\bar{x}$ ± SD.
[8] Estimated median (95% CI).

TABLE 3
Initial anthropometric measurements and changes from baseline, by intervention group, in Ecuadorian children 12–36 mo old with low initial length-for-age during a 6-mo intervention[1]

	Intervention group					
Variable	Placebo	3 mg Zn/d	7 mg Zn/d	10 mg Zn/d	10 mg Zn + 0.5 mg Cu/d	*P*
Subjects						
Baseline (*n*)	127	123	126	126	128	
Change from baseline (*n*)	108	103	100	110	103	
Length						
Initial (cm)	77.6 ± 4.7[2]	77.3 ± 4.5	77.2 ± 4.6	77.2 ± 4.6	77.5 ± 4.7	0.93[3]
Change (cm)	4.9 ± 1.1	4.9 ± 1.2	5.0 ± 1.0	4.8 ± 1.2	4.9 ± 1.1	0.34[4]
LAZ						
Initial	−2.3 ± 0.7	−2.3 ± 0.6	−2.3 ± 0.7	−2.2 ± 0.6	−2.3 ± 0.6	0.74[3]
Change	0.11 ± 0.32	0.11 ± 0.30	0.15 ± 0.29	0.05 ± 0.33	0.10 ± 0.30	0.59[4]
Weight						
Initial (kg)	9.7 ± 1.3	9.8 ± 1.3	9.7 ± 1.2	9.7 ± 1.4	9.8 ± 1.2	0.82[3]
Change (kg)	1.0 ± 0.4	1.1 ± 0.5	1.1 ± 0.4	1.0 ± 0.5	1.1 ± 0.4	0.33[4]
WAZ						
Initial	−1.3 ± 0.8	−1.2 ± 0.8	−1.2 ± 0.7	−1.2 ± 0.8	−1.2 ± 0.7	0.59[3]
Change	0.04 ± 0.33	0.07 ± 0.45	0.06 ± 0.30	0.02 ± 0.41	0.06 ± 0.34	0.66[4]
WLZ						
Initial	−0.2 ± 0.8	−0.1 ± 0.9	−0.1 ± 0.8	−0.2 ± 1.0	−0.0 ± 0.8	0.36[3]
Change	0.04 ± 0.46	0.09 ± 0.65	0.05 ± 0.42	0.07 ± 0.57	0.07 ± 0.46	0.72[4]

[1] LAZ, length-for-age *z* score; WAZ, weight-for-age *z* score; WLZ, weight-for-length *z* score.
[2] $\bar{x}$ ± SD (all such values).
[3] Group means were compared with ANOVA for baseline variables.
[4] Group means were compared with ANCOVA for change variables, after control for significant covariates and baseline values.

very low consumption rates (consumption of at least half the dose on <60% of study days) came from all 5 study groups, but 3 of them were in the placebo group, and 2 left the study before reaching 30 d of data collection. There were no significant differences by treatment group in the percentage of children who were consuming the entire supplement ($P = 0.45$; Table 1) or at least half of the supplement ($P = 0.73$).

Plasma zinc concentration

The mean change in plasma zinc concentrations from baseline increased progressively with higher doses of supplemental zinc ($P < 0.001$) (Table 2). Regression analysis indicated that the increase in plasma zinc concentration did not deviate significantly from linearity within the range of doses provided in this study (**Figure 2**).

Morbidity

Among children consuming the placebo supplement, the estimated median incidence of diarrhea was 1.9 episodes/100 d of observation. The incidence of diarrhea was 21–42% lower in children consuming any dose of zinc than in those consuming placebo ($P < 0.01$ for group-wise comparison). Tukey-Kramer analyses indicated that the effect of treatment group on the incidence of diarrhea was significant for doses of 3 and 7 mg Zn/d ($P = 0.04$ and $P < 0.01$, respectively) relative to placebo (**Figure 3**). Similar patterns were observed among boys and girls (data not shown).

Initial age, WLZ, and parental SES factors were significantly ($P < 0.001$, $P = 0.038$, and $P = 0.028$, respectively) associated with diarrhea incidence. In particular, diarrhea rates decreased as initial age, parental SES factors, and initial WLZ increased. Further analyses showed a significant interaction between treatment group and age ($P < 0.001$ for incidence). To assess these effects, adjusted group means were calculated at ages 17.5 and 23.5 mo, which were the cutoffs for age tertiles (ie, 11.5–17.4, 17.5–23.4, and 23.5–30 mo old at baseline). The general model is shown in **Table 5**.

Subanalyses by age tertile showed a significantly ($P < 0.001$) greater effect of zinc than of placebo on the incidence of diarrhea in children in the youngest age tertile who received any dose of zinc (Tukey-Kramer test; **Figure 4**). No significant group-wise differences were found among children in the 2 older age tertiles (≥17.5 mo at baseline). However, the 11–39% reductions in diarrhea incidence in the older children who received 3 or 7 mg Zn are of a magnitude similar to the changes reported by Bhutta et al (2) in a pooled analysis of zinc intervention studies, although with larger CIs. Thus, the lack of statistical significance among the older children in the present study may be due to an inadequate sample size. These patterns did not differ significantly by study site (data not shown).

The overall mean percentage (and 95% CI) of days on which other morbidity symptoms were present were as follows: 4.4% (3.9, 4.9) for poor appetite, 15.2% (13.9, 16.7) for cough, 2.5% (2.2, 2.7) for reported fever, 2.7% (2.5, 2.9) for purulent nasal discharge, and 2.3% (1.9, 2.7) for acute upper respiratory infection. The overall proportion of days with vomiting, earache or discharge from the ear, respiratory rate > 40 rpm, and acute lower respiratory infection was <1%. There were no significant

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TABLE 4
Initial concentrations, by intervention group, of markers of copper status and lipoprotein concentrations and changes from baseline in Ecuadorian children 12–36 mo old with low initial length-for-age during a 6-mo intervention[1]

Variable	Intervention group			
	Placebo	10 mg Zn/d	10 mg Zn + 0.5 mg Cu/d	*P*
Maximum subjects (*n*)[2]	49	53	49	
Plasma copper concentration (μg/dL)				
Initial[3]	135.8 ± 25.4	131.5 ± 23.1	143.2 ± 30.1	0.10[4]
Change[5]	−14.4 (−20.9, −7.9)	−13.0 (−19.5, −6.4)	−8.9 (−15.6, −2.2)	0.49[6]
Serum ceruloplasmin activity (IU)[7]				
Initial	221.9 (203.6, 241.8)	205.2 (187.8, 224.2)	228.7 (209.7, 249.4)	0.21[4]
Change	−23.4 (−38.3, −7.3)	−18.3 (−34.3, −0.9)	−24.5 (−40.2, −7.3)	0.86[6]
Erythrocyte SOD activity (units/mg Hb)[7]				
Initial	1.65 (1.38, 1.98)	1.48 (1.22, 1.78)	1.66 (1.37, 2.02)	0.61[4]
Change	−0.04 (−0.19, 0.12)	0.21 (0.03, 0.41)	0.12 (−0.06, 0.32)	0.12[6]
Serum HDL-cholesterol concentration (mg/dL)[5]				
Initial	38.2 (33.9, 43.1)	44.6 (39.3, 50.7)	38.1 (33.8, 43.0)	0.14[4]
Change	5.6 (1.4, 10.3)	3.4 (−0.9, 8.2)	6.3 (1.9, 11.2)	0.66[6]
Serum total cholesterol concentration (mg/dL)[7]				
Initial	108.6 (100.8, 116.9)	105.2 (97.4, 113.6)	106.9 (99.3, 115)	0.84[4]
Change	5.6 (1.4, 10.3)	3.4 (−0.9, 8.2)	6.3 (1.9, 11.2)	0.94[6]

[1] SOD, superoxide dismutase; Hb, hemoglobin.
[2] Actual samples analyzed and remaining in final analyses ranged from 39 to 53/group. Smaller sample sizes are due to inadequate sample volumes.
[3] Unadjusted $\bar{x}$ ± SD.
[4] ANOVA.
[5] Estimated median; 95% CI in parentheses.
[6] ANCOVA.
[7] Geometric $\bar{x}$; 95% CI in parentheses. Values were normalized by log transformation for analyses, and change in group means was based on the percentage change multiplied by the baseline overall geometric mean.

group-wise differences in any of these symptoms or illness categories (data not shown) or in any other symptoms or illness categories (**Table 6**).

Anthropometry

During the 6-mo intervention, the enrolled children gained 4.9 ± 1.1 cm in height and 1.1 ± 0.4 kg in weight. LAZ increased by 0.1 ± 0.3, WAZ by 0.05 ± 0.37, and WLZ by 0.07 ± 0.52. However, no significant group-wise differences were found between changes in length, weight, or anthropometric *z* scores, even after control for initial age, LAZ, WLZ, parental SES factor, and study site or when analyses were restricted to children with initial LAZ of <−2.0 (Table 3).

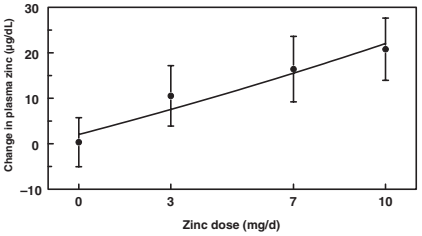

FIGURE 2. Mean (and 95% CI) change in plasma zinc concentration in Ecuadorian children 12–36 mo old with low initial length-for-age during the 6-mo intervention, by dose of zinc; 95% CI and best fit regression line showed no significant deviation from linearity. Group means (and 95% CIs) were based on the percentage change multiplied by the baseline overall geometric mean. $P < 0.001$ overall as analyzed with ANCOVA. The cubed term was not significant, $P = 0.28$. Subjects by group: placebo, $n = 49$; 3 mg Zn/d, $n = 45$; 7 mg Zn/d, $n = 44$; 10 mg Zn/d, $n = 53$.

Markers of copper and iron status and serum lipoprotein concentrations

Plasma copper and serum ceruloplasmin concentrations declined in all children during the course of the study; there were no

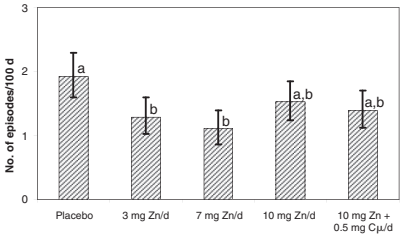

FIGURE 3. Estimated median (and 95% CI) incidence of diarrhea in Ecuadorian children 12–36 mo old with low initial length-for-age during the 6-mo intervention, by intervention group. Values with different letters are significantly different between groups (ANCOVA). Subjects by group: placebo, $n = 116$; 3 mg Zn/d, $n = 117$; 7 mg Zn/d, $n = 116$; 10 mg Zn/d, $n = 118$; 10 mg Zn + 0.5 mg Cu, $n = 121$.

TABLE 5
Results of statistical models testing for main effects of treatment on the incidence of diarrhea with significant covariates and selected interactions, conducted among Ecuadorian children 12–36 mo old with low initial length-for-age[1]

	Main-effects model		Interaction model	
Individual variables or interactions	β coefficient	P	β coefficient	P
Treatment group		0.004		0.032
Initial age (mo)	−0.034	<0.001	−0.063	<0.001
Parental SES factor	−0.114	0.018	−0.120	0.028
Initial WLZ	−0.075	0.025	−0.071	0.038
Initial plasma zinc concentration	—	NS	−0.499	0.071
Initial age × treatment group		—		0.039
Initial plasma zinc concentration × treatment group		—		0.081

[1] SES, socioeconomic status; WLZ, weight-for-length z score; LAZ, length-for age z score. Interactions that were tested but were not significant: initial WLZ × treatment group, parental SES factor × treatment group, and initial LAZ × treatment group.

significant group-wise differences in these changes (Table 4). There was no consistent or significant change in erythrocyte SOD activity among the 3 groups ($P = 0.12$). Mean hemoglobin and ferritin concentrations decreased during the study intervention in all groups, but there were no significant group-wise differences in the change in either of these markers of iron status (Table 2).

The mean serum HDL-cholesterol concentration increased in all groups by 4.9 ± 17.5 mg/dL during the intervention period. There was no change in total cholesterol during the 6-mo intervention, and there were no significant group-wise differences in the mean change in serum HDL or total cholesterol during the 6-mo intervention ($P = 0.83$ and $P = 0.66$, respectively; Table 4).

DISCUSSION

We found that the mean plasma zinc concentrations of the children increased progressively in relation to the dose of supplemental zinc. In addition, there were greater reductions in the incidence of diarrhea in children who received supplemental zinc than in those who received the placebo, and these differences were significant for groups that received 3 or 7 mg supplemental Zn/d. There were no other significant group-wise differences in symptoms of morbidity or rates of growth between children who received any dose of supplemental zinc and those who received the placebo. Finally, there were no adverse effects of 10 mg supplemental Zn/d on markers of copper and iron status or on lipoprotein concentrations. The randomized clinical trial design, blinding of investigators and participants to treatment group, selection of participants on the basis of the likelihood of finding zinc deficiency, and the use of intention-to-treat analyses lend strength to the findings of the present study.

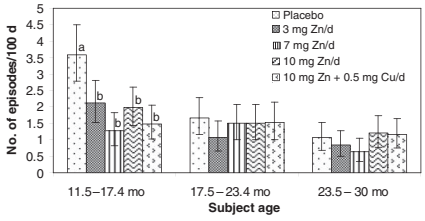

FIGURE 4. Estimated median (and 95% CI) incidence of diarrhea in Ecuadorian children 12–36 mo old with low initial length-for-age during the 6-mo intervention, by intervention group and age tertile. $P < 0.001$ overall. Values with different letters are significantly different between groups (ANCOVA). Subjects by age tertile and group—age 11.5–17.4 mo: placebo, $n = 36$; 3 mg Zn/d, $n = 39$; 7 mg Zn/d, $n = 39$; 10 mg Zn/d, $n = 42$; 10 mg Zn + 0.5 mg Cu, $n = 39$; age 17.5–23.4 mo: placebo, $n = 38$; 3 mg Zn/d, $n = 40$; 7 mg Zn/d, $n = 40$; 10 mg Zn/d, $n = 38$; 10 mg Zn + 0.5 mg Cu, $n = 38$; age 23.5–30 mo: placebo, $n = 42$; 3 mg Zn/d, $n = 38$; 7 mg Zn/d, $n = 39$; 10 mg Zn/d, $n = 38$; 10 mg Zn + 0.5 mg Cu, $n = 44$.

Response of plasma zinc concentration to zinc dose

The increases in plasma zinc concentration that we observed in response to supplemental zinc confirm that the intervention was successfully administered (28, 29). Tracer studies in adults and children have shown that the total amount of absorbed zinc increases linearly in relation to the test dose, with doses ranging from 1 to 6 mg Zn (30–32). However, the magnitude of the increase in zinc absorption is progressively less with higher doses, ie, 9–30 mg Zn/d (30–32). We observed no significant deviation from linearity in the response of plasma zinc concentrations to zinc doses ranging from 3 to 10 mg Zn/d (Figure 2).

Response of diarrhea to zinc dose

The reductions we observed in the incidence of diarrhea after zinc supplementation are consistent with reductions reported in a pooled analysis of zinc intervention studies in which the provided single doses of supplemental zinc ranged from 5 to 20 mg/d to infants and children (2). By simultaneously assessing 3 different doses of zinc, we were able to determine that 3 mg supplemental Zn/d was sufficient to reduce the incidence of diarrhea in the current study population, and the greatest effect occurred with doses of 3 and 7 mg Zn/d. These findings support the recommendation of the IZiNCG (13) to provide 5 mg prophylactic supplemental Zn/d to children at risk of zinc deficiency. This dose-response design also exposed an interesting interaction between age at baseline and the supplemental dose of zinc for rates of diarrhea, which suggested that the greatest effect of supplemental zinc occurred among the children in the youngest age tertile, and that the children responded to the lower doses of supplemental zinc but not to the highest dose.

TABLE 6
Duration of diarrhea and percentage of days with selected symptoms of illness, by intervention group, in Ecuadorian children 12–36 mo old with low initial length-for-age during a 6-mo intervention

	Intervention group					
Variable	Placebo	3 mg Zn/d	7 mg Zn/d	10 mg Zn/d	10 mg Zn + 0.5 mg Cu/d	*P*
Subjects (*n*)	116	117	116	118	121	
Duration of episodes of diarrhea[1]	1.71 (1.57, 1.87)	1.81 (1.63, 2.02)	1.82 (1.65, 2.01)	1.72 (1.59, 1.86)	1.72 (1.56, 1.90)	0.82[2]
Days with low appetite (%)[4]	4.6 (3.6, 5.8)	4.2 (3.2, 5.3)	4.1 (3.1, 5.2)	4.6 (3.6, 5.7)	4.9 (3.8, 6.0)	0.76[3]
Days with cough (%)[4]	18.8 (16.1, 21.7)	14.8 (12.4, 17.5)	15.4 (12.9, 18.1)	14.7 (12.3, 17.3)	10.5 (14.5, 19.9)	0.14[3]
Days with reported fever (%)[4]	2.9 (2.4, 3.4)	2.1 (1.7, 2.6)	2.3 (1.9, 2.8)	2.1 (1.7, 2.6)	2.5 (2.0, 3.0)	0.18[3]
Days with nasal discharge (%)[4]	2.1 (1.5, 2.7)	2.0 (1.5, 2.6)	1.3 (0.9, 1.8)	1.5 (1.1, 2.0)	1.5 (1.1, 2.1)	0.16[3]

[1] Geometric $\bar{x}$; 95% CI in parentheses.
[2] Values were normalized for ANCOVA by log transformation.
[3] Values were normalized for ANCOVA by square root transformation.
[4] Estimated median; 95% CI in parentheses.

In general, the risk of zinc deficiency in children decreases with age, as growth velocity slows and more food sources of zinc are introduced into the diet (33). Thus, there is reason to believe that the younger children in this study were at the greatest risk of zinc deficiency. Moreover, in the placebo groups, the proportion of days with diarrhea was nearly two-thirds less in children ≥17.5 mo old (2.3%) than in those <17.5 mo old (7.1%), which substantially reduces the power to detect differences among the older children. Nevertheless, there were no significant differences in mean baseline plasma zinc concentrations by age tertile, so we cannot confirm whether there were indeed age-related differences in zinc status.

The absence of a reduction in rates of diarrhea in the older children consuming 10 mg supplemental Zn/d could be due to the lack of a beneficial effect or to the presence of an adverse effect of this dose of supplemental zinc in these children. Intestinal perfusion studies (34, 35) showed positive net water and sodium absorption at lower zinc concentrations (≈30–270 μmol Zn/L) and net losses at higher concentrations (≈270–1070 μmol Zn/L), which suggested a possible reversal at high doses of zinc's beneficial effect of reducing diarrhea. Because the incidence of other morbidity symptoms was low in the study population, it is likely that the sample size was inadequate to detect other group-wise differences in morbidity.

Anthropometry

It was surprising that we found no change in growth rate in response to zinc supplementation in these children with presumed zinc deficiency, even after exploring for possible modifying effects of initial age, LAZ, and plasma zinc concentration. Although it is more likely to see a growth response in younger than in older children, we found no significant interaction by initial age. As described previously, the doses of zinc provided were sufficient to increase the plasma zinc concentration (Table 2), which indicated that bioavailability of the zinc provided was not a limiting factor. Previous studies found growth responses in stunted children consuming doses of 10 mg supplemental Zn/d (9), which suggested that the 10-mg dose of supplemental zinc should have been adequate to promote a growth response in the population of the present study. Therefore, it is possible that zinc was not limiting the growth response of the children in the current study or that another factor was preventing these children from responding to zinc with increased growth rates. Analyses of dietary data may provide more insight into other macronutrient or micronutrient deficiencies that could have contributed to the stunting found among the children in the present study.

Possible adverse effects of zinc

Our findings that markers of copper and iron status and lipoprotein concentrations did not differ significantly between children receiving 10 mg supplemental Zn/d, with or without copper, and those receiving placebo are consistent with the results of most studies among young children in which copper markers were analyzed (7, 36–42). Five of these 8 studies provided 10 mg supplemental Zn/d. We do not have an explanation for the downward trend in copper markers observed from baseline to 6 mo among all treatment groups in the current study. However, these results are consistent with the changes observed in the placebo group of other zinc intervention studies that enrolled children 1–3 y old (37–40, 42) and reported 5–13% decreases in erythrocyte SOD activity or serum copper concentrations after 4–15 mo of intervention.

Conclusions

The results of the present study confirm that a rise in plasma zinc concentration may be used as an indicator of successful provision of supplemental zinc in a population of young children, even at doses as low as 3 mg/d. The results also indicate that it may be possible to provide as little as 3–7 mg supplemental Zn/d to reduce the incidence of diarrhea in young children at risk of zinc deficiency. Indeed, there is some suggestion that the beneficial effect of zinc on diarrheal morbidity may not occur with larger doses of zinc, especially in older children. More dose-response studies are needed in other populations of young children to confirm these findings. We observed no adverse effects of 10 mg supplemental Zn/d on indicators of copper and iron status in this study population, which indicates that the tolerable UL of zinc recommended by the Institute of Medicine and the IZiNCG may be set unnecessarily low. Studies reporting the effect of zinc on copper and iron status markers and on lipoprotein concentrations in children should be reviewed to determine whether there is now sufficient

information to reassess the recommended tolerable UL of zinc for young children.

We acknowledge the hard work of all the health workers, office and laboratory staffers, nutritionists, and doctors who were instrumental in completing this study in Ecuador, and we acknowledge Janet Peerson for assistance with the statistical analyses. In particular, we thank each of the children and the parents whose participation made this study possible.

The authors' responsibilities were as follows—SEW: the study design, implementation of the project in the field, data analysis, interpretation of results, and preparation of the manuscript; KHB: the study design, overall supervision of the research team, interpretation of results, and preparation of the manuscript; and FS: contributions to the study design, implementation of the project in the field, and interpretation of the results. None of the authors had any personal or financial conflict of interest.

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Chapter 4

Effects of short-term zinc supplementation on cellular immunity, respiratory symptoms, and growth of malnourished Equadorian children

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European Journal of Clinical Nutrition 1996; 50; 42-46

European Journal of Clinical Nutrition (1996) 50, 42–46

Effects of short-term zinc supplementation on cellular immunity, respiratory symptoms, and growth of malnourished Equadorian children

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Objective: To assess the effect of zinc supplementation on respiratory tract disease, immunity and growth in malnourished children.
Design: A randomized double-blind placebo-controlled trial.
Setting: A day-care center in Quito, Ecuador.
Subjects: Fifty children (12–59 months old) recruited by height-for-age and weight-for-age deficit.
Interventions: Twenty-five children (supplemented, S group) received 10 mg/day of zinc as zinc sulfate, and 25 (nonsupplemented, NS group) received a placebo during 60 days. All were also observed during a 60-day postsupplementation period. Two children of the S group dropped out. Daily the clinical presence of cough, respiratory tract secretions, and fever, was recorded. On days 0, 60 and 120, the cutaneous delayed-type hypersensitivity (DTH) to multiple antigens, and anthropometric parameters were assessed. On days 0 and 60 serum zinc levels were also measured.
Results: On day 60, DTH was significantly larger (20.8 ± 7.1 *vs* 16.1 ± 9.7 mm), and serum zinc levels were significantly higher (118.6 ± 47.1 *vs* 83.1 ± 24.5 µg/dl) in the S group than in the NS group ($P < 0.05$ for each). The incidence of fever [relative risk (RR): 0.30, c.i. = 0.08–0.95, $P = 0.02$], cough (RR): 0.52, c.i. = 0.32–0.84, $P = 0.004$) and upper respiratory tract secretions (RR):0.72, c.i. = 0.59–0.88, $P = 0.001$) was lower in the S group than in the NS group at day 60. At the end of the postsupplementation observation period (day 120), the incidence of fever and upper respiratory tract secretions was the same in both the S and NS groups. The incidence of cough was higher at day 120 in the S group than in the NS group (RR): 2.28, c.i. = 1.37–3.83, $P = 0.001$).
Conclusions: This study supports a role for zinc in immunity, and immunity to respiratory infections, while pointing out the need for larger studies.
Sponsorship: Universidad Central del Ecuador. This work was supported by the Ministry of Public Health of Ecuador. Pasteur–Mérieux Laboratories and QUIFATEX of Ecuador donated skin multitest kits, and Nestlé-Latinreco (Ecuador) provided technical assistance.
Descriptors: immune response, malnourished, respiratory infections, zinc

Introduction

In 1988 the National Council of Development and the Ministry of Public Health of Ecuador determined the deficiency rates of total calories, proteins, vitamins and essential elements such as iron and zinc in malnourished children less than 5 years of age in Ecuador. Forty per cent of children under 5 years of age had low serum zinc levels (< 70 µg/dl). Though children in Quito had an overall zinc deficiency rate of 33%, children in rural and Andean areas were especially deficient (54% and 45%, respectively) (Freire *et al*, 1988). Although low serum zinc is not the best indicator of inadequate zinc status, these published figures suggest that zinc deficit is both more common and more profound in Ecuador than in other countries in Central and South America (Fisberg *et al*, 1984; Rodríguez *et al*, 1984). In addition, C-reactive protein (CRP) and α-1 acid protein were assessed in these children as an indirect measure of infection and inflammation. In 54% of the children with low zinc levels, both CRP and α-1 acid protein serum levels were elevated (> 12 mg/l and > 1.4 g/l, respectively) (Freire *et al*, 1988). Thus in Ecuador zinc deficiency, malnutrition and biochemical indicators of chronic infection and inflammation are associated.

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Received 31 March 1995; revised 12 June 1995; accepted 12 July 1995

Many of the best sources of dietary zinc – meat, milk, milk products and cereals – are not readily available to Equadorian families with low incomes.

Some studies have reported that zinc deficit affects the cellular immunity (Ebadi & Swanson, 1988; Zeng *et al*, 1991). Zinc deficit reduces cutaneous delayed-type hypersensitivity (DTH) (Fraker *et al*, 1987). In addition to these general effects on immunity, low serum zinc has been correlated with respiratory infection (Taneja, 1990). Zinc deficit has been associated with growth retardation in animals and humans (Underwood, 1977). Recently it has been communicated that zinc ions induce monokine secretion and subsequent T-cell activation (Driessen *et al*, 1994). Moreover, zinc supplementation has improved nutritional recovery in malnourished children (Walravens & Hambidge, 1976; Golden & Golden, 1981; Walravens *et al*, 1983).

Since, as in Ecuador, a leading cause of morbidity and mortality in malnourished children under 5 years of age is respiratory infection (Ministerio de Salud Pública, 1992), we evaluated the impact of zinc supplementation in a group of malnourished children from a high Andean urban slum, examining respiratory symptoms, cutaneous delayed-type hypersensitivity and anthropometric parameters before, during and after supplementation with zinc sulfate or a placebo. This study was undertaken in accordance with the nutritional subcommittees of the United Nations recommendations (Tomkins & Watson, 1989).

Subjects, materials and methods

Characteristics of the study population and informed consent

Fifty children between 12 and 59 months of age who attended a day-care center (centro infantil No. 1 CAI. National Institute for the Children and the Family) for at least 6 months were subjects of the study. This number represents the entire day-care center malnourished population in this age. This center is located in the 'Comité del Pueblo' (People's Committee), an urban marginal neighborhood of households of low social economic status in the northeast region of the city of Quito, Ecuador. On day 0 of the study, the children were characterized as malnourished according to height and weight parameters from the National Center for Health Statistics from USA (NCHS) (World Health Organization, 1983*a*). We defined children as acutely malnourished if weight-for-age was under the 10th percentile, and as chronically malnourished if the height-for-age was under the 10th percentile. Weight was measured with a Detecto® (Brooklyn, USA) balance calibrated by INEN (Equadorian Institute for Normalization), and recorded in kilograms. The length of children under 2 years of age, or the height of older children, was obtained with a foot-board or with a calibrated scale, and recorded in centimetres. These devices were also calibrated by INEN. Both height and weight were measured on days 60 and 120.

Children received the same breakfast and lunch at the day-care center. Three direct random observations of dietary intake based on food quantity weighed at one time were carried out during the first 60 days of the study. The estimated daily zinc intake per child at the day-care center was 5.9 mg of elemental zinc. Mean daily kJ provided at the day-care center was 3120 (747.6 kcal), based principally on rice, bread, potatoes, milk and sugar. This estimation did not include the dinner that children probably received at home.

Parents were informed about the investigation aims, objectives, risks and potential benefits before informed consent was sought. Formal written consent was freely obtained from the parents of all children after they had a clear understanding of the study. The protocol was approved by the Ethical Committee from the Ministry of Public Health (National Research Institute of Health, IIDES).

Design

Malnourished children were randomly assigned, by the Moses-Oakford algorithm (Nicole, 1986), to the active supplementation group (S; $n = 25$) or to the placebo, nonsupplemented group (NS; $n = 25$) on day 0 of the study.

Children in the supplemented group received 10 mg/day of zinc as zinc sulphate (American Food and Nutrition Board, 1980), whereas nonsupplemented children received a placebo during the first 60 days of the study (supplementation period). The S and NS groups were observed over both the supplementation period (days 1–60) and the subsequent 60 days (postsupplementation, observation period, days 61–120).

During each weekday of the 120 days of the study, each child was examined clinically by a pediatrician, who was a member of the investigation team, for respiratory signs and symptoms. Individual records were maintained on each child for the following signs and symptoms: upper respiratory tract secretions by the examining of nasal and/or post-nasal discharge, cough, lower respiratory tract secretions as assessed by thoracic ausculation, and fever (rectal temperature $>37.8°C$).

Serum zinc levels were measured on days 0 and 60 (see method below), and cutaneous delayed-type hypersensitivity reactions to multiple antigens were quantified on days 0, 60 and 120 (see method below).

Supplementation

Zinc and placebo syrups had an identical appearance and flavor (pink with strawberry flavor). The syrup with zinc had 10 mg of zinc as zinc sulfate per cc. The syrup was prepared in Quito, at the School of Chemistry at the Central University of Ecuador. The NS group received syrup 'A' that contained placebo. The S group was given syrup 'B' that contained zinc. Monday through Friday the syrups were administered in the day-care center by two pediatricians (FN and LA) who did not know which group was the actively supplemented group until after the study was completed, and who were not involved in the daily clinical examination of the children. The code was kept by the Ethical Committee until the end of the study. During weekends and holidays, the mother of each child administered the syrup. Each mother was instructed in the administration of the syrup and received an eyedropper bottle of syrup (calibrated). The day after weekends and holidays we asked her if the syrup had been given to her child, and also inquired about the child's health over the weekend or holidays. A daily record of supplementation was kept.

Serum zinc measurement
In all children, at the beginning of the study and on day 60, we measured serum zinc levels in 150 µl of serum by atomic absorption spectrometry using a Perkin–Elmer Spectrophotometer (Model 4000, Norwalk, CT). We obtained 2.5 ml of peripheral venous blood after the child had fasted for 12 h. We used a vacutainer tube without anticoagulant and a 21 × 1 needle. Blood was centrifuged at once and serum was disposed into a sterile zinc-free glass tube and frozen at −20°C until analysis. All glassware was treated with a sulfochromic acid, and nitric acid wash (24 h each). For each set of samples distilled water controls were tested for residual zinc. The determination was performed at the National Polytechnic School (JC). We performed interlaboratory quality control studies with the Latinreco Nestlé Laboratory in Quito, Ecuador. A batch of 30 serum samples with a code number was tested in both laboratories ($r = 0.70$, c.i. = 0.44–0.84).

Skin testing for delayed-type hypersensitivity (DTH)
At the beginning of the study, all children had a cutaneous DTH test, and were reassessed on days 60 and 120. We used the CMI skin Multitest kindly provided by Pasteur–Mérieux Laboratories (Paris, France). This multivalent, multi-pronged test contains a trigger dose (recall antigens) of tetanus, diphteria, streptococcus, proteus, tuberculin, trichophyton and Candida, and glycerine as a negative control. We followed Mérieux technical recommendations in applying and reading the test: cleanness and supervision of the puncture area, time and pressure needed, postapplication care, and readings of induration 48 h after the test was applied. The multitest was applied in the dorsal area between the left scapula and the spinal column given the ages of the children involved in the study. In this way we obtained a large and flat area of skin with easy access, so as to minimize any technical errors.

To read the DTH responses, we used a circular ruler calibrated in millimetres. We measured the vertical and horizontal diameters and considered the reaction positive when the mean value was ≥ 2 mm. All children were included in the test because none had cutaneous infection or viral disease in the 15 preceding days.

Standardization
Investigators made repetitive Multitest CMI applications and skin test readings in order to minimize inter-observer variance. The differences between each researcher and a reference observer, who was the most experienced, were examined statistically via Student's *t*-test. The procedures were repeated until P value was >0.05. Height and weight measurements were standardized according to WHO guidelines (World Health Organization, 1983*b*).

Statistical analysis
We calculated the mean ± s.d. of height-for-age Z score (HAZ), and the mean ± s.d. of weight-for-age Z score (WAZ). In addition, we calculated the mean ± s.d. of serum zinc level. The values of the skin responses to the different antigens in each child were summed to determine an individual score. In all groups we obtained a mean score ± s.d. We also calculated the mean ± s.d. of positive antigen tests. The differences between supplemented and placebo groups were examined by ANOVA test. We accepted $P < 0.05$ as a significant value. In each group the incidence rate (%) of respiratory signs and symptoms during the periods of supplementation and postsupplementation was calculated. We used as denominator the number of observations of each group in the periods. Relative risk (incidence rate in the S group/incidence rate in the NS group), 95% confidence interval (c.i.) and significance tests (χ^2) were also calculated. Statistical analysis was performed in an IBM-compatible computer with the Epi Info version 6.0 program.

Results

Forty-eight malnourished children finished the study because two malnourished children from the S group were lost to the follow-up when their families moved to another province. On day 0, the zinc and placebo groups were not significantly different in relation to the variables of the study (Table 1). The proportion of cronically malnourished children was similar in both zinc and placebo groups (92 *vs* 95%, respectively).

On day 60, at the end of the supplementation period, the summed mean cutaneous DTH responses to the multitest antigens, and the mean number of positive tests, were higher in the group that received the zinc supplement when compared to the placebo group ($P < 0.05$ for both, Table 2). In the group that received zinc supplementation the incidence of fever, cough and upper respiratory tract secretions (URTS) was lower than in the placebo group (relative risk = 0.30, 0.52 and 0.72 for each, respectively) for the cumulative period days 1–60 (Table 3). No cases of lower respiratory tract secretions (LRTS) were detected in this period in both S and NS groups. On day 60 serum zinc levels (µg/dl) were significantly higher in the supplemented group than in the placebo group (118.6 ± 47.1 *vs* 83.1 ± 24.5, $P < 0.05$). Serum zinc level increased 34% over the baseline in the supplemented group (88.5 ± 14.9 *vs* 118.6 ± 47.1, $P = 0.04$); there was no change in the placebo group (84.6 ± 17.8 *vs* 83.1 ± 24.5).

On day 120, 60 days after supplementation had ended, the mean summed cutaneous DTH reactions, and the mean number of positive cutaneous tests, remained higher in the zinc supplemented group, but

Table 1 Characteristics of zinc and placebo groups (day 0)

	Zinc (*n* = 23)	*Placebo* (*n* = 25)	*P*
Age (months) (mean ± s.d.)	42.4 ± 14.7	42.3 ± 14.1	n.s.
Sex (females) (%)	43.5	44.0	n.s.
WAZ (mean ± s.d.)	−1.4 ± 0.6	−1.4 ± 0.5	n.s.
HAZ (mean ± s.d.)	−2.1 ± 0.7	−1.9 ± 0.9	n.s.
DTH (mm) (mean ± s.d.)	18.2 ± 6.7	20.7 ± 9.5	n.s.
Positive cutaneous tests (mean ± s.d.)	4.0 ± 1.3	4.0 ± 1.5	n.s.
ZINC (µg/dl) (mean ± s.d.)	88.5 ± 14.9	84.6 ± 17.8	n.s.

DTH = delayed-type hypersensitivity; WAZ = weight-for-age Z score; HAZ = height-for-age Z score.

Table 2 Anthropometric variables, and DTH in children who took zinc or placebo (60 and 120 days)

	Day 60			*Day 120*		
	Zinc (*n* = 23)	*Placebo* (*n* = 25)	*P*	*Zinc* (*n* = 23)	*Placebo* (*n* = 25)	*P*
WAZ (mean ± s.d.)	−1.3 ± 0.6	−1.3 ± 0.5	n.s.	−1.2 ± 0.6	−1.3 ± 0.5	n.s.
HAZ (mean ± s.d.)	−1.8 ± 0.7	−1.7 ± 0.8	n.s.	−1.8 ± 0.7	−1.7 ± 0.8	n.s.
DTH (mm) (mean ± s.d.)	20.8 ± 7.1	16.1 ± 9.7	<0.05	17.0 ± 7.1	15.7 ± 5.5	n.s.
Positive cutaneous tests (mean ± s.d.)	4.4 ± 1.6	3.4 ± 1.9	<0.05	4.1 ± 1.6	3.8 ± 1.3	n.s.

DTH = delayed-type hypersensitivity; WAZ = weight-for-age *Z* score; HAZ = height-for-age *Z* score.

the difference was not significant ($P = 0.4$ and $P = 0.5$, respectively). There was no significant difference in the cumulative incidence of fever or URTS between the supplemented and placebo groups during the period from days 61 to 120 (Table 3). The incidence of cough as an isolated symptom was higher in the formerly supplemented group than in the placebo group (relative risk = 2.28) (Table 3). No cases of LRTS were detected in this period in both S and NS groups.

Neither at 60 days, nor at 120 days, did we find any significant difference in anthropometric characteristics between the supplemented and placebo groups (Table 2).

Discussion

Mean serum zinc levels in the malnourished children who received zinc increased by 34% during the period of supplementation. In contrast, there was no significant change in the mean serum zinc levels of malnourished children who received a placebo. This strongly suggests that increased absorption of zinc occurred during the supplementation period in those children who received zinc supplements. The baseline zinc levels may represent the steady-state levels consistent with the dietary intake of these children.

No differences in growth were seen in the supplemented and placebo groups during the course of this study. This may be due in part to the short period of supplementation and the age of the children involved in the study. Linear growth in children of this age is not so vigorous that a difference may be detected after only 2 months of active supplementation. A number of prior studies on the effects of zinc supplementation on growth are contradictory (Bates *et al*, 1984; Gibson *et al*, 1989; Walravens *et al*, 1989; Cavan *et al*, 1993*a*,*b*).

After zinc supplementation, malnourished children improved their cutaneous DTH responses. Since no other dietary changes were made, it is likely that these improved DTH responses were in fact secondary to zinc repletion. This finding is consistent with other reports on enhanced cellular immunity with zinc supplement (Singh *et al*, 1992; Lincastro *et al*, 1994).

The incidence of upper respiratory secretions, fever and cough was significantly higher in the unsupplemented group of malnourished children than in the supplemented group during the period of zinc supplementation. Children of this age frequently have upper respiratory tract viral infections, marked precisely by these symptoms. While we do not have bacteriologic or viral isolation data to support on etiologic basis to the respiratory tract illnesses suffered by these children, we note that in a number of studies zinc supplementation has assisted in the amelioration of respiratory tract viral disease. For example, zinc gluconate lozenges reduced the severity and common cold duration (Eby *et al*, 1984; Al-Nakib *et al*, 1987; Godfrey *et al*, 1992).

Table 3 Respiratory symptoms in children who took zinc or placebo (60 and 120 days)

	Day 60					*Day 120*				
	Zinc r/o	*Placebo* r/o	*RR*	*95% c.i.*	*P*	*Zinc* r/o	*Placebo* r/o	*RR*	*95% c.i.*	*P*
URTS	272/814	404/983	0.72	0.59–0.88	0.001	205/648	209/665	1.01	0.79–1.28	0.93
IR	33.4	44.1				31.6	31.4			
Cough	27/814	61/983	0.52	0.32–0.84	0.004	53/648	25/665	2.28	1.37–3.83	0.001
IR	3.3	6.2				8.1	3.7			
Fever	4/814	16/983	0.30	0.08–0.95	0.02	9/648	3/665	3.11	0.77–14.52	0.07
IR	0.5	1.6				1.4	0.4			

URTS = upper respiratory tract secretions.
r/o = respiratory symptoms/observations in the period; IR: incidence rate (×100 observations); RR: relative risk of respiratory symptoms, S/NS.

However, others did not show these results (Farr *et al*, 1987; Douglas *et al*, 1987; Weisman *et al*, 1990). It has been suggested that lozenges did not produce sufficient zinc ions in saliva for local antiviral activity (Potter & Hart, 1993). Moreover, zinc's mechanism of action against cold is not clear. Our results suggest a benefit of zinc supplementation by improving cellular immunity.

During the postsupplementation period, the incidence of upper respiratory secretions was similar in both groups, as was fever. The incidence of cough decreased to less than half the rate seen during the supplementation period in the nonsupplemented group, and doubled in the previously supplemented group, suggesting that the natural rate of variation in cough was high. Moreover, cough can signal allergy as well as infection, or signal the presence of environmental pollutants.

Community-based studies on respiratory infections are fraught with difficulties, and are much harder to conduct and interpret than hospital-based studies on respiratory infections. However, our data suggest that respiratory tract symptoms and signs are less frequent during zinc supplementation of malnourished children, and that this supplementation is accompanied by improved DTH reactions and higher serum zinc levels. Our data also suggest that high variance in the incidence of some respiratory tract infection symptoms and signs, such as cough, will require community-based studies to include larger population groups if meaning ful data are to be obtained.

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Chapter 5

Zinc as an adjunct to the treatment of severe pneumonia in Ecuadorian children: a randomized controlled trial

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American Journal of Clinical Nutrition 2014; 99; 497-505

Zinc as an adjunct to the treatment of severe pneumonia in Ecuadorian children: a randomized controlled trial[1–3]

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ABSTRACT

Background: Studies of zinc as an adjunct to treatment of severe pneumonia in children have shown mixed results, possibly because of poor information on zinc status and respiratory pathogens.

Objective: We evaluated the effect of zinc given with standard antimicrobial treatment on the duration of respiratory signs in children with severe pneumonia. Zinc status and pathogens were assessed.

Design: Children aged 2–59 mo with severe pneumonia who were admitted to the main children's hospital in Quito, Ecuador, were given standard antibiotics and randomly allocated to receive zinc supplements twice daily or a placebo. Measurements included anthropometric variables, breastfeeding, hemoglobin, plasma zinc, and common bacteria/viral respiratory pathogens. The primary outcome was time to resolution of respiratory signs. The secondary outcome was treatment failure.

Results: We enrolled 225 children in each group. There was no difference between groups in time to resolution of respiratory signs or treatment failure; pathogens were not associated with outcomes. Tachypnea and hypoxemia resolved faster in older children ($P = 0.0001$) than in younger ones. Higher basal zinc concentration ($P = 0.011$) and better height-for-age z score (HAZ) ($P = 0.044$) were associated with faster resolution of chest indrawing. Better weight-for-height z score (WHZ) ($P = 0.031$) and HAZ ($P = 0.048$) were associated with faster resolution of tachypnea. Increased C-reactive protein was associated with a longer duration of tachypnea ($P = 0.044$).

Conclusions: Zinc did not affect time to pneumonia resolution or treatment failure, nor did type of respiratory pathogens affect outcomes. Higher basal zinc and better HAZ and WHZ were associated with reduced time to resolution of respiratory signs. These results suggest the need for prevention of chronic zinc deficiency and improvement of general nutritional status among Ecuadorian children. This trial was registered at www.clinicaltrials.gov as NCT 00513929.

Am J Clin Nutr 2014;99:497–505.

INTRODUCTION

Community-acquired pneumonia is a major cause of morbidity and mortality in children in resource-limited countries (1, 2) and is responsible for ~18% of deaths in children <5 y of age (1.4 million deaths/y) (2). Zinc, critical for healthy growth and immune function (3) and commonly deficient in low-income countries (4), can improve growth and reduce the frequency and severity of diarrhea and acute lower respiratory tract infections (ALRIs)[4], including pneumonia in children (5–8).

Whereas zinc is an important adjunct to diarrhea treatment, reducing time to recovery among children aged 6 mo to 5 y (9), trials of zinc in children hospitalized with severe pneumonia have yielded mixed results (10–15). In a trial conducted in children aged 2–23 mo in Bangladesh, zinc reduced the duration of clinical manifestations of severe pneumonia, including chest indrawing, tachypnea, hypoxia, and length of hospitalization (12). Another study in Kolkata, India, found that short-course zinc supplementation given to children with severe ALRI significantly reduced fever duration and very ill status in boys but not girls (11). However, studies in India and Nepal found no benefits of zinc supplementation in children with pneumonia (13, 14). Inadequate statistical power, variations in pneumonia case definitions, methodologic differences, and regional differences in undernutrition and zinc deficiency may explain these conflicting results. In addition, given the suggestion in the India study that bacterial infection might influence the treatment effect of zinc and even lengthen duration of hospitalization (13, 16), pneumonia etiology has been discussed as an explanation of these conflicting findings.

Given these inconsistent results, and a lack of prior studies in Latin America where zinc deficiency is widespread (4), we hypothesized that adjunctive zinc supplementation for treatment of pneumonia would reduce both duration of time to resolution of symptoms and treatment failures. Given inconclusive evidence that the impact of zinc supplementation is etiology dependent (16) and limited data that zinc mediates antiviral activity on

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[2] Supported by the Thrasher Research Fund (grant 028257).

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[4] Abbreviations used: ALRI, acute lower respiratory tract infection; CRP, C-reactive protein; DSMB, Data Safety Monitoring Board; HAZ, height-for-age z score; Hib, *Haemophilus influenzae* type b; hMPV, human metapneumovirus; PCR, polymerase chain reaction; RSV, respiratory syncytial virus; SpO_2, oxygen saturation; WHZ, weight-for-height z score; WLZ, weight-for-length z score.

Received June 12, 2013. Accepted for publication December 30, 2013.

First published online January 15, 2014; doi: 10.3945/ajcn.113.067892.

respiratory syncytial virus (RSV) by altering cellular capacity to support RSV replication (17), a secondary hypothesis was that this impact would be etiology independent. To test these hypotheses, we conducted a double-blind, placebo-controlled, randomized trial in Quito, Ecuador, which is an ideal location because severe pneumonia in young children represents a major health threat (18), stunting is common (19), and nearly one-third of children suffer from zinc deficiency (20).

SUBJECTS AND METHODS

This study was a randomized, parallel group, double-blind, placebo-controlled clinical trial in children with severe pneumonia designed to measure the efficacy of daily zinc administration until hospital discharge on the duration of pneumonia symptoms, including hypoxemia and treatment failure. It was conducted from February 2008 to April 2010 in Quito, Ecuador, at the Baca Ortiz Children's Hospital, the main pediatric referral hospital in Ecuador. Baca Ortiz has ~130,000 outpatient and emergency room consultations annually, and ALRI is the most common reason for hospitalization.

The Ethics Committee of the Corporación Ecuatoriana de Biotecnología and the Boston University Institutional Review Board approved the trial. The study was registered at www.clinicaltrials.gov before starting enrollment (NCT 00513929). Written informed consent was obtained from each child's parent or care provider. The Ethics Committee of the Corporación Ecuatoriana de Biotecnología supervised the study to ensure that study procedures were followed and served as the Data Safety Monitoring Board (DSMB). The DSMB conducted 2 interim reviews of safety data and a blinded interim analysis at one- and two-thirds' enrollment. At each review, the DSMB recommended that the study continue without changes until the number of enrolled children met the planned sample size because there was no significant statistical difference in the outcomes between groups.

Eligibility criteria, definitions, and randomization

Children aged 2–59 mo with severe pneumonia admitted to the Baca Ortiz Children's Hospital, whose parents provided written informed consent, were eligible for participation. Children suffering from marasmus, kwashiorkor, measles, pneumonia resulting from reported aspiration, hepatic or renal disease, neurologic disorders, acute diarrhea with dehydration, other viral infections, sepsis, congenital abnormalities, complicated pneumonia, severe anemia (hemoglobin <8 g/dL), inability to take fluids, whose parents refused to provide informed consent, and who had been treated with antibiotics for ˃24 h before admission were excluded.

In the emergency room, a resident physician exclusively dedicated to the screening of potential study subjects evaluated each child with respiratory symptoms. If the child was eligible, a senior study physician performed a thorough clinical examination and rapid hemoglobin test. If pneumonia was confirmed and the child met no exclusion criteria, the study physician enrolled the child after written informed consent was obtained from the care provider.

At enrollment, study physicians collected information on demographic characteristics, current illness, and medical history. A detailed clinical evaluation was performed including weight, height, respiratory rate, oxygen saturation (SpO_2), axillary temperature, and chest indrawing. A calibrated scale (Detecto) was used to measure the child's weight while wearing minimal clothing. Length or height was measured with a footboard (children aged <2 y) or vertical tape measure (children aged ≥2 y). Additional baseline testing included the following: *1*) collection of blood to measure C-reactive protein (CRP), plasma zinc, whole-blood polymerase chain reaction (PCR) for *Streptococcus pneumoniae* and *Hemophilus influenzae* type b (Hib), *2*) pulse oximetry, *3*) nasopharyngeal aspirate for PCR analysis of atypical bacteria and viruses, and *4*) chest X-ray.

Severe pneumonia was based on a modified version of the WHO definition (21) and included the presence of cough and/or chest wall indrawing, tachypnea (>50 breaths/min in children from 2 to <12 mo of age, >40 breaths/min in children from 12 to 59 mo), hypoxemia (SpO_2 <90%), and at least one of the following by thoracic auscultation: rales, diminished breath sounds, bronchial breath sounds, or pleural rub.

Criteria for clinical resolution of pneumonia were remission of tachypnea and hypoxemia for at least 12 h. In children with chest wall indrawing at baseline, resolution also included absence of this sign for at least 12 h. Treatment failure within the first 7 d was defined as follows: *1*) clinical deterioration any time after enrollment, including chest indrawing, at 72 h; *2*) inability to take oral medication because of persistent vomiting; *3*) modification of initial antibiotic choice; or *4*) death.

The Universidad Central del Ecuador, Escuela de Quimica manufactured the zinc and placebo syrups, which had similar appearance, color, odor, and mint flavor. Bottles with syrup were assigned 1 of 4 alphabetical codes (2 corresponding to zinc and placebo, respectively) by the manufacturer. A computer randomization list was generated and converted into a sequence of bottles containing the therapeutic regimen by a statistician who was blinded to the codes. Once an eligible child was identified, the next bottle in the sequence was opened, and the corresponding regimen provided to the child. The local ethics committee held the blinded randomization codes in a secure place. The study code was broken after all data were entered and initial blinded analyses had been performed. Two batches of zinc/placebo were manufactured (one per year), with blinded quality control to determine the zinc concentration. Sealed laboratory reports were sent to the president of the ethics committee for verification of appropriate concentrations.

Intervention

Children were prescribed antibiotics and were randomly allocated to receive 20 mg elemental zinc or placebo, in 2 doses per day to minimize adverse gastrointestinal effects. The active substance concentration was 10 mg elemental zinc (zinc sulfate) in 5 mL. Five milliliters of syrup were administered to each participant twice daily (at noon and at midnight) in addition to standard antibiotic treatment. The same dosing regimen (10 mg of zinc twice a day) was used by the previous studies described above (11–13). If the child vomited within 30 min, the dose was repeated. Care providers and study team members were blinded to the allocation.

Participants were given standard antimicrobial regimens based on national guidelines, which is standard practice in pediatric hospitals in Ecuador (22). Children started with regimen number 1 according to his or her age (ampicillin if <2 y, penicillin if ≥2 y). In cases of deterioration within 48 h or no improvement, the treatment shifted to the next regimen. However, if deterioration was rapid, any other standard regimen was selected. Additional

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therapeutic measures were provided as needed, including oxygen, intravenous hydration, nothing by mouth other than the study supplement if the respiratory rate was >60/min, and salbutamol nebulizer every 6 h for wheezing.

Follow-up procedures

Study physicians performed standard procedures for all clinical assessments every 6 h. When the participant was calm, respiratory rate was determined by counting respiratory abdominal movement for 60 s. Lower chest wall indrawing was evaluated by direct observation. SpO_2 was measured with a pulse oximeter (OxiMax N-65; Nellcor), which performs an internal calibration when turned on and when the oxygen transducer is applied. For readings <90%, the observer reapplied the transducer to the child and repeated the procedure. An average of 2 measurements was documented.

For participants receiving oxygen therapy, we withdrew the oxygen cannula for 2 min and assessed respiratory rate, lower chest indrawing, and pulse oximetry. When oxygen withdrawal could not be tolerated, it was immediately readministered.

Axillary temperature was measured with digital electronic thermometers, confirmed by second recordings. Thorough examinations were performed every 24 h. At remission of tachypnea, hypoxemia, and chest wall indrawing, blood was drawn for measurement of final plasma zinc concentration.

In cases of suspected treatment failures, the principal investigator or a senior pediatrician was contacted to confirm the treatment failure. At this time, antibiotic therapy was changed and another appropriate therapy was started. All cases of treatment failure were followed up until resolution.

Laboratory analyses

At enrollment and time of remission, blood samples (4 mL for the basal and 3 mL for the final sample) were obtained by venipuncture at the forearm. Because the time of enrollment and pneumonia resolution were different for each child, it was not possible to obtain samples under fasting conditions. Hemoglobin was measured at enrollment by finger prick by using a portable HemoCue Hb 201+ machine. A nasopharyngeal aspirate was obtained and divided into 2 samples: one for bacteria detection and another for viruses. Samples were stored at –20°C until processing.

Standard chest X-rays were obtained at enrollment by using a Gentron 650 radiographic machine (General Electric) and interpreted by an experienced senior radiologist.

A photometric-turbidimetric test was performed for determination of plasma CRP. The assay Dimension Clinical Chemistry System Flex reagent cartridge (Siemens) was performed according to the manufacturer's instructions.

For plasma zinc analysis, blood was collected in standard disposable plastic syringes (nonheparinized) with stainless steel needles. Blood was transferred to a polystyrene tube containing zinc-free heparin (300 μL/10 mL blood). Blood samples were centrifuged immediately to obtain plasma, which was stored in nitric acid–treated Eppendorf tubes at –20°C until analysis. Zinc concentrations were measured by atomic absorption spectrometry at the Chemistry School of Central University, as previously described (23). Zinc deficiency was defined as a plasma concentration <70 μg/dL after adjustment by CRP concentration (24).

Microbiological studies

The presence of *Mycoplasma pneumoniae*, *Chlamydiophila pneumoniae*, and human metapneumovirus (hMPV); RSV; parainfluenza virus 1, 2, 3; influenza A virus; influenza B virus; and adenovirus was determined by PCR in nasopharyngeal aspirates (F Sempértegui, B Estrella, O Rodríguez, L Ortíz, C Cruz, DH Hamer, unpublished observations, 2013). Plasma PCR was performed for detection of *S. pneumoniae* and Hib.

Sample size

In previous research at the Baca Ortiz Children's Hospital, the average (±SD) duration of hospitalization of children with pneumonia was 5.07 ± 2.87 d (22). With 80% power, a 2-sided significance of 0.05, and a mean reduction in duration of pneumonia signs by 1 d (12), the required sample size was 174 children per study arm. Assuming a 15% loss to follow-up (22), the total sample size for this primary outcome was 200 children per group. Assuming treatment failure of 20% in the placebo group (22), a 50% reduction in treatment failure in zinc-supplemented children, 80% power, and 2-tailed α of 0.05, the required sample size was 199 children in each group. With no published data on the etiology-dependent duration of pneumonia in young children, we assumed a similar duration of pneumonia episode for all etiologies.

Because of a very heavy volume of pneumonia-related hospitalizations in the winter of 2008, several participants were discharged before completion of all study procedures. The Boston University Institutional Review Board and local ethics committee recommended increasing the sample size to 450 or 225 per group to ensure complete data on 200 children per group, the original sample size.

Statistical analysis

Project-specific case report forms were used for data collection, all of which were standardized in a previous vitamin A study (22). All forms were checked twice weekly by the study supervisor for completion and discrepant, inappropriate, and illogical responses. Double data entry was performed with Microsoft Access 2000 (Microsoft Corporation). Microsoft Excel was used for validation and cleaning, and SPSS (version 11.5.0; SPSS, Inc) was used for all analyses.

Descriptive statistics were calculated for all baseline measurements. Because decreased plasma zinc concentration has been found during acute infections in children (20, 24), we performed linear regression between CRP and plasma zinc concentrations to derive the constant for adjusting the baseline zinc concentration. Intent-to-treat and per-protocol analyses were performed. Zinc and placebo groups were compared with regard to primary and secondary outcome variables: time to remission (in h) of the 2 clinical signs together (tachypnea and hypoxemia), the 3 signs together when chest wall indrawing was present, and treatment failure. The 2 groups were also compared for time to remission of each sign (tachypnea, hypoxemia, and chest wall indrawing). Differences between means and proportions were analyzed by using Student's t and chi-square tests, respectively, with level of significance at ≤0.05. The log-rank test for

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homogeneity for Kaplan-Meier survival curves was used to test differences in time to resolution of all 3 signs.

To assess the effect of zinc on hours to resolution of clinical signs of pneumonia, while controlling for predictor variables [age, sex, breastfeeding, adjusted basal zinc concentration, basal CRP concentration, height-for-age z score (HAZ), weight-for-height and weight-for-length z scores (WHZ and WLZ, respectively), hemoglobin, and presence of pathogens] as potential confounders, we developed a multiple linear regression model. To evaluate the effect of zinc on treatment failure, we developed a logistic regression model that controlled for the same predictor variables. Associations between the intervention and outcomes of interest in the regression models were considered to be significant at $P \leq 0.05$.

RESULTS

Of 2768 screened potential study candidates, 662 children met the eligibility criteria, and 450 children were enrolled with 225 children assigned to each study group—zinc and placebo. The principal reasons for exclusion were ambulatory treatment, admission to nonstudy wards, no study ward beds available, refusal of parental consent, and readmission for pneumonia (**Figure 1**). During the study, 69 children (15.3%) did not complete all study procedures because of early hospital discharge due to ward overcrowding, parental requests for discharge against medical advice, and development of complicated pneumonia. There were 4 deaths, 1 in the intervention group and 3 in the placebo group. Children with incomplete follow-up were distributed evenly in both groups (Figure 1). Five children in the zinc group and 4 in the placebo group who were referred immediately after enrollment to other wards because of either erroneous enrollment or per hospital request were not included in the analysis. There were no significant differences between zinc and placebo groups with regard to baseline variables in children who did not complete all study procedures.

Of the 450 children enrolled, 40.4% were girls and 59% were infants aged 2–12 mo. Eighteen percent of children were underweight, 19% were stunted, and 14% were wasted. At enrollment, no significant differences were observed between the zinc and placebo groups in baseline characteristics, including zinc deficiency (plasma zinc concentration ≤70 μg/dL), respiratory signs, chest X-ray findings, or pathogens (bacteria or virus) (**Table 1**).

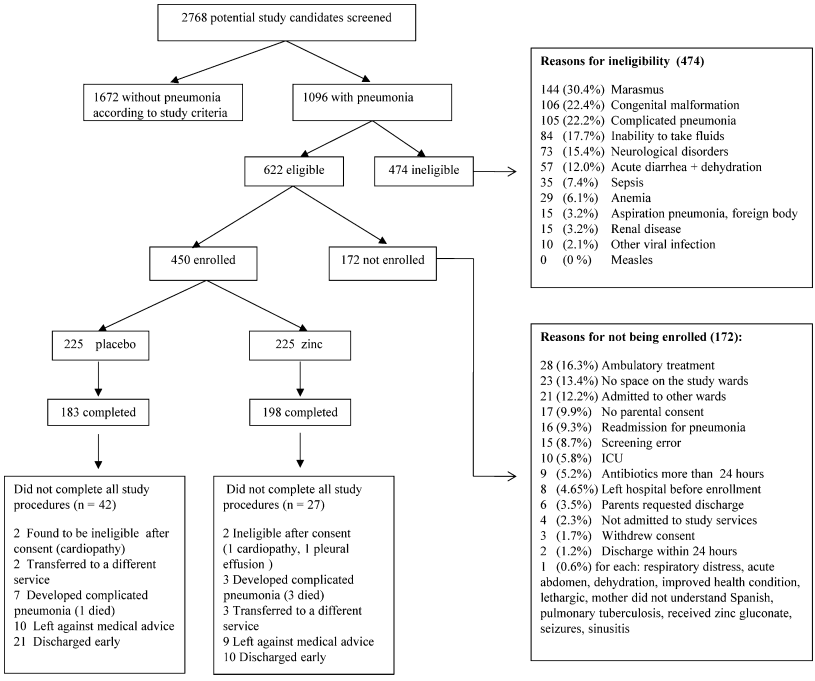

FIGURE 1. Study profile. ICU, intensive care unit.

Pneumonia pathogens

A total of 407 samples (404 plasma and 407 nasopharyngeal samples) were analyzed for determination of all pathogens except for Hib (299 tested, 0 positive). Pathogens were detected in 297 of 407 samples (73%) including viruses in 63% (257 of 407) and bacteria in 2.7% (11 of 406). Some samples were positive for both viruses and bacteria (7.1%; 29 of 407). Two or more pathogens were identified in 34.7% (103 of 297) of children with pathogen-positive results. RSV was the most frequently identified virus (39.2%), followed by hMPV (17.5%) and adenovirus (15.3%). *S. pneumoniae* was the most prevalent bacteria (9.2%).

There were no differences in the frequency or type of pathogen between the zinc and placebo groups. Moreover, baseline-adjusted zinc concentration and zinc deficiency were not associated with specific pathogens.

Clinical findings

There were no significant differences between zinc and placebo groups for the primary outcome: time to remission of tachypnea and hypoxemia (**Figure 2**). Moreover, there were no significant differences in the duration of individual respiratory signs (**Table 2**) or in prevalence of treatment failure (placebo compared with zinc: 34.4% compared with 34.5%; OR: 1.00; 95% CI: 0.68, 1.50) (**Table 3**). Clinical deterioration and change in antibiotics were the most common causes of treatment failure; there was no difference in the incidence of these 2 causes between the 2 groups.

There were no differences in the time to resolution of respiratory signs either combined or individually between the zinc and placebo groups when stratified by the presence of specific pathogens or by the presence of virus or bacteria at enrollment (data not shown).

Predictors of primary outcomes

Multiple linear regression models showed no effect of zinc on time to remission of the 2 clinical signs together when predictor variables were controlled for. However, higher age was associated with decreased time to resolution of tachypnea and hypoxemia together (**Table 4**). The results were similar when assessing remission of the 3 signs together (including chest wall indrawing) (data not shown).

Higher baseline zinc concentration was associated with a reduction in time to remission of chest indrawing ($P = 0.011$) (**Table 5**). Higher HAZ was also associated with reduction in time to remission of chest indrawing ($P = 0.044$). There was no significant interaction between basal plasma zinc and HAZ on time to resolution of chest indrawing. Better WHZ/WLZ was associated with a reduction in time to remission of tachypnea ($P = 0.031$). Better HAZ was also associated with a reduction in time to remission of tachypnea ($P = 0.048$). Higher CRP concentration was associated with increased time to resolution of tachypnea ($P = 0.044$). Breastfeeding, exclusive or nonexclusive, was associated with a trend toward reduced time to remission of chest indrawing

TABLE 1
Baseline demographic, clinical, and laboratory characteristics[1]

	Placebo group (n = 225)	Zinc group (n = 225)
Age (mo)	12.99 ± 11.24[2]	13.06 ± 10.32
2–12 mo [n (%)]	137 (60.9)	132 (58.7)
>12 mo [n (%)]	88 (39.1)	93 (41.3)
Female [n (%)]	101 (44.9)	105 (46.7)
WAZ	−1.01 ± 1.21	−1.05 ± 1.30
HAZ	−1.05 ± 1.36	−0.93 ± 1.39
WHZ or WLZ[3]	−0.19 ± 1.90	−0.08 ± 1.99
Hemoglobin (g/L)	10.99 ± 1.31	11.16 ± 1.49
CRP (mg/dL)	4.23 ± 5.34	3.94 ± 5.84
Zinc adjusted[4] (μg/dL)	74.2 ± 24.9	76.40 ± 27.23
Zinc-adjusted deficiency[4] (<70 μg/dL) [n (%)]	99 (44.2)	92 (40.9)
Breastfeeding [n (%)]	151 (67.1)	157 (69.8)
Respiratory signs		
Pulse oximetry (%)	77.96 ± 7.02	78.04 ± 6.51
Rales [n (%)]	199 (88.4)	202 (89.8)
Diminished breath sounds [n (%)]	105 (46.7)	110 (48.9)
Bronchial breath sounds [n (%)]	23 (10.2)	36 (16.0)
Wheezing [n (%)]	34 (15.1)	36 (16.0)
Chest X-ray [n (%)]		
Consolidation	6 (2.7)	10 (4.4)
Infiltrate	130 (57.8)	150 (66.7)
Other[5]	38 (16.9)	28 (12.4)
Presence of pathogens [n (%)]	143 (63.3)	154 (68.4)

[1] P values were nonsignificant for all comparisons (Student's t test and chi-square test). CRP, C-reactive protein; HAZ, height-for-age z score; WAZ, weight-for-age z score; WHZ, weight-for-height z score; WLZ, weight-for-length z score.
[2] Mean ± SD (all such values).
[3] Wasted (WHZ or WLZ < −2): 13.9% of the zinc group, 14.4% of the placebo group.
[4] Zinc concentration was adjusted by using the following equation: 0.36 × CRP concentration + baseline zinc concentration.
[5] Air trapping, pleural effusion, pneumatocele, pneumothorax, necrosis, atelectasis, and abscesses.

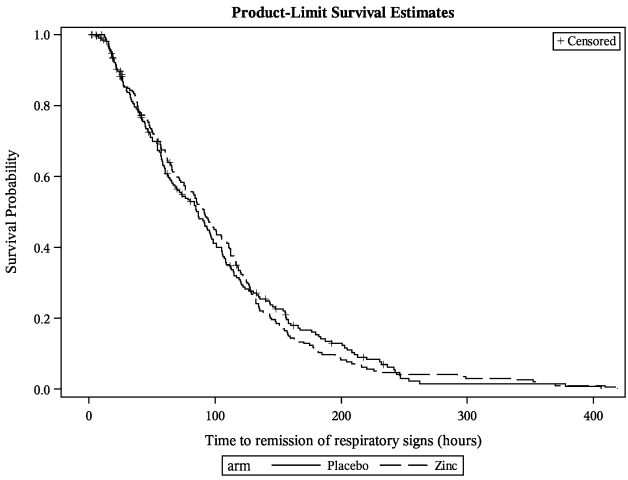

FIGURE 2. Kaplan-Meier survival curves for time to remission of both tachypnea and hypoxemia in the placebo group (solid line) and the zinc-supplemented group (dashed line).

(P = 0.074). The analysis of time to remission of hypoxemia showed that only age was significantly associated with this outcome.

DISCUSSION

Zinc as an adjunct to the standard treatment of severe pneumonia in hospitalized Ecuadorian children had no effect on time to resolution of clinical signs or the proportion with treatment failure. Children with better baseline zinc status had more rapid resolution of chest indrawing, which was independent of the intervention. Previous studies, which also failed to show that zinc supplements reduced time to resolution, did not provide data on baseline zinc status or the association of baseline zinc status with time to resolution of pneumonia (12–14, 25–30). After adjusting for inflammation, we found that >40% of enrolled children were zinc deficient. Because plasma zinc concentrations significantly increased in the supplemented group, the lack of effect of zinc on duration of respiratory signs suggests that the systemic impact of chronic zinc deficiency, including the impairment of immune response, is not reversed with short-term supplementation.

Most previous therapeutic trials of zinc in children with pneumonia did not identify pathogens associated with episodes of severe pneumonia. Although Coles et al (16) suggested a pathogen-dependent effect of zinc, they used CRP as a proxy for bacterial compared with viral infection. This inflammatory marker is nonspecific and has not been shown to reliably distinguish between these 2 pneumonia etiologies (31–33). We found that higher CRP was associated with delayed remission of tachypnea. Higher CRP may be associated with more severe disease and therefore could serve as a nonspecific marker of risk for prolonged illness.

TABLE 2
Effect of intervention on the remission of respiratory signs[1]

	Placebo group		Zinc group	
Experienced remission of	n	Time to remission	n	Time to remission
		h		h
Two signs[2]	183	93.2 ± 69.5[3]	198	101.3 ± 75.5
Three signs[4]	178	93.9 ± 69.8	191	102.6 ± 76.1
Tachypnea	197	69.5 ± 61.9	205	69.2 ± 68.5
Hypoxemia	185	92.5 ± 69.5	198	100.5 ± 75.5
Chest indrawing	200	45.7 ± 49.4	199	43.1 ± 41.1

[1] P values were nonsignificant for all comparisons (Student's t test).
[2] Tachypnea and hypoxemia.
[3] Mean ± SD (all such values).
[4] Tachypnea, hypoxemia, and chest indrawing.

TABLE 3
Effect of intervention on treatment failure

	Placebo group (n = 221)	Zinc group (n = 220)	OR (95% CI)
	n (%)	n (%)	
Treatment failure	76 (34.4)	76 (34.5)	1.00 (0.68, 1.50)
Clinical deterioration	39 (17.6)	35 (15.9)	0.88 (0.53, 1.46)
Change in antibiotics[1]	52 (24.8)	56 (25.9)	1.06 (0.69, 1.65)
Death	3 (1.4)	1 (0.5)	0.33 (0.03, 3.20)
Unable to drink fluids	1 (0.5)	2 (0.9)	2.00 (0.18, 22.3)

[1] n = 210 and 216 for placebo and zinc groups, respectively. Fifteen children were not prescribed antibiotics.

TABLE 4
Time to remission of 2 respiratory signs together: tachypnea and hypoxemia[1]

	β (95% CI)[2]	*P*
Constant	125.73 (53.8, 197.6)	<0.01
Zinc/placebo	5.06 (−10.5, 20.6)	0.52
Age (mo)	−1.76 (−2.67, −0.87)	<0.01
Female sex	10.29 (−5.44, 26.0)	0.19
HAZ	−5.94 (−12.2, 0.30)	0.06
WHZ or WLZ	−4.22 (−9.22, 0.78)	0.09
Hemoglobin (g/L)	−2.27 (−7.82, 3.28)	0.42
CRP (mg/dL)	0.84 (−0.58, 2.25)	0.24
Basal zinc adjusted[3] (μg/dL)	−0.03 (−0.33, 0.26)	0.82
Breastfeeding	−7.75 (−28.09, 12.6)	0.45
Pathogen[4]	14.68 (−3.49, 32.9)	0.11

[1] CRP, C-reactive protein; HAZ, height-for-age *z* score; WHZ, weight-for-height *z* score; WLZ, weight-for-length *z* score.
[2] Based on a multilinear regression analysis.
[3] Zinc concentration was adjusted by using the following equation: 0.36 × CRP concentration + baseline zinc concentration.
[4] The presence of at least one microorganism.

We did not find any differences in the prevalence or type of pathogen between the 2 groups. Furthermore, time to resolution of pneumonia episodes did not differ between groups for each of the most frequent pathogens (RSV, hMPV, adenovirus, and *S. pneumoniae*). Therefore, these findings suggest that there is not a pathogen-specific effect of zinc on pneumonia.

We found that better basal plasma zinc concentration was associated with shorter time to remission of chest indrawing. This suggests that zinc-deficient children suffer from impaired immune function, which affects their ability to fight infection, as has been reported previously (3, 34, 35). In fact, zinc supplementation has been shown to prevent pneumonia episodes (7, 8, 36). Thus, a policy aimed at improving the zinc status of young children continues to be a high priority in developing countries, especially where dietary zinc intake is limited and phytate intake is high, as in Andean countries.

We also found that better HAZ was associated with reduced time to remission of chest indrawing and tachypnea, in contrast to previous zinc treatment studies (12–14, 25–29). Although there was no significant interaction between HAZ and basal plasma zinc in reducing time to remission of chest indrawing, it is possible that nutritional status could be improved either via a direct zinc-mediated effect on growth (5) or via rapid clearance of pathogens (to prevent their deleterious effect on growth). In fact, we have found that linear growth is significantly reduced by one or more episodes of pneumonia in young children (F Sempértegui, B Estrella, J Egas, et al, unpublished observations, 2013). The association between better WHZ and faster resolution of tachypnea suggests that acute malnutrition could lead to increased vulnerability of children to pneumonia. This finding is consistent with a study from Bangladesh, which found that wasted children have impaired immune function and are at higher risk of upper respiratory infections (37).

The association between basal zinc concentration and time to remission of respiratory signs does not necessarily imply a direct cause and effect. Similarly, the association between nutritional status (HAZ, WHZ) and duration of respiratory signs is suggestive of an overall effect of generalized nutritional status, which, in the case of stunted or wasted children, is likely to be associated with multiple micronutrient deficiencies as a causal factor. Although there was a trend to faster resolution of chest indrawing only in breastfed children, it is important to emphasize that breastfeeding has been found to be associated with improved innate immunity (38) and interferon production in children with influenza (39).

Strengths of this study included its adequate power, careful attention to allocation and blinding, close monitoring of respiratory signs, training of the study team, and standardization of measurement techniques. The study also benefited from inclusion of critical baseline variables to control for potential confounders of the effect of zinc. Weaknesses included a dropout rate that was higher than expected, especially during the initial weeks of the study, because of overcrowded wards. Additional limitations were related to the lack of certain routine bacteriologic test procedures (blood cultures, induced-sputum Gram stain and culture).

TABLE 5
Time to remission of each respiratory sign[1]

	Chest indrawing		Tachypnea		Hypoxemia	
	β (95% CI)[2]	*P*	β (95% CI)[2]	*P*	β (95% CI)[2]	*P*
Constant	56.29 (12.6, 100.0)	<0.01	104.43 (39.6, 169.3)	<0.01	119.93 (48.43, 191.83)	<0.01
Zinc/placebo	−1.09 (−10.5, 8.28)	0.82	1.86 (−12.1, 15.8)	0.79	4.76 (−10.71, 20.24)	0.54
Age (mo)	−1.09 (−1.65, −0.53)	<0.01	−0.76 (−1.58, 0.07)	0.07	−1.76 (−2.67, −0.84)	<0.01
Female sex	5.48 (−4.05, 15.0)	0.26	13.27 (0.86, 27.4)	0.06	10.87 (−4.84, 26.57)	0.17
HAZ	−3.87 (−7.64, −0.10)	0.04	−5.64 (−11.2, −0.06)	0.04	−6.11 (−12.33, 0.11)	0.05
WHZ or WLZ	−1.92 (−4.74, 0.90)	0.18	−4.65 (−8.86, −0.44)	0.03	−4.30 (−9.30, 0.69)	0.09
Hemoglobin (g/L)	1.12 (−2.29, 4.53)	0.51	−3.19 (−8.21, 1.83)	0.21	−2.04 (−7.57, 3.49)	0.46
CRP (mg/dL)	0.53 (−0.32, 1.38)	0.22	1.31 (0.03, 2.58)	0.04	0.89 (−0.53, 2.30)	0.21
Basal zinc adjusted[3] (μg/dL)	−0.23 (−0.40, −0.05)	0.01	−0.13 (−0.39, 0.13)	0.33	−0.02 (−0.31, 0.27)	0.87
Breastfeeding	−11.31 (−23.7, 1.12)	0.07	−8.04 (−24.6, 10.4)	0.39	−6.62 (−26.12, 13.64)	0.52
Pathogen[4]	8.54 (−2.37, 19.5)	0.12	9.30 (−6.92, 25.3)	0.26	15.15 (−2.96, 33.26)	0.10

[1] CRP, C-reactive protein; HAZ, height-for-age *z* score; WHZ, weight-for-height *z* score; WLZ, weight-for-length *z* score.
[2] Based on a multilinear regression analysis.
[3] Zinc concentration was adjusted by using the following equation: 0.36 × CRP concentration + baseline zinc concentration.
[4] The presence of at least one microorganism.

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In conclusion, we found no beneficial effect of zinc on the duration of respiratory signs or risk of treatment failure. Although previous studies that failed to show a benefit of zinc in the treatment of pneumonia had emphasized the lack of baseline information on zinc status and associated pathogens, our study suggests that these variables do not explain the absence of benefit. The association of zinc deficiency and stunting/wasting with prolongation of respiratory rates and hospitalization suggests a need for greater efforts directed at improving the overall nutritional status of Ecuadorian children, in particular their zinc status.

We thank the resident physicians—Mónica Paladines, Martha Almeida, and Guadalupe Segura—for their kind assistance with data collection and Ramiro Estrella and Edgar Játiva for their collaboration in the screening at the emergency room. We are also grateful to Lenis Ortiz and engineer Cecilia Cruz for their work in the Microbiology Laboratory at Catholic University.

The authors' responsibilities were as follows—FS, BE, OR, and DHH: conceived, designed, and implemented the study; FS and BE: were responsible for patient enrollment, data collection, and monitoring; MC, DG, and GS: contributed to the clinical monitoring of the subjects; BE: supervised the data management; FS, BE, and DHH: conducted the analysis and interpretation of the data and writing of the manuscript; and LLS: contributed to the development and design of the trial and to the interpretation of the study results. All authors reviewed and approved the manuscript. The sponsors of the study had no role in the study design, data collection, data analysis, or manuscript writing. All of the authors stated that they had no potential conflicts of interest.

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Chapter 6

Nutritional, immunological and health status of the elderly population living in poor neighbourhoods of Quito, Ecuador

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British Journal of Nutrition 2006; 96; 1-10

British Journal of Nutrition (2006), **96**, 845–853 DOI: 10.1017/BJN20061802

Nutritional, immunological and health status of the elderly population living in poor neighbourhoods of Quito, Ecuador

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(Received 8 July 2005 – Revised 21 February 2006 – Accepted 13 March 2006)

The number of elderly people is increasing in less-developed countries. Although nutritional deficiencies and infectious diseases are generally more prevalent in resource-poor countries, the health and nutritional status of the elderly in South America in general, and in Ecuador, in particular, remains largely unstudied. The objective of the present study was to assess the nutritional, immunological and health status of elderly Ecuadorians. A cross-sectional study was conducted to evaluate a sample of elderly Ecuadorians with 24 h dietary recalls, biochemical and anthropometric measurements, delayed type hypersensitivity skin response and a health questionnaire. The 145 elders who enrolled had a mean age of 74·3 (SD 6·9) years. Of the subjects, 52 % exhibited BMI $\geq 25\,kg/m^2$, whereas 9·1 % had BMI $\leq 20\,kg/m^2$. Means of dietary intakes were below recommendations for most nutrients; exceptions were carbohydrate, fat, Fe and Se. Serum nutrient levels indicated that 50, 44, 43, 19 and 18 % of participants had deficiencies of Zn, Fe, vitamins B_{12} and D, and folate, respectively. The mean number of positive responses to seven recall antigens was 2·1 (SD 1·7) with an induration diameter of 9·9 (SD 7) mm, which are substantially lower than those reported for elders in developed countries. During the previous 6 months, 54 and 21 % of subjects reported at least one episode of respiratory infection or diarrhoea, respectively. Of these, 47 % sought care at a hospital or from a physician and 96 % from a relative or friend. In conclusion, while few elderly Ecuadorians were underweight, obesity was common. Micronutrient deficiencies were prevalent and may contribute to reduced immunological responses in this population.

Elderly: Immune senescence: Less-developed countries: Micronutrients: Body mass index

The number of elderly individuals is increasing in both developed and less-developed countries. Populations in less-developed countries are currently undergoing rapid and unprecedented changes in their age structure, which will have a dramatic impact on the number of aged in this century. The percentage of the total worldwide population over 60 years of age is expected to more than double from 8 to 19 % by 2050 (United Nations, 2001). The increase in the proportion of elderly in less-developed countries is projected to grow 1·5 times faster than that in developed countries, and by 2050, approximately 80 % of all individuals over the age of 60 years will be living in less-developed countries (United Nations, 2004). For the last two decades, developed nations have gradually increased the proportion of their national health resources designated for the elderly, but very little of the limited and already strained health resources of less-developed countries have been devoted to ageing members of their societies. Because the elderly have a decreased capacity to fight disease without adequate nutritional, economical, and social support, they face the risk of living their lives burdened by disease and dysfunction. This threatens to put an even greater strain on their countries' resources.

Age-associated physiological, psychological, social, and economic changes may adversely affect the nutritional and immunological status of older individuals. These changes are often reflected in poor health and a reduced quality of life. The incidence of infections (Crossley & Peterson, 1996) and diseases such as cancer, atherosclerosis and autoimmunity (Cross *et al.* 1987) have been shown to increase with age, reflecting, in part, alterations in immune function seen in the elderly. The elderly are at greater risk for low consumption

Abbreviations: DTH, delayed type hypersensitivity; PLP, pyridoxal-5-phosphate.

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of several micronutrients such as vitamins E, C, and B_6, folate, Se, Fe and Zn, which have been shown to play important regulatory roles in maintaining the function of the immune system (Meydani & Santos, 2000). Malnutrition in the elderly has been shown to be associated with impairment of the immune response and decreased functional status, and this has been shown to respond to nutrient repletion (Lesourd, 1995; Meydani & Santos, 2000; High, 2001).

The health and nutritional status of the elderly in South America, in general, and in Ecuador, in particular, remains largely unevaluated. According to estimates based on a census conducted in 2000 (Instituto Nacional de Estadistica y Censos, 2001), the total population of Ecuador is approximately 12 million with an estimated 6·7 % (813 624) of inhabitants over the age of 65 years. The elderly population of Quito, the capital of Ecuador, is estimated to be 122 417, which represents a significant number of urban inhabitants whose health and nutritional status remains largely uncharacterised. This cross-sectional study of an independently living elderly population in a low-income urban area of Quito presents, for the first time, detailed anthropometric, dietary recall, nutritional, biochemical and immunological parameters in elderly Ecuadorians.

Methods

Study site and population

The present study was conducted in elderly Ecuadorians who live in poor urban areas in northwestern Quito (Ecuador), 2800 m above sea level. The neighbourhoods are located on a hill and are structurally similar. There is only one principal paved road and electricity is present in all homes. Some households have a municipal source of potable water and sewerage. Most houses consist of two rooms of cement block construction. Inhabitants are mainly poor immigrants from small cities and rural areas of Ecuador. To identify eligible elderly individuals, we carried out a census in three neighbourhoods. During household visits we provided potential study candidates with detailed information on the study and invited those who were willing to participate. A total of 145 elderly individuals aged 63–92 years, verified with their national identification card, who were willing to provide written informed consent, were enrolled.

The Ethical Committee of the Corporación Ecuatoriana de Biotecnología approved the protocol and the informed consent forms. During meetings at the fieldwork station, each subject was given detailed information on the study objectives and the procedures to be performed. In the instance of a subject having a health condition such as deafness, we used the assistance of a relative to help the subject to understand the study purpose and procedures. After potential participants had had an opportunity to ask questions, written informed consent was obtained. Biochemical analysis of serum or plasma samples was approved by the Tufts – New England Medical Center Institutional Review Board.

Nutritional profile

Individual dietary intake was estimated with a modified 24 h recall–weighing method (Zamora & Valverde, 1983). The interview was carried out in each household. Each subject was given an explanation on the importance of answering as truthfully and accurately as possible. In order to help the subject recall the previous day, we asked her/him about her/his activities, such as the time of awakening, daytime activities and when he or she went to bed. This approach helped the participants to remember the foods ingested. During the interview, the amounts of food consumed were verified by asking the subject the size of the household measures used to prepare the consumed food. The recall questionnaire was applied once per week to each subject. Household measures and the weights of food most frequently consumed in the neighbourhood were standardised. The questionnaire had two parts: (1) general information on health status, regular daily activities, special diet, type of lipids, use of salt in cooking and at the table, consumption of vitamins, supplements and alcohol; (2) specific information on food ingested at different times of the day (breakfast, lunch and dinner, and between meals). These data were collected with a questionnaire standardised during a pilot phase in a neighbourhood close to those where the present study was conducted. Prepared food and the ingredients used in each preparation were recorded, including their weight. Generally, recipes were obtained for foods served to the entire family, from which the amount of food ingested for each member was calculated. We made adjustments based on the portion he/she effectively consumed from what they recalled. Finally, each food and its ingredients were coded according to United States Department of Agriculture Food Codes and, when available, for specific Latin American Foods (United States Department of Agriculture Agricultural Research Service, 2004). We used release 17, which is no longer available, though release 18 can now be accessed (United States Department of Agriculture Agricultural Research Service, 2006). Foods not included in this file were coded according to the Ecuadorian Table of Foods (Instituto Nacional de Nutrición, 1965).

When available, we used the estimated average requirement (Food and Nutrition Board & Institute of Medicine, 2000; Institute of Medicine, 2000*b*) to determine the percentage of subjects consuming below or above the recommendation of a nutrient, and the adequate intake when an estimated average requirement was not available. The acceptable macronutrient distribution range (Food and Nutrition Board & Institute of Medicine of the National Academies, 2002) was used to determine adequacy of macronutrient intakes. It is recommended as the best method for assessment of population-based macronutrient adequacy, based on intervention and epidemiological evidence of reducing risk of chronic diseases (Food and Nutrition Board & Institute of Medicine of the National Academies, 2002).

Standard anthropometric measurements were performed including weight, height, knee height, skinfold thickness, and waist circumference for each participant according to the procedures outlined by Gross (1997) as follows. Weight was recorded to the nearest 0·1 kg using a Detecto scale (Detecto®, Webb City, MO, USA) with the least amount of clothes possible in all elderly participants, except one who could not stand on the scale and another one who refused to take her clothes off. The scale was calibrated daily with standard weights. Height was measured in all subjects but one who was unable to stand. A fibreglass tape measure right next to a wood structure was used. Measurements were recorded to the

nearest cm. Knee height was measured in all subjects using a fibreglass tape while the participant was sitting comfortably in a chair, with the knee bent at 90° and with the foot at 90° relative to the leg (Blaum *et al.* 1995). The procedure was repeated and recorded twice, to the nearest cm. Waist circumference was obtained between the border of the right anterior superior iliac crest and the umbilicus. It was recorded twice and measured to the nearest 0·1 cm with a non-stretchable measuring tape. Triceps skinfold was measured with a Caliper® (Beta Technology Incorporated, Cambridge, MD, USA) in a vertical fold at the midline posterior over the triceps muscle, between the acromion and the olecranon, with the elbow extended and the arm relaxed. Two measurements were taken, and recorded to the nearest mm.

Nutrient analysis and clinical chemistries were assessed in serum or plasma samples of a random sub-sample of sixty-five subjects. A 10 ml venous blood sample was drawn from each subject, after an overnight fast, into an EDTA-treated vacutainer tube and a vacutainer tube without anticoagulant. The samples were immediately transported to the laboratory and centrifuged. Serum or plasma was collected in plastic tubes, frozen at − 20°C, and then shipped to Boston for analysis. Lipids, glucose, homocysteine, B vitamins, vitamin E, vitamin D, Ca, Se, Zn, Fe and Cu were analysed according to standard procedures used at the Nutritional Evaluation Laboratory of the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University as previously described (Meydani *et al.* 1994). Reference ranges for vitamins A, D and E, pyridoxal-5-phosphate (PLP), Fe, Zn and Se were based on those recommended in the *Textbook of Clinical Chemistry* (Tietz, 1986) or by the manufacturer of the kits used to analyse for the nutrients (vitamin B_{12}, folate, Na, K, Ca, P, Mg, Cl), and verified by the Nutritional Evaluation Laboratory at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging, using data generated in healthy elderly people over the last 25 years. Modifications to these recommendations were made for some of the nutrients, based on new reports related to serum level of nutrients and risk for prevention of chronic diseases. These include vitamins B_6 and B_{12} and folate. Although clinical cut-off points have been set at 20 nmol/l, it has been argued that plasma PLP below 30 nmol/l may be a better indicator of low vitamin B_6 (Driskell, 1994; Bailey *et al.* 1997). The sensitivity and specificity for cut-offs for vitamin B_{12} concentrations are relatively poor. Although a clinical cut-off point has often been set at $<$ 148 pmol/l (200 pg/ml), vitamin B_{12} deficiency has been documented with serum concentrations as high as 258 pmol/l (350 pg/ml) (Lindenbaum *et al.* 1994). Therefore, for estimates of population deficiency, the cut-off of 185 pmol/l (250 pg/ml) has been used (Tucker *et al.* 2000). Cut-off points for folate have also varied considerably. Sauberlich (1990) defines plasma levels of $>$ 5 ng/ml as low risk for deficiency, 3–5 as moderate risk and $<$ 3 as high risk. Selhub & Rosenberg (1996) refer to these same cut-off levels as adequate, low and deficient in the 7th edition of *Present Knowledge in Nutrition*. Thus, we used 5 ng/ml as a cut-off point for folate adequacy.

Health profile

An interview was performed using a questionnaire to obtain information on diagnosed non-communicable diseases, respiratory tract infections, and diarrhoeal diseases within the previous 6 months.

Immunological assessment

Delayed type hypersensitivity (DTH) was assessed using a cell-mediated immunity kit (Multitest CMI; Pasteur-Merieux, Paris, France), a single-use, disposable applicator of acrylic resin with eight heads loaded with a glycerine control and the following seven recall antigens: tetanus toxoid, diphtheria toxoid, S*treptococcus* (group C), *Mycobacterium tuberculosis*, *Candida albicans*, *Trichophyton mentagrophytes* and *Proteus mirabilis*. We followed the product's technical recommendations in applying and evaluating the results, including cleanliness and supervision of the puncture area, time and pressure needed at the application site, and reading of the local reaction at 24 and 48 h after the test was applied. The DTH test was administered on the volar surface of the right arm and evaluated by the same investigator (F. S.) for all subjects. To read the DTH responses, we used a circular ruler calibrated in mm. We measured the vertical and horizontal diameters of induration, and considered the reaction positive when the mean value was $\geq$ 2 mm. Averages for each individual antigen were calculated and a composite score based on the results of all of the antigens in each subject was determined.

Statistical analysis

Data entry and management were done with Epi-Info software, version 6.04d (CDC, Atlanta, GA, USA). Statistical analyses were performed with SPSS, version 11.5 (Lead Technologies Inc., Haddonfield, NJ, USA; SPSS Inc., Chicago, IL, USA). The mean intake and SD of energy, macronutrients, vitamins and minerals were calculated from the 24 h dietary recall survey at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging, Dietary Assessment and Epidemiology Research Program using the compiled database described earlier. Mean intakes were compared with estimated average requirements or adequate intakes for micronutrients and to the acceptable macronutrient distribution range reference values for macronutrients (Institute of Medicine, 1997, 1998, 2000*a*,*b*; Food and Nutrition Board & Institute of Medicine, 2000; Food and Nutrition Board & Institute of Medicine of the National Academies, 2002; Food and Nutrition Board, 2004).

Descriptive statistics for anthropometric measurements, BMI, DTH response, and health profile survey responses were calculated globally and by sex. BMI was calculated as the weight in kg divided by height in metres squared (Garrow & Webster, 1985). Weight was classified as normal (BMI 20–24·9 kg/m^2), underweight (BMI $<$ 20 kg/m^2) and overweight (BMI $\geq$ 25 kg/m^2). Differences in means and percentages were evaluated by Student's t test and the χ^2 test, respectively. A linear multiple regression analysis was done to test the correlation between plasma micronutrient concentrations and DTH response at 48 h. The model included age, sex and BMI as confounders. The models for vitamins A and E were also adjusted for cholesterol concentrations. To evaluate the association between dietary micronutrients and serum micronutrient concentrations, a similar model was applied. The energy intake was also included as confounder.

A logistic regression model was developed to evaluate the association between plasma micronutrient concentrations and respiratory and diarrhoeal infection during the previous 6 months. The model included age, sex and BMI as potential confounders. A similar model was used to evaluate the association between dietary micronutrients and respiratory and diarrhoeal infection. The energy intake was also included as confounder. The model for dietary Fe intake also included dietary Ca, vitamin C and fibre as confounders (Fleming *et al.* 1998). Regression model diagnostics were done and the adequacy of covariate functional forms was examined.

Results

Nutritional profile

One hundred and forty-three elderly subjects were enrolled, of whom ninety-six (67 %) were female (Table 1). There was no substantially significant difference in weight between men and women (58 (SD 10) *v.* 53 (SD 9) kg). Female subjects had significantly lower values for height and knee height, and higher triceps skinfold values than men. Of the subjects, 39 % had normal BMI, while 9·1 % were underweight and 51·7 % were overweight. The mean BMI for women was higher than that of men ($P=0·007$). Dietary recall assessments in fifty-two participants showed that most participants consumed less than the recommended estimated average requirement or adequate intake for vitamins A, D, E, K, B_2, B_6 and B_{12}, folate, Zn, Ca, Mg and K. Although the mean total energy intake was lower than generally recommended (7840–9226 kJ) (Food and Nutrition Board & Institute of Medicine of the National Academies, 2002), there was a wide range of reported intakes (899–16 677 kJ). Furthermore, in the absence of information on the level of physical activity, it is difficult to determine adequacy of energy intake. Carbohydrate consumption was higher than recommended in 86·5 % of subjects, while protein and fat intakes were low in 53·8 and 92·3 %, respectively (Table 2). Laboratory micronutrient analysis in sixty-five subjects revealed that about 50 % of these elderly Ecuadorians had low concentrations of plasma vitamin B_{12}, Zn and Fe, 30 % had low PLP (vitamin B_6) and 19 % had low folate and vitamin D (Table 3).

Multiple linear regression analysis in a sub-group of subjects for whom both dietary intake and serum nutrient concentrations were available (*n* 52 and *n* 65, respectively) showed a positive correlation between dietary Zn and PLP levels and their corresponding serum level ($P<0·01$ and $P<0·05$, respectively). A significant correlation between dietary Fe and serum Fe levels was found ($P=0·007$), but the significance was lost after correcting for sex, age, BMI, energy intake, Ca, vitamin C and fibre levels (Fleming *et al.* 1998). The dietary Fe levels were split into haeme and non-haeme Fe, but no significant correlation between serum Fe levels and either dietary form was observed.

About 20 % of the subjects had high triacylglycerol and VLDL concentrations, while 19 % had low HDL concentrations; however, the LDL:HDL ratio was within the reference range. Consistent with the observation of low B vitamin concentrations, homocysteine concentrations were higher than the reference value in 25 % of the subjects (Table 4).

Health profile

The most frequent non-communicable diseases reported by the subjects were hypertension (19 %), arthritis (19 %), heart disease (12 %) and osteoporosis (10 %). Other medical problems included impaired vision, cerebrovascular accident, and type 2 diabetes (6, 5 and 4 %, respectively). The most common infectious diseases recalled during the previous 6 months were upper respiratory infections (54 %), diarrhoea (21 %) and bronchitis (7 %). Approximately 47 % of those with infections had visited a physician or hospital emergency room, and 96·2 % had asked for help from relatives or friends. Women suffered more frequently from diarrhoea ($P<0·02$) and sought care at emergency rooms more often than men ($P<0·01$).

Logistic regression analysis in a sub-group of subjects for whom dietary and serum nutrient levels were available (*n* 52 and *n* 65, respectively) showed no significant correlation between plasma nutrient concentration and respiratory infections or diarrhoea. Similar analysis showed that only dietary vitamin C was significantly correlated with respiratory infections (relative risk 1·101 (95 % CI 1·004, 1·208)). There was no significant association between dietary nutrients and diarrhoea. These results, however, need to be interpreted with caution due to the low

Table 1. Anthropometric measurements in elderly Ecuadorians
(Mean values and standard deviations)

	Men (*n* 47)		Women (*n* 96)		
Variable	Mean	SD	Mean	SD	*P*
Weight (kg)	58	10	53	9	0·09
Height (cm)	155	8	143	4	<0·0001
Knee height (cm)	40	2	35	3	<0·0001
Waist (cm)	86	8	88	12	0·327
Triceps skinfold (mm)	6	2	11	4	<0·0001
BMI (kg/m^2)	24	3	26	4	0·007
Underweight (BMI < 20 kg/m^2; %)	13		7		0·285
Normal (BMI 20–24·9 kg/m^2; %)	47		36		0·190
Overweight (BMI ≥ 25 kg/m^2; %)	40		57		0·058
High waist circumference*	4·3		44·9		<0·0001

* Men > 102 cm; women > 88 cm.

Table 2. Dietary macronutrient and micronutrient intake of elderly Ecuadorians (*n* 52)
(Mean values and ranges)

Nutrient	Mean	Range	EAR, AI or AMDR reference values	Below reference range (%)	Above reference range (%)
Energy (kJ)	5862	899–16 677			
Total fat (% energy)	14·1	0·71–34·1	25–35*	92·3	
Carbohydrate (% energy)	76·7	47–100	45–65*		86·5
Protein (% energy)	10·3	0·76–20·81	10–30*	53·8	
Total vitamin A (μg RE)	406	0·1–5031	500–625†	88·5	
Vitamin D (calciferol) (μg)	1	0·00–12	10–15‡	98·1	
Total vitamin E activity (mg)	2	0·04–5	12†	100	
Vitamin K (phylloquinone) (μg)	32	0·1–226	90–120‡	92·3	
Riboflavin (mg)	0·78	0·01–2·8	0·9–1·1†	67·3	
Vitamin B_6 (mg)	1	0·1–3	1·3–1·4†	53·9	
Folate (μg)	359	2–859	320†	50·0	
Vitamin B_{12} (μg)	1·4	0·00–30	2·0†	82·7	
Ca (mg)	238	2–1667	1200‡	98·1	
P (mg)	553	12–1638	580†	61·5	
Mg (mg)	174	5–367	265–350†	92·3	
Fe (mg)	10	0·1–23	5·0–6·0†	19·2	
Zn (mg)	5	0·1–14	6·8–9·4†	76·9	
Cu (mg)	1	0·1–3	700†	34·6	
Se (μg)	64	0·4–163	45†	28·9	
Na (mg)	2686	244–11 654	1500‡	30·8	69·2
K (mg)	1651	83–4548	4700‡	100	

EAR, estimated average requirement; AI, adequate intake; AMDR, acceptable macronutrient distribution range; RE, retinol equivalents.
* AMDR (Food and Nutrition Board & Institute of Medicine of the National Academies, 2002).
† EAR (Food and Nutrition Board & Institute of Medicine, 2000; Institute of Medicine, 2000*b*).
‡ AI; EAR not available (Institute of Medicine, 2000*b*; Food and Nutrition Board, 2004).

number of subjects for whom all data were available, as well as the recall nature of data collected for infection.

Immunological assessment

DTH skin tests were performed on fifty-two randomly selected participants. The majority (69 %) had positive responses to tuberculin, 41 % to diphtheria and less than 40 % to the other antigens at 48 h. The composite score and the mean number of positive responses were similar at 24 and 48 h. Similar patterns were seen in both women and men. The response to tetanus toxoid was significantly higher at 48 h than at 24 h, whereas the response to *Proteus* was significantly lower at 48 h than at 24 h (Table 5). Logistic regression analysis in a sub-group of subjects for whom dietary and serum nutrient concentrations as well as DTH were available (*n* 52) showed a positive correlation between plasma PLP concentration and DTH score at 48 h after correcting for sex, age and BMI ($P=0·002$), and a positive correlation between dietary intake of vitamin B_6 ($P=0·005$), vitamin A ($P=0·02$), Fe ($P=0·02$) and Cu ($P=0·01$) after correcting for energy intake, BMI, sex and age. There was also a trend for correlation between dietary vitamin D levels and DTH ($P=0·057$). These results, however, need to be interpreted with caution because of the limited number of subjects for whom all values were available.

Discussion

This sample of elderly Ecuadorians had multiple nutritional deficiencies, as assessed by both dietary recall and laboratory analyses. This population had lower than recommended dietary intake of vitamins A, D, E, B_2, B_6 and B_{12}, and folate, Zn, Ca and Mg. Although the mean total energy intake appeared to be low, total energy intake varied widely. Furthermore, in the absence of information on physical activity, it is difficult to determine adequacy of energy intake. In addition, despite several quality-control measures included in the collection of these dietary recall data, the possibility that some subjects underreported their intake could not be ruled out. Notably, carbohydrate intake was higher than recommended (Food and Nutrition Board & Institute of Medicine of the National Academies, 2002) in the majority of subjects. In addition, 69·2 % had higher than recommended intake of Na. It is, however, difficult to obtain accurate estimates of salt intake from the dietary intake data. Thus, these results should be confirmed by 24 h urinary Na excretion analysis. Although the proportion of overweight and obese elderly people in this peri-urban, poor community was less than that in the USA (Flegal *et al.* 1998), the fact that 51·7 % had BMI greater than 25 kg/m^2, in the presence of low concentrations of several micronutrients, suggests that these elderly Ecuadorians suffer from the increasingly common double burden of diseases associated with nutrition transition in less-developed countries and consumption of diets with poor quality.

We did not specifically evaluate the metabolic syndrome in the present study, but based on triacylglycerol and HDL levels, approximately 20 % of these Ecuadorian elders met the proposed limit of these indicators of the metabolic syndrome (Hall *et al.* 2003). This proportion is similar to those reported in overweight American adults (Park *et al.* 2003). Of the women, 45 % had waist circumference that was

Table 3. Blood vitamin and trace element concentrations in elderly Ecuadorians (*n* 65)
(Mean values, standard deviations and ranges)

Variable	Mean	SD	Reference range	Reference method	Below reference range (%)
Vitamin A (μg/l)	57·8	15·0	30·0–90·0*	Bieri *et al.* (1979)	3
Vitamin D (ng/ml)	19·03	5·6	15–38*	Anonymous (2001)	19
Vitamin E (μg/l)	11 621·1	330·0	500·0–1800·0*	Bieri *et al.* (1979)	0
PLP (nmol/l)	42·49	23	30–117†·	Hamfelt (1962); Maruyama & Coursin (1968); Sundaresan & Coursin (1970); Reynolds (1987)	31
Vitamin B_{12} (pg/ml)	323·44	256	250–1200†	‡	43
Folate (ng/ml)	6·57	2	5–30†	‡	18
Cu (μg/l)	128·11	22·0	55·0–175·0*	Dawson *et al.* (1968)	0
Fe (μg/l)	64·34	34·0			39
Females			50·0–170·0*	Olson & Hamlin (1969)	
Males			65·0–175·0*		
Zn (μg/l)	70·38	13·0	70·0–150·0*	Smith *et al.* (1979)	50·0
Se (ng/ml)	142·24	17·81	33–180*	Brumbaugh & Walther (1991)	0
Na (meq/l)	141·93	3	135–150§	Anonymous (1988)	0
K (meq/l)	4·69	0·5	3·5–5·3§	Tietz (1986); Anonymous (1988)	0
Ca (mg/l)	9·34	0·3	8·3–10·2§	Michaylova & Ilkova (1971)	0
P (mg/l)	3·30	0·4	2·3–4·7§	Daly & Ertingshausen (1972)	1·5
Mg (mg/l)	1·70	0·2	1·3–2·6§	Ingman & Ringbom (1966); Gindler & Helth (1971)	0
Cl (mmol/l)	105·64	3	96–110§	Anonymous (1988)	0

PLP, pyridoxal-5-phosphate.
* Tietz (1986).
† Tietz (1986), modified; see p. 847.
‡ Quantafhase II B_{12}/Folate Radioassay (Bio-Rad Laboratories Inc., Hercules, CA, USA).
§ Reference ranges recommended by the kit used for analysis or as indicated (Cobas Mira Chemical System; Roche Diagnostics, Nutley, NJ, USA).

higher than the limit value (Hall *et al.* 2003), whereas only 4·3 % of men had values above the limit, suggesting that elderly women in Quito are at higher risk for the metabolic syndrome than men.

A substantial proportion of the subjects had low plasma Zn and Fe concentrations. Consistent with this, 76·9 % of the elderly had a low dietary intake of Zn due to the limited consumption of food of animal origin in this population. A positive correlation between dietary intake and plasma Zn concentration was observed even after adjustment for Cu. Although only 19 % of subjects had low daily intake of total Fe according to the dietary recall survey, most of the consumed foods are grains and vegetables, which are not a good source of bioavailable Fe. Similar results have been found in Ecuadorian children (Sempertegui *et al.* 1996). In addition, high consumption of phytates from barley and other local cereals, which reduces mineral absorption, has been reported in this region (Holt & Brown, 2004). Logistic regression analysis indicated significant positive correlation between dietary Fe and serum Fe levels, but the significance was lost after correcting for sex, age, BMI, energy

Table 4. Fasting blood lipid, glucose and homocysteine concentrations in elderly Ecuadorians (*n* 65)
(Mean values and standard deviations)

	Total				
Variable	Mean	SD	Above upper limit (%)	Reference range	Reference method
Cholesterol (mg/l)	190·0	32·0	2	130·0–278·0	Allain *et al.* (1974)
Triacylglycerol (mg/l)	167·0	83·0	21	41·0–204·0	Trinder (1969); Spayd *et al.* (1978); Esders & Michrina (1979)
HDL (mg/l)	52·0	10·0	0*	37·0–82·0	Trinder (1969); Allain *et al.* (1974); Spayd *et al.* (1978); Esders & Michrina (1979); Warnick *et al.* (1982)
LDL (mg/l)	106·0	29·0	2	62·0–189·0	Friedewald *et al.* (1972)
VLDL (mg/l)	34·0	15·0	20	5·0–31·0 or 3·0–34·0	Friedewald *et al.* (1972)
LDL:HDL	2	0·6	0	1·1–5·9 or 0·8–4·9	–
Glucose (mg/l)	86·0	14·0	3	64·0–112·0	Barthelmai & Czok (1962)†
Homocysteine (mol/l)	12	4	25	5·5–15·0	Araki & Yoshiyasu (1987)

* 19 % Below limit.
† As modified by Roche Diagnostic Systems, Inc. (Nutley, NJ, USA); technical procedure number 44 557 (1985).

Table 5. Delayed type hypersensitivity response to multitest CMI (Pasteur-Merieux, Paris, France) at 24 and 48 h in elderly Ecuadorians (*n* 50)

(Mean values and standard deviations)

	Diameter of induration (mm)					
	24 h		48 h		Positive (%)	
Antigen	Mean	SD	Mean	SD	24 h	48 h
Tetanus toxoid	0·70	1·5	1·43*	2·3	20	30
Diphtheria toxoid	2·02	2·3	2·30	2·7	47	41
Streptococcus	0·70	1·2	0·71	1·2	24	20
Tuberculin	2·90	2·4	3·30	2·5	65	69
Glycerin	0·02	0·14	0·02	0·14	0	0
Candida	0·62	1·0	0·92	1·3	12	28
Trichophyton	0·18	0·52	0·23	0·58	2	4
Proteus	2·25	1·9	0·88*	1·2	51	22
Total diameter of induration	9·68	6·09	9·94	7·03		
Total number of positive responses	2·20	1·41	2·12	1·69		

Mean value was significantly different from that at 24 h *$P<0·01$.

intake, Ca, vitamin C and fibre levels (Fleming *et al.* 1998). The dietary Fe levels were split into haeme and non-haeme Fe, but no significant correlation between serum Fe levels and either dietary form was observed. Given the small number of subjects in whom both measurements were available, it is likely that a larger number of subjects are needed to observe significant correlation when confounders are included. Alternatively, since the diet of the elderly is low in Zn and B_6, and they have low animal protein consumption, we suggest that the low Fe status is mainly due to the poor quality of their diet; however, the elderly Ecuadorians might have large day-to-day variation in haeme intake not well captured in a few days represented by dietary records.

Of these elders, 43 % had low plasma vitamin B_{12} concentrations and 18 % had low folate. This could be related to low dietary intake, although vitamin B_{12} deficiency might also have been secondary to diminished absorption due to gastric atrophy associated with ageing (Wolters *et al.* 2004). Approximately 25 % of our cohort had high homocysteine concentrations. Plasma homocysteine is related to the cellular availability of both vitamin B_{12} and folate (Wolters *et al.* 2004). The plasma PLP was low in 31 % of these elders, which might be due to poor consumption of vitamin B_6 (53 % had below recommended dietary intake of B_6). This is further supported by the observation that dietary vitamin B_6 levels positively correlated with serum level of PLP ($P<0·05$). To our surprise, and despite numerous sunny days throughout the year, 19 % of these Ecuadorian elders had low vitamin D concentrations. It is possible that those subjects who were deficient tended to stay inside their households most of the time.

About 20 % of these elders had high triacylglycerol and VLDL concentrations. This could be related to the high intake of carbohydrates, which are converted into lipids in the liver. A similar percentage had low HDL concentrations. Underlying dyslipidaemias in this population may contribute to the self-reported moderate levels of hypertension and heart disease (19 and 12 %, respectively) of the studied subjects. These figures are lower than those reported for the US population (Lethbridge-Cejku *et al.* 2004; National Center for Health Statistics, 2004), but this could be related to under-reporting and infrequent visits to health providers.

DTH reaction to seven recall antigens showed an average diameter of induration, which was about one-third of that reported in developed countries (Meydani *et al.* 1997). Although a positive association was found only between plasma PLP and dietary PLP, Fe, Cu, vitamin A, and DTH, and between dietary vitamin C and respiratory infection, the impaired cellular immunity might also be due to other critical deficiencies such as that of Zn, protein (Pepersack *et al.* 2001) and/or metabolic changes related to being overweight. However, a larger sample size than that of the present study as well as interventional clinical trials would be needed before these associations can be established. Several factors contribute to resistance to infectious diseases. However, immune response is an important determinant. Thus, the low immunological status of these elderly Ecuadorians could contribute to the high frequency of upper respiratory and diarrhoeal infections reported (54 and 21 %, respectively) during the previous 6 months. However, these figures are based on a 6-month recall survey focused on infectious diseases and thus should be interpreted with caution, given potential problems with recall bias.

In conclusion, micronutrient deficiencies and inadequate consumption of protein and fat are common in elderly Ecuadorians and might contribute to their low immunological status and high prevalence of infectious diseases. These findings need to be confirmed in larger cross-sectional and interventional studies. Nevertheless, this initial report of nutrition and health status in elderly Ecuadorians suggests that an argument similar to those used in support of increasing children's nutriture in the less-developed countries could begin to be made for elderly people in the less-developed countries. Furthermore, these results suggest that nutrition intervention to address both under- and over-nutrition in older individuals in Ecuador may play a crucial role in preventing death and disability, promoting successful ageing, and reducing the burden on the limited healthcare resources of the country.

Acknowledgements

The present study was presented in part at Experimental Biology 2004, Washington, DC, 17–21 April 2004

(Sempértegui *et al.* 2004). It was supported by Fogarty no. 1R03 TW00577901A1 and USDA contract no. 58-1950-9-001.

We greatly appreciate the technical assistance of Mercy Sempértegui and Ximena Narváez, and the logistical collaboration of the field nurses of the Vitamin A and/or Zinc Supplementation for the Prevention of Pneumonia in Children (VAZPOP study). In addition, we appreciate the dietary data analysis by Janice Maras and Peter Bakun in the Dietary Assessment and Epidemiology Research Program. Special recognition also goes to Dr Jeffrey K. Griffiths, principal investigator of the VAZPOP study, for his kind assistance. We are very grateful to the elderly participants.

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Chapter 7

Micronutrient deficiencies are associated with impaired immune response and higher burden of respiratory infections in elderly Ecuadorians

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Nutrition 2009; 139; 113-119

The Journal of Nutrition
Nutritional Epidemiology

Micronutrient Deficiencies Are Associated with Impaired Immune Response and Higher Burden of Respiratory Infections in Elderly Ecuadorians[1,2]

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Abstract

The proportion of the Latin American population above age 65 y is expected to rise substantially. To better define the prevalence of infectious diseases and micronutrient deficiencies, assess immunological status, and evaluate associations between nutritional status and infection, we performed a cross-sectional study of elderly Ecuadorians in a low-income peri-urban community in Quito, Ecuador. Culturally adapted questionnaires, delayed type hypersensitivity (DTH) skin response, micronutrient, and immunological assays were performed in randomly selected Ecuadorians aged ≥65 y. Multiple linear and logistic regression models were developed to assess relationships between micronutrient concentrations and history of infection, DTH, and immune function. Participants ($n = 352$; mean age ± SD, 74.4 ± 6.4 y) recalled recent episodes of colds/influenza-like syndromes (62.8%), cough (61.0%), urinary tract infection (37.9%), diarrhea (32.2%), fever (24.1%), and pneumonia (3.5%). A prospective substudy of respiratory infections (RI) in 203 elderly revealed similar findings. Colds and pneumonia occurred in 42.8 and 7.9% of participants, respectively, during 737 person-weeks of observation (3.6 ± 1.1 wk per person). Anemia and micronutrient deficiencies, especially for vitamins C, D, B-6, and B-12 and folic acid and zinc, were common. Plasma vitamin C was associated with interferon-γ (IFNγ) ($P < 0.01$) and zinc with IFNγ and interleukin-2 (each $P < 0.0001$). RI history was associated with any micronutrient deficiency ($P < 0.001$). The burden of infectious diseases, micronutrient deficiencies, and anemia was substantial in this elderly Ecuadorian population. Deficiencies of essential vitamins and minerals place these elderly adults at risk for infections through their negative impact on immune function. J. Nutr. 139: 113–119, 2009.

Introduction

There has been a steady increase in the proportion of the elderly population in both developed and developing countries during the last few decades. As a result of decreased infant mortality, lower fertility rates, and improved longevity, developing country populations will soon undergo rapid and unparalleled changes in their age structure that will result in a substantial increase in the percentage of the aged population (1). The increase in the proportion of the population over 60 y of age in developing countries is estimated to be 1.5 times faster than that in developed countries (1).

In Latin America and the Caribbean, the number of people aged ≥60 y is projected to increase from ~40 million in 2004 to 100 million in 2025 (2). Because many parts of Latin America are poor, the elderly in Latin American countries are likely to have more infectious and chronic diseases, greater disability, and fewer resources available for their health care needs. Unlike many developed nations, few of the limited health resources of developing countries have been devoted to the elderly, despite their diminished capacity to fight disease without adequate nutritional, economic, and psychosocial support. They therefore

[1] Supported by the NIH Fogarty International Center (1 R03 TW005779-01A1), the National Institute on Aging (2R01 AG009140-10A1), National Institute of Child Health and Development (R01 HD38327), and cooperative agreement no. 58-1950-7-707 with the USDA.
[2] Author disclosures: D. H. Hamer, F. Sempértegui, B. Estrella, K. L. Tucker, A. Rodríguez, J. Egas, G. E. Dallal, J. Selhub, J. K. Griffiths, and S. N. Meydani, no conflicts of interest.
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0022-3166/08 $8.00
Manuscript received 24 June 2008. Initial review completed 15 August 2008. Revision accepted 17 October 2008.
First published online December 3, 2008; doi:10.3945/jn.108.095091.

risk living their lives burdened by disease and disability, which threatens to put an even greater strain on the limited health care resources of their countries.

The elderly in Latin America suffer from the same age-associated physiological, psychological, social, and economic changes that adversely affect the nutritional and immunological status of older individuals in industrialized nations. The incidence of chronic medical conditions such as cardiovascular disease, type 2 diabetes mellitus, malignancy, arthritis, autoimmune disorders, and infections increases with age (3–5). Aging is associated with impaired regulation of the immune system (6–9), contributing to a higher incidence of morbidity and mortality from infectious, autoimmune, and neoplastic diseases. Prospective studies indicate greater morbidity and mortality in elderly subjects with low delayed-type hypersensitivity (DTH)[11] responses, an in vivo measure of cell-mediated immune responses (10–13). Subtle subclinical deficiencies of micronutrients, such as zinc, selenium, and vitamin E, and inadequate macronutrient intake contribute to the decline in immune function in the elderly (14–17).

Limited information is available on the nutritional, immunological, and general health status of the aged in Latin America. Preliminary evidence from a previous, smaller study of 145 elderly in Quito, Ecuador revealed inadequate intake of protein and multiple micronutrients, including vitamins B-6, B-12, and D, folate, iron, and zinc (18). Infectious diseases were a major cause of morbidity and of physician or hospital visits for the elderly in this study. To better define the prevalence of infectious diseases and micronutrient deficiencies, determine the immunological status of the elderly using DTH skin responses, and evaluate the association between nutritional status and infection, we performed a cross-sectional survey of elderly Ecuadorians living in a peri-urban slum community in Quito, Ecuador.

Methods

Study site and population. This cross sectional study was conducted in 2 consecutive rainy seasons from September 2003 to December 2004 in poor peri-urban areas in northwestern Quito, at an altitude of 2800 meters above sea level. The study area had an estimated population of 19,000 and, based on electoral results, 5% (~950 individuals) were above the age of 65 y. The 3 study neighborhoods, which included only residents of low socioeconomic status (mean monthly income was US$54, which is <50% of the basic income in Ecuador), were located on a hillside and were structurally similar, with 1 main paved road and electricity present in all homes. More than 40% of the elderly individuals in these neighborhoods were illiterate. Some households had a municipal source of potable water and sewerage. Inhabitants of this community were originally from small cities and rural areas of Ecuador. This area of Quito was typical of poor, under-resourced neighborhoods within the city. This population is similar to those one would encounter in peri-urban areas in other Andean countries, such as Puno, Peru and La Paz, Bolivia.

Screening and enrollment. The Ethical Committee of the Corporación Ecuatoriana de Biotecnología and the Tufts-New England Medical Center Institutional Review Board approved the protocol and informed consent form. During meetings at the field work station, each subject was given detailed information on the study objectives and procedures. If the potential study participant was deaf, we used the assistance of a relative to help the participant understand the study purpose and procedures. After potential participants had an opportunity to ask questions, written informed consent was obtained. If the participant could not read, the form was read to them in the presence of a literate family member. The participant was then asked to place an X on the signature line and the form was cosigned by a witness.

To identify eligible elderly people, we conducted a census in the 3 neighborhoods. During household visits, we provided potential study candidates ($n = 413$) with detailed information on the study and a subset of participants ($n = 352$) were selected, using a random numbers table, to participate. Eligibility criteria included age ≥65 y, mental competence, and willingness to provide written informed consent. Participants' age was verified with their national identification card. The study nurses determined mental competence by means of a brief set of simple questions related to memory and cognitive skills for daily decision making.

Study procedures. Questionnaires and study procedures were performed by trained nurses at the field work stations, which were located in each of the 3 study neighborhoods and were accessible to all study participants. Anthropometric measurements, DTH, and phlebotomy for micronutrient, hematological, and immunological assays were performed at the field work stations.

Participants were interviewed to assess their social, economic, housing, sanitation, and general health status using a Spanish version of the Cross Cultural Research on the Nutrition of Older Subjects questionnaire (19), which has been used in Guatemala. The questionnaire was adapted by including Spanish idioms used in Ecuador and reviewed to ensure that they were relevant and culturally appropriate. A pilot test was conducted to assess the adequacy of words and questions for the local culture. The questionnaire was finalized using the pilot study results (18). A week-long pilot study with 30 elderly participants was performed to allow study personnel to familiarize themselves with the study procedures and to finalize data collection instruments. The results of the pilot study were not included in the final analysis.

Active surveillance of respiratory infections substudy. To assess accuracy of infection data collected through recall by participants, 203 elderly participants were randomly selected from the study neighborhoods to participate in a substudy of the incidence of respiratory infections (RI). After obtaining informed consent, participants were evaluated at a study clinic based in a local community center once per week during a 5-wk period from September until early October 2004. During the visit, a history of RI symptoms during the previous week was obtained and then the participants were examined for signs of respiratory and eye infections. Using standardized definitions of infections (20), the field physicians determined diagnoses of RI and conjunctivitis based on their history and physical examination findings.

Anthropometry. Anthropometric measurements were taken as described by Gross (19); these included weight, height, knee height, and waist circumference. Weight was recorded to the nearest 0.1 kg using a Detecto scale with a minimal amount of clothing. Height was measured to the nearest 0.1 cm using a steel fiberglass measuring tape affixed to a wooden rod, with a sturdy straight edge used as a headpiece. Because it can be difficult to measure standing height in some elderly individuals, we also measured knee height to the nearest 0.1 cm, using a special knee-height anthropometer (21). We used knee height in the BMI calculation for 23 women (10% of female participants) and 17 men (13% of male participants) who were not able to stand fully erect. Waist circumference was assessed with a nonstretch measuring tape between the upper edge of the right iliac crest and the umbilicus and was recorded twice to the nearest 0.1 cm.

DTH skin response. DTH was assessed using the Mantoux technique with 4 recall antigens (22): tetanus toxoid, tuberculin, *Candida albicans*, and *Trichophyton mentagrophytes*, as well as a glycerin control administered on the volar surface of the right arm. The same investigator evaluated the DTH response for all participants. We measured the vertical and horizontal diameters of induration after 24 and 48 h and considered the reaction positive when the mean value was ≥5 mm. Means for each antigen were calculated and a composite score, based on the results of all of the antigens in each participant, was determined.

[11] Abbreviations used: DTH, delayed type hypersensitivity; IFNγ, interferon-γ; IL-2, interleukin-2; PHA, phytohemagglutinin; RI, respiratory infection.

Laboratory procedures. A 10-mL venous blood sample was drawn from each participant, after an overnight fast, into an EDTA-treated tube and a tube without anticoagulant. Samples were immediately transported to the laboratory and centrifuged. Plasma samples for vitamin C were promptly deproteinized using perchloric acid and EDTA. Serum or plasma was collected in plastic tubes, frozen at −20°C, and shipped to Boston, MA for analysis of B vitamins; vitamins A, C, D and E; and calcium, zinc, iron, and copper according to standard procedures at the Nutritional Evaluation Laboratory of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University as previously described (18). Full details of the derivation of the reference ranges are described in Sempértegui et al. (18).

Lymphocyte proliferation and cytokine measurements were conducted using a modified whole-blood assay (23,24) phytohemagglutinin (PHA) was used to stimulate the whole blood as follows. In each well, 100 μL of a PHA 200 mg/L solution was added to 900 μL of blood. We measured PHA-stimulated interleukin-2 (IL-2) and interferon-γ (IFNγ) production in culture supernatant (25,26) using commercially available ELISA kits (R&D Systems). Complete blood counts and a blood smear were done to determine the white blood cell differential. Participants were asked to provide a fecal sample, which was analyzed for ova and parasites by saline wet mounts under 40× microscopy.

Statistical analysis. The sample size was based on the assumptions that nutritional deficiencies are prevalent in poor elderly Ecuadorians living in Quito, that these nutritional deficiencies contribute to the age-associated decline in T cell-mediated function, and that this results in an increase in infectious diseases. We therefore hypothesized that elderly Ecuadorians with more severe nutrient deficiencies would have the lowest T cell-mediated function and the highest prevalence of infectious diseases. As data on prevalence of infectious diseases in this population were not available, variability observed in DTH was used for sample size calculation. A sample of 350 participants would yield an 80% chance that the hypothesis of a 0 population correlation coefficient or partial correlation coefficient would be rejected at the 0.05 level, as long as the magnitude of the underlying correlation coefficient was ≥0.15.

Data entry and management were done using Epi-Info software, version 6.04d (CDC, Atlanta, GA). Statistical analyses were performed using SPSS, version 11.5. Descriptive statistics for anthropometric measurements, BMI, DTH response, and health profile survey responses were calculated globally and by sex. BMI was calculated as kg/m^2 (27). Weight was classified as underweight (BMI <20), recommended (BMI 20–24.9), and overweight (BMI ≥25). RI incidence was calculated by dividing the number of diagnoses by person-weeks of observation. Differences in means and percentages between male and female participants were evaluated by Student's *t* and chi-squared tests, respectively. Values in the text are means ± SD.

Multiple linear or logistic regression models were developed to assess the relationship between micronutrient concentrations likely to have an effect on immune function (vitamins A, B-6, C, and E and iron and zinc) and the main outcome measures: DTH, IL-2, IFNγ, history of diarrhea, pneumonia, common cold, and RI (defined as a history of either common cold or pneumonia). Potential confounders for models in which diarrhea was an outcome included age, sex, housing score (based on a composite score of type of walls, floors, and roofs, number of bedrooms, kitchen location, cooking fuel, food storage, drinking water source, and toilet facilities), education, income, BMI, and fecal carriage of pathogenic (*Giardia lamblia*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Hymenolepsis nana*, *Balantidium coli*, and *Strongyloides stercoralis*) or non-pathogenic parasites (e.g. *Entamoeba coli*, *E. dispar*, *Chylomastix mesnili*). The RI model included the same variables, with the exception of the fecal parasites, and also included a variable for crowding (defined as the number of persons living in the household divided by the number of bedrooms). Response variables were log transformed to improve model fit, as needed.

Results

There were 225 women and 127 men enrolled. The majority of the participants, 95.2%, were enrolled in 2 sequential rainy seasons. The age of the participants was 74.4 ± 6.4 y. Male participants were older and had higher educational levels than female participants, although few men and no women had attended secondary school (**Table 1**). A greater percentage of men than women were currently married, whereas more women were divorced. Most participants were Catholic and had originated from rural areas of Ecuador. Overall, 11% currently smoked some form of tobacco and 21% had smoked in the past for more than 1 y. Only 7.7% acknowledged current consumption of alcoholic beverages, although 50% used to occasionally drink, 10% most days, and only 4.6% daily. Women were significantly more likely than men to be overweight and to have a high waist circumference.

Recall of recent infections. During the previous month, only 12% of participants denied having had 1 of the following: diarrhea, fever, cough, cold or influenza-like syndrome, pneumonia, or urinary tract infection. Of those who recalled 1 of these syndromes in the last month, 19% had 1 episode, 25% had 2 episodes, and 44% had 3 or more. Participants recalled having had colds or influenza-like syndromes (62.8%), cough (61.0%), urinary tract infection (37.9%), diarrhea (32.2%), fever (24.1%), and pneumonia (3.5%). Overall, 42% had seen a health care provider, 25% had spent time in bed, and 4.6% had been hospitalized overnight because of illness in the last 3 mo. Common types of infections that prompted visits to health workers were pneumonia (0.85%), cough (1.7%), cold or flu (0.85%), urinary tract or kidney infection (1.1%), and diarrhea (0.85%). Common infectious diseases that resulted in spending time in bed at home included colds or flu (4.8%) and pneumonia (0.85%).

TABLE 1 Demographic characteristics and anthropometric measurements of elderly Ecuadorians[1]

Variable	Men	Women	*P*-value
n	127	225	
Age, *y*	75.8 ± 6.5	73.7 ± 6.1	0.003
(range)	(65 – 94)	(65–97)	
Education level, %			
No school	29.4	52.5	<0.0001
<3 y primary school	23.0	24.2	0.8
3–6 y primary	42.9	22.9	<0.0001
Some secondary	4.0	0	
Completed secondary	0.8	0	
Marital status, %			
Married	69.8	37.5	<0.0001
Single	2.4	7.6	0.07
Widowed	22.8	49.6	<0.0001
Divorced or separated	5.6	4.5	0.62
Religion, *% Catholic*	91.3	91.0	0.4
Birthplace, *% rural*	69.0	73.9	0.31
Anthropometrics			
Weight, *kg*	59.9 ± 9.5	54.0 ± 9.80	<0.0001
Height, *cm*	156 ± 7	144 ± 6	<0.0001
BMI, *kg/m^2*	24.8 ± 3.2	26.1 ± 4.0	0.0037
Underweight: BMI <20, %	2.7	4.5	0.444
Normal: BMI 20–24.9, %	57.3	39.8	0.003
Overweight: BMI ≥25, %	40.0	55.7	0.008
High waist circumference,[2] %	8.8	46.7	<0.0001

[1] Values are mean ± SD or %.
[2] Men >102 cm; women >88 cm.

Substudy of active RI surveillance. There were 203 participants (65.5% women) in the RI substudy who were observed for a 3.6 ± 1.1 wk per person for a total of 737 person-weeks of observation. Nearly one-third (29.6%) had a RI or acute conjunctivitis. The common cold was the most frequently encountered RI, occurring at a rate of 115 per 1000 person-weeks of observation, followed by conjunctivitis (71 episodes), pharyngitis (53 episodes), pneumonia (38 episodes), bronchitis (7 episodes), otitis media (5 episodes), and 2 cases of asthma per 1000 person-weeks of observation. When all RI were combined (pharyngitis, common cold, otitis, bronchitis, and pneumonia), 50.5% of the elderly had some type of RI in the preceding month. Common colds and pneumonia occurred in 42.8 and 7.9% of participants, respectively.

Micronutrient blood and serum concentrations. The mean concentrations of vitamins A and D and of iron were significantly lower in women than in men (**Table 2**). In contrast, male participants had significantly lower concentrations of vitamins C, E, and B-6 and folate than women. Substantial deficiencies (≥5%) were noted for vitamins C, D, B-6, and B-12 and folate, zinc, and copper (Table 2). Men were more likely than women to be deficient in vitamins C, D, and B-6 and tended to have lower folate ($P = 0.06$).

DTH responses, hematological measurements, and immunological assays. More than 50% of participants had positive DTH responses to *Candida* and tuberculin, whereas only 22 and 16% responded to *Trichophyton* and tetanus toxoid, respectively (**Table 3**). The mean diameter of induration was significantly greater at 48 h relative to 24 h for *Candida* and tuberculin but not for the other antigens tested. Men and women did not differ in total white blood cell count (6876/μL ± 1992 for men vs. 6588/μL ± 1810 for women), IFNγ (4889 ± 5224 ng/L supernatant for men vs. 5464 ± 5789 ng/L supernatant for women), and IL-2 (3375 ± 4382 ng/L supernatant for men vs. 4022 ± 4796 ng/L supernatant for women). Hemoglobin tended to be higher in men (157 ± 29.1 g/L) than in women (136 ± 25.8 g/L) ($P = 0.08$). Using a standard cutoff point for anemia at sea level (men <120 g/L and women <115 g/L), 17.3% of men and 27.1% of women were anemic ($P = 0.038$). However, because Quito is at 2800 m above sea level, the altitude-adjusted threshold for anemia was higher (men <136 g/L and women <131 g/L) (28,29). Using this cutoff point, 29.9% of men and 39.6% of women were anemic ($P = 0.08$).

Stool parasites. Of 343 participants (97.4%) for whom stool samples were submitted, 6.4% were positive for potentially pathogenic parasites. Although 36% of the participants had *E. histolytica*/*E. dispar*, specialized tests were not performed to distinguish between these species. Because there were no correlations between the presence of these organisms and blood/fecal leukocytes and a history of diarrhea in the last month, they most likely had the nonpathogenic *E. dispar*. Whereas 36.7% had no parasites in stool specimens, 63.3% had 1 or more parasites (pathogenic and nonpathogenic combined) and 42.6% had 2 or more. Sixty-one percent of the elderly had 1 or more nonpathogenic parasites identified in their fecal specimens.

Logistic and linear regression analysis. None of the potential confounding variables, specific micronutrient deficiencies, or the presence of any type of micronutrient deficiency were associated with a history of recent diarrhea or pneumonia in logistic regression models. However, a history of RI (defined as either pneumonia or cold) and a cold were strongly associated with the presence of any micronutrient deficiency ($P < 0.001$) and were inversely associated with education ($P = 0.03$) (**Table 4**).

Plasma vitamin C and IFNγ were associated ($P < 0.01$) and serum zinc and IFNγ were associated ($P < 0.0001$) in linear models, adjusting for age, sex, and BMI (**Table 5**). Serum iron and IL-2 concentrations were also positively associated ($P = 0.02$), as were serum zinc and IL-2 ($P < 0.0001$).

Discussion

This cross-sectional study of elderly Ecuadorians residing in a peri-urban community in Quito, characterized by low socioeconomic status, revealed a high burden of infectious diseases, especially RI, and numerous deficiencies of essential vitamins and minerals. DTH testing revealed that a minority of the study population mounted responses to tetanus toxoid and *Trichophyton*, whereas more than one-half responded to tuberculin and *Candida* test antigens. Although the majority of the elderly participants had normal white blood cell and platelet counts, a moderate proportion was anemic. Given the high-altitude

TABLE 2 Circulating vitamin and trace mineral concentrations in elderly Ecuadorians stratified by gender[1]

Micronutrient	Reference range[2]	Men ($n = 125$)	Women ($n = 224$)	Men	Women
				% deficient	
Serum vitamin A, *μmol/L*	1.05–3.14	1.92 ± 0.48*	1.78 ± 0.43	0.8	4.1
Plasma vitamin D, *nmol/L*	39.0–98.7	57.1 ± 15.7**	49.3 ± 13.6	18.8¶	9.4
Serum vitamin E, *μmol/L*	11.6–41.8	27.4 ± 7.0†	30.4 ± 9.1	0	0
Plasma vitamin C, *μmol/L*	11.4–125	11.4 ± 9.1**	17.0 ± 10.2	59.8**	32.6
Plasma vitamin B-6, *nmol/L*	30–117	42.6 ± 20.0†	52.3 ± 28.5	27	16.1
Plasma vitamin B-12, *pmol/L*	184–884	300.4 ± 157.7	325.5 ± 169.9	20.5	19.6
Plasma folate, *nmol/L*	11.3–68.0	13.13 ± 4.7††	15.0 ± 6.1	37	27.4
Serum copper, *μmol/L*	Female 13.2–24.0 Male 13.2–21.7	18.5 ± 5.2	19.4 ± 5.3	16.7	12.4
Serum iron, *μmol/L*	Female 8.95–30.4 Male 11.6–31.3	23.4 ± 9.0**	19.9 ± 8.2	4	5
Serum zinc, *μmol/L*	10.7–19.9	11.6 ± 3.4	11.4 ± 3.6	41.3	45.4

[1] Values are mean ± SD. *$P = 0.004$, **$P < 0.001$, †$P = 0.001$, ††$P = 0.003$, ¶$P = 0.02$.
[2] 18.

TABLE 3 Delayed type hypersensitivity response to 4 test antigens at 24 and 48 h in elderly Ecuadorians[1]

	Diameter of induration[1]		Timepoint	
	24 h	48 h		
Antigen	($n = 346$)	($n = 343$)	24 h	48 h
	mm		*% Positive*	
Tetanus toxoid	1.94 ± 3.76	2.03 ± 4.40	15.6	15.7
Tuberculin	7.69 ± 5.90	9.08 ± 7.15*	59.5	61.5
Glycerin	0.04 ± 0.32	0.02 ± 0.24	0	0
Candida	5.97 ± 3.67	6.54 ± 4.28*	64.6	65.4
Trichophyton	3.43 ± 3.40	3.27 ± 3.56	22.8	22
Total diameter of induration, *mm*	19.0 ± 9.05	21.0 ± 10.2*		
Total positive responses, *n*/person	1.61 ± 0.96	1.64 ± 0.97		

[1] Values are mean ± SD or %. *Different from 24 h, $P < 0.05$.

setting where this study occurred, these participants are at risk for morbidity associated with decreased functional capacity secondary to the anemia.

We found several sex-specific differences in demographic, anthropometric, and nutritional measures in this elderly population. More men than women had at least 3–6 y of primary education. Some men had attended secondary school. In contrast, more than one-half of the women had not attended any school and none had attended secondary school. More women than men were overweight based on BMI and waist circumference. Men were more likely than women to be deficient in vitamins B-6, C, and D, and folate.

DTH testing demonstrated that the elderly Ecuadorians had substantially lower numbers of positive responses and mean diameters of induration for common skin test antigens than has been observed in elderly in the United States (30). However, DTH responses to tuberculin were common. This is not surprising given the relatively high prevalence of tuberculosis in Andean populations. In contrast to the tuberculin results, only a minority of the study participants mounted DTH responses to tetanus toxoid. Similarly, in a study of United States war veterans, elderly participants were significantly more likely than younger participants to have negative tetanus skin test responses (31). This finding suggests that there may be a loss of tetanus reactions with ageing. Alternatively, the elders living in these relatively poor communities, many of whom had migrated to Quito from rural farming communities, may never have been immunized with tetanus toxoid. These elderly Ecuadorians may therefore be at risk for the acquisition of tetanus.

Recall of RI during the last month showed a substantial burden of cold and influenza-like syndrome and a small but meaningful number of recent episodes of pneumonia. As impaired memory may limit the ability of elders to recall recent events, the similar levels of RI, especially common cold and pneumonia, in the prospective active surveillance substudy suggests that recall bias did not adversely impact the infection history data, as collected with the modified Cross Cultural Research on the Nutrition of Older Subjects questionnaire. In fact, the active surveillance substudy revealed even higher rates of pneumonia than recall; 7.9% of participants had pneumonia during the period of observation, yielding an incidence of 38 episodes of pneumonia per 1000 person-weeks of observation, whereas 3.5% recalled having had pneumonia during the last month. Although this difference is relatively small, active surveillance yielded pneumonia rates that were more than double the recall rates. This suggests that the burden of pneumonia in this elderly Ecuadorian population is substantial. Given the morbidity and mortality from pneumonia in the aged (5), the negative impact of pneumonia in this poor Latin American population is likely to be substantial. However, in contrast to the pneumonia results, the rate of recall was higher for common colds/influenza-like illness than the rates in the prospective study.

Measurements of micronutrient concentrations in blood samples revealed substantial deficiencies of vitamins C, D, B-6, and B-12 and folic acid and zinc. Vitamin C and zinc and IFNγ were associated, as were zinc, iron, and IL-2. The essentiality of these nutrients for normal function of the immune response is well recognized. Both IL-2 and IFNγ play critical roles in resistance to infectious diseases. Although we did not observe an association between any of the individual micronutrients with a history of recent pneumonia, influenza-like illness, cold, or diarrhea, the presence of some type of micronutrient deficiency and a history of recent infection were associated. Subclinical

TABLE 4 Logistic regression analysis for RI and diarrhea in elderly Ecuadorians

	RI		Pneumonia		Cold		Diarrhea	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Sex, *male*	0.83	0.46, 1.48	2.44	0.57, 10.4	0.82	0.46, 1.45	1.15	0.64, 2.05
Age, *y*	0.97	0.93, 1.02	0.91	0.80, 1.04	0.97	0.93, 1.02	0.97	0.93, 1.01
BMI, *kg/m*^{2}	0.97	0.90, 1.03	1.12	0.96, 1.32	0.96	0.90, 1.03	1.01	0.94, 1.08
Income, *US dollars/mo*	1.00	0.99, 1.00	0.98	0.95, 1.00	1.00	0.99, 1.00	1.00	0.99, 1.00
Housing score[1]	1.00	0.93, 1.08	1.12	0.93, 1.35	0.99	0.92, 1.07	1.02	0.95, 1.10
IL-2, *ng/L*	1.00	1.00, 1.00	1.00	1.00, 1.00	1.00	1.00, 1.00	1.00	1.00, 1.00
IFNγ, *ng/L*	1.00	1.00, 1.00	1.00	1.00, 1.00	1.00	1.00, 1.00	1.00	1.00, 1.00
Education, *y*	0.74[2]	0.56, 0.97	1.07	0.51, 2.23	0.74[2]	0.56, 0.98	0.84	0.63, 1.12
DTH score at 48h, *mm*	0.98	0.95, 1.00	0.95	0.89, 1.02	0.98	0.95, 1.00	1.00	0.97, 1.02
Crowding[3]	0.87	0.70, 1.07	0.78	0.40, 1.51	0.89	0.72, 1.09		
Any micronutrient deficiency[4]	4.00[5]	1.76, 9.06	1.51	0.17, 13.7	3.30[5]	1.51, 7.21	1.04	0.47, 2.28

[1] Housing score based on a composite score of type of walls, floors, and roofs, number of bedrooms, kitchen location, cooking fuel, food storage, drinking water source, and toilet facilities.
[2] $P = 0.03$.
[3] Ratio of number of persons to the number of rooms in the participant's home.
[4] Defined as the presence of a deficient blood or plasma level of any of the following micronutrients: vitamin C, vitamin E, pyridoxal 5′-phosphate (PLP), folate, iron, or zinc.
[5] $P < 0.001$.

TABLE 5 Linear regression analysis for IL-2 and INFγ in elderly Ecuadorians

	IL-2			INFγ		
	β[1]	SE[2]	*P*	β[1]	SE[2]	*P*
Age, *y*	−48.9	46.3	0.29	3.0	57.2	0.96
Sex, *male*	−474.4	614.6	0.44	−423.0	759.7	0.58
BMI, *kg/m²*	−1.5	71.7	0.98	44.4	88.7	0.62
Vitamin E, *μmol/L*	−1.0	0.74	0.17	−0.68	0.91	0.46
Vitamin C, *μmol/L*	2144.1	1577.6	0.18	5345.1	1950.1	0.007
PLP, *nmol/L*	−19.5	11.5	0.09	−19.8	14.2	0.16
Folate, *nmol/L*	−1.1	109.6	0.99	112.5	135.5	0.41
Iron, *μmol/L*	−15.1	6.4	0.02	−3.7	7.95	0.65
Zinc, *μmol/L*	75.5	13.3	<0.0001	84.2	16.4	<0.0001

[1] The β coefficient is the estimated variation of the dependent variable (IL-2 or INFγ) by the effect of the independent variables.
[2] SE, Standard error.

micronutrient deficiencies in general, and that of zinc in particular, have been associated with impairment of immune function (15,32) and increased risk of infection in the elderly population (15,33–35). However, the current study did not demonstrate an association of zinc deficiency with infections. This may have stemmed from the use of recall as the primary method for determining recent infection history or from an insufficient sample size. Given the anticipated rapid growth of the elderly portion of the total population in Latin America during the next few decades (2), there is a need to focus resources on more rigorous, prospective evaluations including interventions to improve the health of the elderly in Latin America.

We found a significant association between recall of a RI (either pneumonia or the common cold) and the presence of some type of micronutrient deficiency. Given the proportion of elderly men and women with micronutrient deficiencies, this finding suggests that inadequate nutrition places elderly Ecuadorians at increased risk for infection. A prior preliminary study conducted by our group in a similar elderly population in Quito revealed that dietary intake of total energy, protein, and several micronutrients, including vitamins A, D, B-6, and B-12 as well as zinc, was substantially below the reference range for usual dietary intake (18). Based on the findings of this earlier study and the present study, the overall nutritional status of the elderly in this peri-urban, marginal community appears suboptimal. Given the well-described immunosenescence associated with aging (36,37) and deficiencies of essential micronutrients such as zinc and vitamins C, D, and B-6, which play important roles in immune function, this population is at high risk for morbidity, and potentially mortality, from infectious diseases.

The fecal parasitology results suggest that the majority of the elderly Ecuadorians in this study population are living in a "contaminated" environment due to inadequate hygiene, sanitation, water sources, or contaminated food sources. Consequently, in addition to the high burden of RI, they are at increased risk for food- and water-borne disease.

Because, with the exception of the RI substudy, this was a cross-sectional study, the study design limited our ability to interpret some of the findings. For instance, each participant was interviewed, examined, and had blood work conducted at only 1 point in time so we cannot comment on longitudinal changes in immune function or micronutrient status over time. We also were limited to noting associations between micronutrient deficiencies and a history of illness such as diarrhea and thus were unable to determine the directionality of causation. Because we staggered enrolment of study participants over a 15-mo period, there is a possibility that there were seasonal fluctuations in the types of local foods available that might have influenced the micronutrient status of the participants. However, by virtue of its location near the equator, agricultural production is relatively stable throughout the year in Quito and its surrounding rural areas, which provide food to the city. In addition, most participants were enrolled and had their micronutrient and immunological status evaluated during the rainy season. Due to the small number enrolled in the sunny season, it was not feasible to evaluate the potential impact of seasonal fluctuations in food availability on the micronutrient status of the elderly in this study.

Given the evidence of substantial deficits in essential nutrients, along with high infection rates, this marginalized elderly population in Ecuador would benefit from improvements in overall food security and sanitation as well as focused interventions to address specific micronutrient deficiencies. In view of the rising proportion of the population that is aged in Ecuador and other countries in Latin Americ, and the anticipated increased health care costs associated with this demographic transition, there is a need to develop and test low-cost, simple nutritional and behavior change interventions to improve nutritional status, strengthen immune function, and minimize the risk of acquisition and severity of infectious diseases of the elderly in this region.

Acknowledgments
We thank Drs. Hooman Poor and Jatin Roper for help with field data collection.

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Chapter 8

Metabolic syndrome in the elderly living in marginal peri-urban communities in Quito Ecuador

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Public Health Nutrition 2010; 14; 758-767

Public Health Nutrition: 14(5), 758–767 doi:10.1017/S1368980010002636

Metabolic syndrome in the elderly living in marginal peri-urban communities in Quito, Ecuador

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Submitted 14 December 2009: Accepted 7 August 2010: First published online 19 October 2010

Abstract

Objective: The proportion of the Latin American population aged >60 years is expected to double during the next few decades. Metabolic syndrome (MetS) is associated with high morbidity and mortality worldwide. However, little is known about MetS in Latin America in general, and in Ecuador in particular. The present study aimed to examine the prevalence of MetS and its association with blood micronutrient, homocysteine (Hcy) and C-reactive protein (CRP) concentrations in the elderly living in a low-income urban area.

Design: We performed a cross-sectional study. MetS, using the International Diabetes Federation definition, dietary intake and plasma micronutrient, CRP and Hcy concentrations were assessed.

Subjects: A total of 352 elderly (≥65 years) Ecuadorians.

Setting: Quito, Ecuador.

Results: MetS was prevalent (40 %) – considerably more so among women (81 %) than men (19 %; $\chi^2 = 32{\cdot}6$, $P < 0{\cdot}0001$). Further, 53 % of those without MetS exhibited two or more of its components. Micronutrient deficiencies were prevalent, including those of vitamin C, zinc, vitamin B_{12} and folate. Vitamin C and E concentrations were inversely (OR = 0·78, 95 % CI 0·71, 0·86; OR = 0·16, 95 % CI 0·03, 0·81, respectively) and CRP (OR = 1·79, 95 % CI 1·04, 3·06) was positively associated with MetS.

Conclusions: The coexistence of MetS with micronutrient deficiencies suggests that elderly Ecuadorians suffer from the double burden of diseases that are increasingly being observed in less developed countries. More research is needed to determine the causal factors, but results presented suggest that these older adults would benefit from interventions to reduce the risk factors for MetS, in particular higher consumption of micronutrient-rich foods.

Keywords
Elderly
Metabolic syndrome
Ecuador
Micronutrient deficiency
C-reactive protein

The number of older persons is projected to more than double worldwide over the next half century[1]. Most of this elderly population will be living in developing countries, which are the least prepared to deal with the challenges of an ageing society[2]. According to the estimates of the United Nations, the population of Latin American and Caribbean adults aged >60 years will almost double, from about 59 million (10·0 % of the total population) in 2010 to 101 million (15·1 % of the total population) in 2025[3]. Currently, those aged >60 years in Ecuador represent 9·5 % of the population (1 303 000 inhabitants), and this is predicted to rise to 14·1 % (2 262 000 inhabitants) by 2025[3]. A recent report from the Pan American Health Organization and Merck Institute of Aging and Health calls for increased surveillance to identify the extent and causes of morbidity and mortality in older adults[4].

Ecuador, like much of Latin America, has not experienced improvements in living standards to the same degree as most developed nations. Older adults in Latin

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American countries are likely to have comparatively more diseases, greater disability and fewer resources for their health-care needs. Although developed nations have gradually increased their national health resources for older adults, few of the limited health resources of the less developed countries have been devoted to their ageing populations. Since older adults face high burdens of disease and disability, this threatens to increase the strains on the limited health-care resources of their countries.

Metabolic syndrome (MetS) is characterized by disturbed glucose and insulin metabolism, central adiposity, dyslipidaemia and high blood pressure (BP), and is associated with type 2 diabetes, CVD and mortality[5]. Although the aetiology of MetS has not been fully elucidated, available evidence suggests that it is the result of a complex interaction between genetic, metabolic and environmental factors[6]. Nutritional factors are the most prominent environmental influences, including obesity, dietary glycaemic index (GI)[7], fruit and vegetable intake[8], total and type of fat intake[9,10], antioxidant nutrients[11], B vitamins and dairy products[12]. Intake of high-GI carbohydrates was positively associated with insulin resistance and prevalence of the MetS in participants of the Framingham Offspring Study[7]. Higher intake of fruit and vegetables was shown to be inversely associated with plasma C-reactive protein (CRP) concentrations, as well as likelihood of having the MetS in a cross-sectional study of 486 Iranian women[8]. Data from the Third National Health and Nutrition Examination Survey (NHANES III) showed that participants with MetS had significantly lower circulating concentrations of vitamin C, carotenoids and vitamin E[11]. High total fat intake, mainly that of saturated fat, has been linked to lower insulin sensitivity, whereas increased proportions of MUFA improved insulin sensitivity in adults[9]. Higher relative intake of vegetable oils and lower intake of foods containing saturated fat at 50 years of age were protective against developing sustained hypertension 20 years later[10]. In a recent study, ferritin and transferrin concentrations were shown to be associated with MetS abnormalities[13].

Our previous preliminary work in elderly Ecuadorians living in poor peri-urban communities showed a high prevalence of elevated waist circumference and low HDL cholesterol concentration, both components of MetS[14]. Energy intake was mainly dependent on high carbohydrate consumption (76·7% of total energy). Furthermore, a high prevalence of several micronutrient deficiencies was found[14]. CVD is now the primary cause of morbidity and mortality in Ecuadorian elders[15]. These findings suggest that the increasing elderly population of Ecuador is at risk for MetS, although no specific information is available for them. Given that the low status of micronutrients such as antioxidants and B vitamins has been suggested to be associated with increased risk for MetS, the present study was conducted using a much larger sample size than that in our preliminary study to determine the prevalence of MetS in elderly Ecuadorians and its association with blood micronutrient concentrations.

In our previous study, 25% of sampled older adults showed blood homocysteine (Hcy) concentration above the upper limit[14]. Markers of low-grade inflammation, such as CRP[16] and Hcy[17], have been shown to be associated with CVD. Therefore, we also aimed to determine the association of these inflammation markers with micronutrient concentrations and with MetS. The present study presents the first report on MetS prevalence in elders living in the poor peri-urban communities of Quito, Ecuador and its relationship to blood micronutrient concentrations and inflammation markers.

Experimental methods

Study site and population

This cross-sectional study was conducted from September 2003 to December 2004 in three adjacent poor peri-urban neighbourhoods in north-western Quito (2800 m above the sea level). The study area had an estimated population of 13 000 and, based on electoral results, 5% were above the age of 65 years. The neighbourhoods are located on a hillside and are structurally similar, with one main paved road and electricity present in all homes. According to the baseline information collected during the screening phase, just over half of the households had a municipal source of potable water (52%) and sewerage (62%). The inhabitants were primarily poor immigrants from small cities and rural areas of Ecuador. Their mean monthly income was US$54, which is <50% of the basic income in Ecuador, and >40% of the elderly individuals were illiterate. The living conditions of this population are similar to those of the poor urban slums of other Andean countries such as Peru and Bolivia.

Screening and enrolment

To identify the eligible participants, we carried out a census in the three neighbourhoods. Eligibility criteria included age ≥ 65 years, mental competence and willingness to provide written informed consent. Age was verified via national identification cards. Mental competence was determined with a variation of the Mini Mental State Examination (MMSE) that has been used in several countries previously, including Spain and Guatemala[18]. We identified and provided detailed information about the study to 413 potential participants, who were asked for consent to participate. If the individual was illiterate, the form was read to them in the presence of a literate family member. Of the 413 elderly invited, 352 (85%) agreed to participate via witnessed informed consent. The Ethics Committees of the Corporación Ecuatoriana de Biotecnología and the Tufts-New England Medical Center Institutional Review Board approved the study protocol and informed consent.

Study procedures

The participants were asked to visit one of the three field stations centrally located in each neighbourhood (a church, a school or a communitarian house). Two physicians and two nurses collected anthropometric data and blood samples. Before the study, all study personnel received training and conducted data collection on the same test participants to check for inter-observer differences. Two nutritionists collected dietary data through household visits. Previously, they also administered the dietary survey jointly to other older adults to close the inter-observer differences.

Anthropometry

Anthropometric measurements were obtained using methods described by Gross[18] and included weight, height, knee height and waist circumference. Weight was recorded to the nearest 0·1 kg using a Detecto scale (Detecto®, Webb City, MO, USA). The participants were asked to wear the least amount of clothing possible. Height was measured to the nearest 0·1 cm, using a steel fibreglass measuring tape affixed to a wooden rod, with a sturdy straight edge used as a headpiece. We also measured knee height to the nearest 0·1 cm, using a knee-height anthropometer[19]. This measurement can be used to estimate standing height for older participants who are unable to stand erect, using published equations[19]. Waist circumference was measured between the border of the right anterior superior iliac crest and the umbilicus, and was recorded to the nearest 0·1 cm using a fibreglass measuring tape. BMI was calculated as weight in kilograms divided by the square of height in metres (kg/m^2)[20]. The following BMI classifications were used: underweight (BMI $<20\,kg/m^2$), normal weight (BMI 20–24·9 kg/m^2), overweight (BMI 25–29·9 kg/m^2) and obese (BMI $\geq 30\,kg/m^2$). Elevated waist circumference was classified using the International Diabetes Federation (IDF) values: men >90 cm, women >80 cm[21].

Blood pressure

Systolic and diastolic BP were measured in mm Hg by detection of Korotkoff sounds, using a conventional certified sphygmomanometer (CE 0483; Riester, Jungingen, Germany). Right arm measurements were obtained while seated, after resting for at least 15 min. Participants with values >130 mm Hg for systolic or >85 mm Hg for diastolic BP were classified as having high BP[22].

Laboratory procedures

Blood samples were obtained at the field stations. A 10 ml venous blood sample was drawn after an overnight fast, into an EDTA-treated tube and a tube without anticoagulant. Samples were immediately transported to the laboratory and centrifuged. Plasma samples for vitamin C were promptly deproteinized using perchloric acid and EDTA. Serum or plasma was collected in plastic tubes, frozen at −20°C, and transported to Boston for micronutrient analysis according to standard procedures at the Nutritional Evaluation Laboratory of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University as described previously[23].

Total plasma Hcy was determined by a method derived from Araki and Sako[24]. Controls included pooled plasma samples spiked with different amounts of cysteine and Hcy. The CV for this assay in our laboratory is 7·8%. A cut-off point of >15 nmol/ml[25] was considered as high Hcy concentration.

CRP was measured in serum, using an immunoturbidimetric reaction in a Cobas Fara II Centrifugal Analyzer with DiaSorin CRP SPQ Test System Antibody Reagent Set II (Item no. 86083, Document AM-0039 (rev 8.27.90); Stillwater, MN, USA). A cut-off point of >3 mg/l was considered as high CRP concentration[26,27].

Chemical analyses for fasting glucose, TAG, LDL and HDL cholesterol were assessed in Quito, using a conventional autoanalyser (HITACHI 911, Roche, Germany). Daily quality control of the precision of the equipment was determined using the Westgard multi-rules[28].

Classification of metabolic syndrome

We used the MetS definition of the IDF and also report the prevalence of MetS using the Adult Treatment Panel III (ATP III) definition[29]. The IDF definition[21] requires participants to have central obesity defined by ethnic and sex-specific waist circumference cut-points (men >90 cm, women >80 cm), plus two of the four other components (elevated TAG (>1·7 mmol/l or >150 mg/dl), elevated BP (systolic BP ≥130 mm Hg or diastolic BP ≥85 mm Hg), elevated fasting blood glucose (≥5·6 mmol/l or ≥100 mg/dl) and low HDL cholesterol (<1·03 mmol/l or <40 mg/dl for men or <1·30 mmol/l or <50 mg/dl for women)[21]. The ATP III definition requires the presence of three or more of the following criteria: (i) elevated waist circumference (>102 cm for men and >88 cm for women); (ii) elevated TAG (>1·7 mmol/l or ≥150 mg/dl); (iii) low HDL cholesterol (<1·03 mmol/l or <40 mg/dl for men and <1·30 mmol/l or <50 mg/dl for women); (iv) elevated BP (systolic BP ≥130 mm Hg or diastolic BP ≥85 mm Hg); and (v) elevated fasting glucose (≥6·1 mmol/l or ≥110 mg/dl).

Definitions of micronutrient deficiencies

The cut-off points for plasma vitamin inadequacies were defined as follows: vitamin A ≤30 μg/dl[30], pyridoxal phosphate (PLP) ≤ 30 nmol/l[14,31,32], vitamin B_{12} ≤ 250 pg/ml[33], folate ≤5 ng/ml[14,34], vitamin C ≤0·2 mg/dl[30], vitamin E ≤500 μg/dl[35] and vitamin D ≤25 ng/ml for mild deficiency and ≤10 ng/ml for severe deficiency[30]. For mineral plasma inadequacies, the cut-off points were: zinc ≤70 μg/dl[30], copper ≤85 μg/dl[30], iron ≤65 μg/dl for men and ≤50 μg/dl for women[30] and calcium ≤8·6 mg/dl[30].

Dietary intake analysis

Individual dietary intake was estimated with a modified 24 h recall or weighing method[36], as described previously[14]. Dietary recall questionnaires were applied two times with each participant, on different working days within a week. Briefly, the interview was carried out in each household, and each participant was given an explanation on the importance of answering as truthfully and accurately as possible. In order to help the participant recall the previous day, we asked him or her about his or her activities, such as the time of awakening, daytime activities and when he or she went to bed. This approach helped the participants to remember the foods ingested. During the interview, the amounts of food consumed were verified by asking the participant the size of the household measures used to prepare the consumed food. More details for this dietary method have been published[14]. The most experienced observer performed quality control with a thorough review of all recalls to ensure consistency of the data. Two independent data entry staff entered the data into a pre-specified Excel spreadsheet. These data were sent to Tufts University, where they were linked to the USDA (US Department of Agriculture) nutrient database, with the addition of food codes for specific Latin American Foods[37]. Foods not included in this file were coded according to the Ecuadorian Table of Foods[38]. The 2 d of dietary measures were averaged to obtain the most stable measure for each individual. The intra- to inter-person variance for the nutrient intakes was also calculated and used in evaluating correlations between nutrient intakes and blood concentrations.

Statistical methods

Data entry and management were carried out using Epi-Info software, version 6·04d (Centres for Disease Control and Prevention, Atlanta, GA, USA). Statistical analyses were performed using the Statistical Package for Social Sciences statistical software package version 11·5 (Lead Technologies Inc., SPSS Inc., Chicago, IL, USA). The prevalence of MetS and of its components was calculated overall and by sex. We also calculated the frequency of MetS components in participants with and without MetS syndrome. Differences in means and percentages by sex, and between participants with or without MetS, were evaluated by the Student's t and χ^2 tests, respectively.

Multiple logistic regression models, also controlling for age and sex, were fitted to determine whether selected blood micronutrient status, high Hcy or high CRP (as binary variables) were associated with the presence of MetS.

Regression analysis was used to assess associations between blood measures and the mean of the two dietary recalls. Day-to-day within-person variation in the reference method (mean of the two 24 h dietary recalls) can attenuate the correlations between nutrient intakes derived from dietary recalls and serum measures. The intra- to inter-person variance for the nutrient intakes, as estimated by the two 24 h dietary recalls, was also calculated and reviewed. The formula used to calculate the de-attenuated regression coefficient is $b_t = b_0(1 + \text{intra}_x/\text{inter}_x/n_x)$, where b_O is the observed coefficient (slope) of the linear regression of the serum measure on the mean of the two 24 h dietary recalls, adjusted for energy intake, BMI, smoking, age and sex; intra_x is the intra-person variation for the intake variable; inter_x is the inter-person variation for the intake variable; and n_x is the number of days of dietary recall, which was two in the present study[39].

Results

Demographic and anthropometric measurements for this population are shown in Table 1. A total of 352 participants were enrolled in the study, consisting of 225 women (64 %) and 127 (36 %) men. The mean age of the study participants was 74·4 (SD 6·4) years. Male participants were older and most had attended some elementary school. The majority of women were illiterate. Most participants were of rural origin (Table 1). More than 11 % admitted to current tobacco use, and 21 % had previously smoked for more than 1 year. Only 7·7 % acknowledged current alcohol consumption; 50 % used to drink occasionally, 10 % most days and only 4·6 % daily. A considerable percentage of the participants were overweight or obese. A higher percentage of women were overweight than men (Table 1).

On the basis of the IDF definition, the majority of MetS components were more frequent in women than in men; women had elevated waist circumference ($P < 0{\cdot}05$), elevated TAG ($P < 0{\cdot}001$) and lower HDL cholesterol than men ($P < 0{\cdot}0001$; Table 2). Hypertension and elevated blood glucose concentrations did not differ by sex. Similar results were found using the ATP III criteria (data not shown).

On the basis of IDF criteria, 40 % of participants had MetS. Among these participants, 19 % were men and 81 % were women ($\chi^2 = 32{\cdot}6$, $P < 0{\cdot}0001$), whereas 33 % (18·3 % men and 81·7 % women) had MetS on the basis of the ATP III definition. In addition, 52 % (110/210) of the participants without MetS (defined by IDF) exhibited two or more of its components. In participants without MetS, a higher percentage of women than men exhibited two or more components of MetS (68/110 *v.* 42/110; $\chi^2 = 9{\cdot}094$, $P = 0{\cdot}002$).

On the basis of serum or plasma concentrations, micronutrient inadequacies were common for men and women, respectively (values in parenthesis are the median and 10th and 90th percentiles followed by the percentage of participants who had low values): vitamin C (0·17 (0·02–0·44), 0·26 (0·09–0·56) mg/dl; 60 % and 33 %), vitamin B_6 (39·9 (23–64), 48·1 (27·3–78·3) nmol/l; 27 % and

Table 1 Demographic characteristics and anthropometric measurements of elderly Ecuadorians

	Men (*n* 127)		Women (*n* 225)		
Variables	Mean	SD	Mean	SD	*P*
Age (years)†	75·8	6·5	73·7	6·1	<0·001
Education level					
No school (%)	29·4		52·5		<0·0001
Birth place (% rural)	69·0		73·9		NS
Anthropometrics					
Weight (kg)	59·9	9·5	54·0	9·8	<0·0001
Height (cm)	156·0	6·7	144·0	6·0	<0·0001
Height by knee height (cm)‡	158·0	6·4	144·0	5·3	<0·0001
Knee height (cm)	48·0	3·5	44·5	2·8	<0·0001
Waist circumference (cm)	87·5	9·4	87·3	11·6	NS
BMI (kg/m^2)	24·8	3·2	26·1	4·0	<0·001
Underweight (<20 kg/m^2; %)	2·7		4·5		NS
Normal (20·0–24·9 kg/m^2; %)	57·3		39·8		<0·001
Overweight (25·0–29·9 kg/m^2; %)	29·5		41·2		<0·05
Obese (≥30·0 kg/m^2; %)	3·3		14·0		<0·01

†Range is 65–94 years for men; 65–97 years for women.
‡Calculated as described in Blaum *et al.*[19].

Table 2 Distribution of MetS components in elderly Ecuadorians by IDF definition

	Total (*n* 301–352)†	Men (*n* 111–127)	Women (*n* 190–225)	
MetS components	%	%	%	*P*
High waist circumference (men >90 cm, women >80 cm)	61	37	75	<0·0001
Blood pressure (systolic ≥130 mmHg or diastolic ≥85 mmHg)	51	55	49	NS
Fasting glucose (≥100 mg/dl)	11	9	13	NS
TAG (≥150 mg/dl)	40	28	46	<0·001
HDL cholesterol (men <40 mg/dl, women <50 mg/dl)	73	56	83	<0·0001

IDF, International Diabetic Federation; MetS, metabolic syndrome.
†Total numbers are 352, 301, 351, 351 and 351 for waist circumference, blood pressure, fasting glucose, TAG and HDL cholesterol, respectively.

16%), vitamin B_{12} (360 (216–676), 390 (208–729) pg/ml; 21% and 20%), folate (5·5 (3·4–8·3), 6·2 (3·7–10·5) ng/ml; 37% and 27%) and zinc (75 (48–106), 71 (47–108) ug/dl; 41 and 45%). Deficiencies of vitamins A (54 (39–72), 50 (36–66) μg/dl) and D (severe <10 ng/ml; 22 (14–29), 19 (12–26) ng/ml), iron (123 (79–188), 107 (62–161) μg/dl) and calcium (9·2 (8·6–10·2), 9·3 (8·6–10) mg/dl) were present in <15% of participants. Mild vitamin D deficiency (<25 ng/ml) was present in 65% and 87% of men and women, respectively.

After adjusting for age and sex, plasma vitamin E:TAG ratios and vitamin C concentrations were inversely associated with MetS (OR = 0·78, 95% CI 0·71, 0·86; OR = 0·16, 95% CI 0·03, 0·81, respectively; Table 3). High plasma CRP (>3 mg/l) was present in 48·9% of the participants and was positively associated with MetS (OR = 1·79, 95% CI 1·04, 3·06; Table 3). High plasma Hcy (>15 nmol/ml), or low zinc, copper or B vitamins, was not associated with MetS (Table 3).

After adjusting for age and sex, low plasma vitamin E:TAG ratios were associated with high plasma glucose (OR = 0·74, 95% CI 0·63, 0·86), low HDL cholesterol (OR = 0·84, 95% CI 0·78, 0·92) and high waist circumference (OR = 0·91, 95% CI 0·85, 0·98). Low plasma vitamin C was also associated with hypertension (OR = 0·15, 95% CI 0·03, 0·67), high waist circumference (OR = 0·15, 95% CI 0·03, 0·68) and low HDL cholesterol (OR = 1·94, 95% CI 1·07, 3·50).

Table 3 Association of age, sex, blood micronutrients, CRP and homocysteine with MetS using the IDF definition

Variables	OR†	95% CI
Age (years)	0·99	0·95, 1·06
Sex (male)	0·18	0·10, 0·40
Vitamin C (mg/dl)	0·16	0·03, 0·81
PLP (nmol/l)	1·01	1·00, 1·02
Vitamin B_{12} (pg/ml)	1·00	0·99, 1·01
Folate (ng/ml)	1·04	0·94, 1·16
Cu (μg/dl)	0·99	0·98, 1·00
Zn (μg/dl)	1·00	0·99, 1·02
High plasma CRP (>3 mg/l)	1·79	1·04, 3·06
High plasma homocysteine (>15 nmol/ml)	1·73	0·94, 3·18
Vitamin E:TAG ratio	0·78	0·71, 0·86

CRP, C-reactive protein; PLP, pyridoxal phosphate; MetS, metabolic syndrome; IDF, International Diabetic Federation.
†OR is based on a multiple logistic regression model, adjusting for age and sex.

Analysis of the dietary intake from the participants is shown in Table 4. In general, the diets were of poor quality – high in carbohydrate and sodium (>50% had intake higher than recommended) and low in protein (about 23% had intake below that recommended), fat

Table 4 Dietary macronutrient and micronutrient intake of elderly Ecuadorians (average of two 24 h recalls)

		Women				Men			
		Intake (*n* 218)				Intake (*n* 124)			
Nutrients	AMDR†, EAR‡ or AI§	Mean	Range	Below ref. (%)	Above ref. or UL (%)	Mean	Range	Below ref. (%)	Above ref. or UL (%)
Energy (kcal)‖		1187	327–2805			1159	166–2428		
Fat (% energy)	20–35†	21·9	5·3–53·6	41·2	5·1	21·9	6·0–40·6	44·4	0·8
Carbohydrate (% energy)	45–65†	65·8	10·3–87·6	2·3	55·5	65·9	36·0–88·8	0·8	55·7
Protein (% energy)	10–35†	13·2	5·3–33·9	20·6	0·0	13·0	5·7–25·9	27·4	0·0
Total vitamin A (μg RAE)	500–6252	396	0·2–3588	74·3	0·0	429	1·7–2696	78·2	0·0
Vitamin D (μg calciferol)	15§	1·35	0·00–7·57	100·0	0·0	1·53	0·00–11·0	100·0	0·0
α-Tocopherol (mg)	12‡	3·10	0·35–8·47	100·0	0·0	3·24	0·21–8·78	100·0	0·0
Vitamin K (μg phylloquinone)	90–120§	29·4	1·0–429	95·4		26·1	1·2–283	97·6	
Vitamin C (mg)	60–75	50·3	0·2–390	74·8	0·0	49·88	1·0–394	84·7	0·0
Riboflavin (mg)	0·9–1·1‡	0·85	0·06–2·61	64·2		0·89	0·13–2·49	70·2	
Folate (μg)	320‡	208	0–646	82·1	0·0	227	18·7–743	76·6	0·0
Vitamin B_6 (mg)	1·3–1·4‡	1·24	0·21–4·59	58·3	0·0	1·25	0·10–2·80	62·9	0·0
Vitamin B_{12} (μg)	2·0‡	2·60	0·00–19·0	56·0		3·02	0·0–18·2	54·8	
Ca (mg)	1200§	247·00	21·3–908	100·0	0·0	248	30–928	100·0	0·0
P (mg)	580‡	575·00	77–1470	58·3	0·0	605	121–1364	52·4	0·0
Mg (mg)	265–350‡	160·00	13–432	93·6	1·4	167	23–415	99·2	0·8
Fe (mg)	5·0–6·0‡	6·29	0·02–17·6	39·5	0·0	6·76	1·37–22·0	50·0	0·0
Zn (mg)	6·8–9·4‡	5·30	0·63–16·8	73·9	0·0	5·59	0·75–13·1	92·7	0·0
Cu (μg)	700‡	862	50–4005	38·1	0·0	886	110–4150	34·7	0·0
Se (μg)	45‡	64·20	7·8–210	28·0	0·0	68·6	12·0–218	27·4	0·0
K (mg)	4700§	1651	134–4036	100·0	0·0	1659	184–3820	100·0	0·0
Na (mg)	1200§ UL 2300	3094	69–20 342	15·6	53·1	3287	200–13 255	13·7	62·1

AMDR, Acceptable Macronutrient Distribution Range; EAR, Estimated Average Requirement; AI, Adequate Intake; ref., reference; UL, Upper Limit; RAE, retinol activity equivalents.
†Adults[50].
‡EAR for women–men aged >70 years[51,52].
§AI for women–men aged >70 years; EAR not available[52–56].
‖1 kcal = 4·184 kJ.

Table 5 Correlation between dietary and blood nutrient concentrations

Dietary variable	Serum variable	Observed regression coefficient	Corrected regression coefficient†
Vitamin C (mg)	Vitamin C (mg/dl)	0·0005*	0·001
Vitamin B_6 (mg)	PLP (nmol/l)	5·6	11·10
Vitamin B_{12} (μg)	Vitamin B_{12} (pg/ml)	9·0	21·70
Zn (mg)	Zn (μg/dl)	0·19	0·40
FV servings	Folate (ng/ml)	0·18*	0·39
FV servings	Vitamin B_{12} (pg/ml)	3·3	7·00
FV servings	PLP (nmol/l)	1·6*	3·40
FV servings	Vitamin C (μg/dl)	0·02*	0·04
Animal protein (g)	Zn (μg/dl)	0·01	0·02
Animal protein (g)	Vitamin B_{12} (pg/ml)	2·4*	5·70

PLP, pyridoxal phosphate; FV, fruit and vegetables.
*$P < 0·05$ adjusted for age, sex, smoking, BMI and energy intake.
†Also adjusted for intra- or inter-individual variation in dietary intake.

(about 42% had intake below that recommended) and several micronutrients (see Table 4), consistent with the low blood micronutrient status exhibited.

The main sources of energy were white rice (16%), potatoes (13%), sugar (8%) and bread (7·5%). Milk, eggs, cheese, beef, chicken giblet and chicken breast contributed 4·5%, 1%, 0·6%, 3·7% and 2·6%, respectively. Intakes of grains other than rice and wheat (barley, oats, 2·3% of energy), legumes (mainly black beans, 0·7%), vegetables other than potato (corn, onions, cassava, plantains, total 4·5%) and of fruit (mainly banana, 0·6%) were low. Protein consumption was low and was derived mainly from beef (11%), rice (9%), chicken giblet (9%), milk (7%) and potatoes (7%). Fish provided 3% of the protein. The main source of fat was from palm oil (16%); the main sources of carbohydrate were rice (20%), potatoes (16%) and white sugar (13%).

Glycaemic load (GL) was mainly from white rice (25%), potatoes (21%), white granulated sugar (12·5%), bread (9·7%) and pasta (2·7%). The diet of 20% of participants was of high GI, using glucose as the reference[40]. Based on the percentage of total energy intake, according to the Acceptable Macronutrient Distribution Range[41], 93% of the participants consumed low linoleic and α-linolenic acid. However, there was no correlation between high GI, GL or low unsaturated fat with MetS (data not shown) in this population. Sodium intake was mainly from regular salt (76%), boiled potato (4·4%) and bread (4·3%). The average intake of dietary salt was 3·2 g. As indicated above, the consumption of fruit and vegetables and animal products was low, and most likely contributing to low serum micronutrient status, as indicated by positive correlations between the intake of fruit and vegetables and plasma vitamin C, vitamin B_6 and folate concentrations ($P < 0·05$; Table 5). A significant correlation between plasma vitamin B_{12} and animal products in the diet was also observed ($P < 0·05$; Table 5). We observed positive correlations between dietary intakes of vitamin C, vitamin B_6, PLP and zinc with respective plasma concentrations, but these reached statistical significance for vitamin C only ($P < 0·05$; Table 5). The lack of statistically significant association between the dietary and blood levels of these nutrients could be due to high day-to-day variation in dietary intake, as indicated by substantially higher regression coefficients after adjustment for intra- or inter-individual variation (Table 5). A larger number of dietary recalls might be needed to obtain accurate dietary intake for these nutrients.

Discussion

We report that MetS, a constellation of conditions associated with substantial morbidity and mortality worldwide, is prevalent (40% and 33%, respectively, using the IDF and ATP III definitions) among the poor elderly Ecuadorians living in Quito, Ecuador. This prevalence is comparable to that reported for elderly adults living in the USA (40%)[5]. Similar to observations in the USA, prevalence is higher among women than men. Furthermore, a significant proportion of elderly participants without MetS had two of the MetS components. Despite their low socio-economic status, 33% of elderly men and 55% of elderly women were overweight. A significant proportion of these adults exhibited low concentrations of blood micronutrients (15%, 82%, 15%, 20%, 29% and 37% had vitamins D, C, B_6, B_{12}, folate and zinc inadequacy, respectively, and 88% exhibited at least one vitamin or mineral deficiency), indicating that they suffer from the increasingly common double burden of malnutrition and chronic disease associated with food insecurity and nutrition transition in less developed countries. Although historically malnutrition was defined as undernutrition, in recent years a situation has been described that links poverty, food insecurity and malnutrition to obesity and associated diseases[42]. This paradoxical condition has been attributed to the fact that the diet of most of the world's poor consists of 'empty energy', i.e. a diet of poor quality. This diet is low in essential nutrients, resulting in the coexistence of both over- and undernutrition in those

living in poverty. The absence of a diversified nutrient-dense diet can lead to energy overnutrition and obesity as well as micronutrient deficiencies. Related to this, households characterized as food-insecure have been shown to have the highest BMI[43]. In fact, the analysis of dietary intake of the participants in the present study supports the contribution of poor-quality diet in this population to the prevalence of under- and overnutrition in the Ecuadorian elderly. Our results show, for the first time, the existence of this paradoxical condition in elderly Ecuadorians. These findings may be relevant to 30% of the elderly in Ecuador who live in the Andean region, and to the elderly of other developing Latin American countries living in similar conditions.

There was a 12% difference in MetS prevalence using the IDF or ATP III definitions. This appears to be due to the lower cut-off points for waist circumference used in the IDF definition. Among MetS components, low HDL cholesterol, high waist circumference and hypertension were present in >50% of the participants, while high fasting glucose concentrations were only observed in 11%. We present most of our results using the worldwide IDF MetS consensus statement definition[23]. This definition uses South Asian recommendations for waist circumference, which may be more appropriate for this population than those used in APT III. No cut-off points are presently available for Central and South Americans. More research is needed to determine the most appropriate cut-off points for this important indicator in different populations.

Although the aetiological pathways that lead to MetS have not been fully determined[6], it is clear that nutritional status and intake are important contributors[12]. Limited evidence suggests that circulating concentrations of antioxidants may be lower among people with MetS than those without this condition[44]. Furthermore, low vitamin B concentrations and high Hcy[45,46] and CRP[8,47] have been associated with MetS. We found high prevalences of low blood concentrations of vitamins C, B_6, B_{12}, folate and zinc and elevated CRP and Hcy concentrations in this population. However, only plasma vitamin C, vitamin E and CRP were significantly associated with MetS in this relatively small sample. As MetS appears to be linked to oxidative stress[48], it is plausible that deficiencies of micronutrients with antioxidant properties, including vitamins C and E, could be related to MetS, although, given the cross-sectional design of the study, it is not possible to draw conclusions on the potential pathogenic mechanisms of those deficiencies. Vitamin C deficiency was the most commonly identified deficiency in our participants. Low plasma vitamin C may reflect low fruit and vegetable intake, which has been shown to increase the risk of MetS[8,49]. In support of this, fruit and vegetable servings correlated negatively with high systolic BP, although this did not reach statistical significance.

Dietary analysis showed that, as a group, these elderly individuals consumed diets of poor quality, with the majority consuming higher than recommended levels of carbohydrates, mostly of high or intermediate GI, and lower than recommended levels of fat, especially poly-unsaturated fat, and micronutrients, consistent with the observed low BP status seen for several micronutrients. More than 50% of these older adults had hypertension, which may be related to the high daily sodium intake and/or prevalence of vitamin C deficiency. Although we observed a significant association between serum vitamin C concentration and hypertension, the association between dietary sodium intake and BP did not reach statistical significance. This may reflect the difficulty in obtaining accurate dietary sodium intake. We found a strong inverse correlation between vitamin E concentrations and risk of MetS. The underlying mechanisms for the apparent protective effect of vitamin E need to be determined and may differ from those of vitamin C, as they were associated with different sets of MetS components (BP for vitamin C; and HDL cholesterol, waist circumference and glucose for vitamin E).

Inflammation, and in particular high CRP concentration, has been implicated in MetS[47]. More than 48% of the elderly in the present study had high CRP concentrations. Inflammatory mechanisms may modulate the nutritional, metabolic and other factors contributing to MetS.

There are limitations to the present study that should be noted. First, this was a cross-sectional study, and as such cannot determine causality. Whether the micronutrient status, particularly that of vitamins C and E, is causally related to the high prevalence of MetS needs to be further determined. Second, the study population included only the poor elderly Ecuadorians living in peri-urban, low-income communities. Although this population may be representative of the elderly in other Andean countries such as Bolivia and Peru, the results may not be generalizable to those in coastal areas or those who live in better socio-economic conditions.

In conclusion, MetS is highly prevalent in these poor peri-urban elderly Ecuadorians. Our findings suggest that poor nutritional status is an important contributor to the high prevalence of MetS in this population. As MetS is highly correlated with morbidity and mortality from chronic diseases such as diabetes and CVD, and relatively few resources are available for the health care of elders in Latin American countries, measures to prevent MetS may reduce the burden of these diseases and improve the quality of life in this growing segment of the population. Given the high prevalence of micronutrient deficiencies in the presence of obesity, nutrition interventions could have a significant impact on health outcomes in this population. More research is needed to determine causal factors, but these data suggest that elderly Ecuadorians would benefit from dietary interventions to normalize blood lipids, reduce hypertension and weight gain and

improve micronutrient status. Given that the diet of these elderly appeared to be high in salt and low in fruit and vegetables, good-quality protein and essential fatty acids, this population is likely to benefit from reduced salt intake, higher consumption of fruit and vegetables, foods with better-quality protein and fatty acid profiles such as fish, chicken, low-fat dairy and legumes.

Acknowledgements

The present study was supported by grants from the US National Institutes of Health (Fogarty International Center R03 TW005779; the National Institute of Aging, 2R01 AG009140; and the National Institute of Child Health and Development R01 HD38327); and the US Department of Agriculture cooperative agreement no. 58-1950-7-707. Any opinions, findings, conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the US Department of Agriculture. No conflict of interest exists between any of the authors or contributors to the study. F.S. contributed to the design of the study, organization of the study team, preparation of the informed consent, supervision of the enrolment, general supervision of the study and preparation of the entire manuscript; B.E. contributed to the design of the study, standardization of the study team in all measurements, database preparation, analysis of the data and preparation of the entire manuscript; K.L.T. contributed to the analysis, design and interpretation, guidance for field data collection and editing of the final manuscript; D.H.H. contributed to the development of the study design, initial training of data collectors and pilot testing of the data collection instruments, data analysis and assisted with the writing and editing of the manuscript; X.N. contributed to the clinical examination, D.T.H. to application and reading and to the preparation of the manuscript; M.S. contributed to the enrolment and anthropometric measurements, CRONOS application and to the preparation of the manuscript; J.K.G. contributed to the design of the study and review of the manuscript; S.E.N. contributed to the review of the literature and to the Introduction and Methods section of the manuscript, besides assisting in the editing of the Results and Conclusion sections; G.E.D. contributed to the analysis and interpretation of the data, and critically reviewed the manuscript; J.S. contributed to the analysis of the samples and review of the manuscript; S.N.M. contributed to the design of the study, obtained funds to conduct the study, developed the questionnaires and data collection form and conducted the overall supervision of the study with F.S., besides helping with data analysis, data interpretation and manuscript preparation. The Nutritional Evaluation Laboratory of HNRCA conducted the biochemical analysis and outside colleagues Dr Hooman Poor and Dr Jatin Roper helped with field data collection. Alicia Rodriguez and Monica Sanaicela applied the dietary recall.

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Chapter 9

Low concentrations of zinc in gastric mucosa are associated with increased severity of Helicobacter pylori-induced inflammation

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Helicobacter 2007; 12; 43-48

Helicobacter ISSN 1523-5378

Low Concentrations of Zinc in Gastric Mucosa are Associated with Increased Severity of *Helicobacter pylori*-Induced Inflammation

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Keywords
zinc, *Helicobacter pylori*, gastritis.

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This study was supported by a grant from the Panamerican Health Organization (PAHO). HDP/HDR/RGP//F-ECU/3033.

Abstract

Background: Chronic *Helicobacter pylori* infection is the most common cause of gastric cancer. *H. pylori* induces oxidative stress while zinc deficiency results in increased sensitivity to it. In Ecuador, the prevalence of gastric cancer and zinc deficiency are high. We hypothesized that zinc deficiency in Ecuadorian people would cause increased *H. pylori*-induced inflammation in the gastric mucosa associated with lower tissue zinc concentrations.

Methods: Three hundred and fifty-two patients with dyspepsia underwent endoscopy to obtain gastric mucosa biopsies. Diagnosis of *H. pylori* infection and its severity, histopathology, mucosal zinc concentration, and inflammation intensity were determined.

Results: *H. pylori*-infected patients with non-atrophic chronic gastritis had lower concentrations of zinc in gastric mucosa than uninfected patients with the same type of gastritis (251.3 ± 225.3 vs. 426.2 ± 279.9 ng/mg of protein; $p = .016$). Considering all patients, the more severe the *H. pylori* infection, the higher the percentage of subjects with infiltration by polymorphonuclear (PMN) cells ($p = .0001$). Patients with high PMN infiltration had lower mucosal zinc concentrations than patients with low PMN infiltration (35.2 ± 20.7 vs. 242.9 ± 191.8 ng/mg of protein; $p = .021$).

Conclusions: The degree of inflammation in *H. pylori*-induced gastritis appears to be modulated by gastric tissue zinc concentrations.

More than 50% of the population worldwide is infected by *Helicobacter pylori* [1]. Chronic *H. pylori* infection is associated with gastritis and peptic ulcer disease [2], and is also the single most common cause of gastric cancer [3]. Gastric cancer is the second most common cause of cancer-related mortality worldwide [4]; however, there are important regional differences in gastric cancer prevalence. Western Europe and the United States have a low incidence, whereas Japan, China, and South America have a higher incidence [3,4]. In Ecuador, gastric cancer is a serious public health problem that represents 12.7% of all cancer cases, with a prevalence of 29 cases per 100,000 inhabitants [5].

H. pylori is successful at surviving the effects of many components of the innate and adaptive host immune response [2,6,7]. *H. pylori* possesses many virulence factors that allow it to survive in this hostile environment, such as urease, flagella, *cag* type IV secretion system, and neutrophil-activating protein (NAP) [2]. The *cag* system allows *H. pylori* to transfer into host cell cytoplasm different bacterial proteins that modulate host-cell functions [8]. One of the principal functions of *cag* is the activation of interleukin-8 (IL-8) gene expression. IL-8 is a potent neutrophil (polymorphonuclear – PMN) chemotactic factor [9–11]. Once attracted, these PMN are activated by NAP to perform their inflammatory functions [12]. Activated PMN cells produce large quantities of reactive oxygen species (ROS) that facilitate bacteria killing.

It has been demonstrated that damage to the gastric mucosa during *H. pylori* infection is mainly caused by increased ROS and consequent oxidative stress [13–15]. Catalase, superoxide dismutase (SOD), thioredoxin, and glutathione reductase have been shown to serve important roles in the mucosal defense against *H. pylori* infection [16–19]. *H. pylori*-induced gastric mucosa inflammation is characterized by an increase of manganese SOD and reduction of copper/zinc SOD [17]. It has also been shown

that after *H. pylori* eradication, the gastric mucosa content of copper/zinc SOD returns to normal levels [18]. Furthermore, a recent study demonstrated that the overexpression of the antioxidant enzyme thioredoxin, in a mouse model of *H. pylori* infection, not only reduces oxidative stress but also inhibits PMN chemotaxis [20]. All these findings emphasize the role of oxidative stress in *H. pylori*-induced gastric pathogenesis.

Zinc is an abundant intracellular micronutrient that participates in a wide range of cellular processes [21]. Zinc deficiency results in an increased sensitivity to oxidative stress [22–24]. As zinc participates in several critical cellular functions, especially DNA repair, it has been suggested that zinc deficiency may increase the risk for cancer [23,25,26]. Zinc deficiency is a major public health problem in Ecuador [27,28]. There is evidence that zinc deficiency in Latin American people, particularly from the Andean regions as Ecuador, is due to both lack of nutritional sources of zinc in their diet and increased consumption of cereal bran containing phytates, which are strong chelators of minerals and therefore prevent zinc absorption [29]. Given the risk of zinc deficiency in Andean populations and the role of zinc in the response to oxidative stress, we hypothesized that a poor population living in an Andean region of Ecuador would have increased *H. pylori*-induced inflammatory damage in the gastric mucosa associated with lower mucosal zinc concentrations.

Subjects and Methods

Study Design and Population

The study was performed in Hospital Pablo Arturo Suarez, in the city of Quito, Ecuador. This hospital serves a low-income population that is mainly of Spanish-Indian (mestizo) background. Subject enrolment took place from 2000 to 2003. The study design and protocol were approved by the Bioethical Committees of the Corporación Ecuatoriana de Biotecnología and that of the Pan American Health Organization (PAHO).

Patients of either sex seeking care in the hospital aged 35–69 years old with a diagnosis of dyspepsia and who were willing to provide written informed consent were eligible for participation. Exclusion criteria included previous treatment for *H. pylori* in the last 6 months, regular use of local or systemic antimicrobial agents (e.g. antibiotics and bismuth), and the presence of other severe diseases (cancer, cardiac, renal or respiratory failure).

After enrolment, study participants underwent endoscopy to obtain gastric mucosa biopsies. From these biopsies the diagnosis of *H. pylori* infection and its severity, histopathologic changes, zinc concentration, and intensity of inflammation were determined.

Definitions

Dyspepsia

Dyspepsia was defined as the presence of epigastric pain shortly after eating or continuous epigastric pain with nausea and/or vomiting and/or abdominal discomfort.

Histologic Gastritis

The histologic observations were classified according to the International Workshop that assessed and revised the Sydney System for the reporting of gastritis with slight variations [30]. Chronic gastritis was determined by the presence of mononuclear cells (lymphocytes, plasma cells, and macrophages). Histologic diagnoses were classified as: normal, non-atrophic chronic gastritis, atrophic chronic gastritis, intestinal metaplasia, or dysplasia. If PMN cells were present, the gastritis was defined as chronic active gastritis. PMN infiltration was graded as mild, moderate, and severe according to the Sydney System.

H. pylori Colonization

Colonization by *H. pylori* was determined by the presence of characteristic short bacilli as evidenced by Giemsa staining either in the crypts of the mucosa or attached to the epithelium. Three categories of *H. pylori* infection were determined [30]: 1, mild, if a low number of bacteria was present; 2, moderate, if higher number of dispersed bacteria was present; and 3, severe, if small colonies or aggregated bacteria were present.

Endoscopic Procedures and Laboratory Evaluations

During the gastroscopy nine biopsies were obtained: three from the incisure-antrum (1, 2 and 3), three from the greater curve of the antrum (4, 5, and 6), and three from the corpus (7, 8, and 9). Biopsies 1, 2, 3, 4, 7, and 9 were used to identify *H. pylori* and for histopathology. Biopsies 5, 6, and 8 were used to measure tissue zinc concentrations.

Histopathology

A total of 12 slides per subject were studied. Six slides were stained with Giemsa to identify *H. pylori*. The other six slides were stained with hematoxylin–eosin for histopathology. For quality control, an experienced observer who was blinded to the first reading performed a second reading of the slides. In the case of disagreement between both observations, the diagnostic criteria of the second observer (MD) prevailed.

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Determination of Zinc Concentration in Gastric Tissue

Zinc mucosal concentrations were determined in the United States Department of Agriculture, Grand Forks Human Nutrition Research Center in North Dakota. The biopsies were preserved at −20 °C until analysis. Three mucosal samples for each individual were pooled and solubilized in sodium hydroxide/sodium dodecyl sulfate solution for 24 hours at room temperature. Five hundred microlitres of solubilized cells was added to 2 mL of nitric acid. The mixture was processed in the AIM500 block digester (A.i. Scientific, Saxonburg, PA, USA) as follows: 100 °C for 35 minutes, 120 °C for 120 minutes, 130 °C for 90 minutes, and 145 °C for 140 minutes. This cycle was repeated once. When samples were dry and cool, 1 mL of hydrogen peroxide was added. The samples were again processed in the AIM block digester under the same conditions as above. Before analysis, 2 mL of 5% nitric acid was added and incubated at room temperature for 1 hour. Every sample was analyzed in the PerkinElmer Optima 3300 DV Inductively Coupled Argon Plasma Atomic Emission Spectrometer (Perkin Elmer, Wellesley, MA, USA). Biopsies were coded, and the researcher performing zinc determinations was blinded to the histopathologic diagnosis. The zinc mucosal concentration was expressed in ng/mg protein. Protein concentration was determined on the solubilized samples by the bicinchoninic acid method of Smith et al. [31].

Statistical Analysis

Data entry and management was carried out using EPI-INFO software version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA, USA). Statistical analyses were carried out using the software SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

Descriptive statistics for histologic evidence of *H. pylori* infection, histopathologic diagnoses, presence of PMN in biopsies, and zinc concentration in gastric mucosa, were calculated. The mean zinc concentration in gastric mucosa was compared between *H. pylori*-positive and -negative biopsies, between *H. pylori* infected patients with non-atrophic gastritis and uninfected patients with nonatrophic gastritis, and between the degrees of PMN infiltration in gastric mucosa. In addition, the relationship between severity of *H. pylori* infection and gastric infiltration by PMN was evaluated. Differences of means and percentages were evaluated by Student's *t*-test and chi-squared test, respectively. A significance level of ≤ 0.05 was accepted.

Data of zinc concentration in gastric mucosa were not normally distributed. Non-parametric analysis produced the same results as those of parametric.

Table 1 Histopathologic diagnosis in Ecuadorian *H. pylori*-infected patients with clinical gastritis

Histopathologic diagnosis	n = 328 %
Normal mucosa	0
Non-atrophic chronic gastritis	48.5
Atrophic chronic gastritis	22.9
Metaplasia	24.7
Dysplasia	4.0

Results

Three hundred and fifty-two subjects were enrolled. Although biopsies were obtained from 352 patients for histopathology, *H. pylori* colonization and for determination of mucosal zinc concentration, only 338 determinations of zinc concentration are included in the analysis because the initial attempt to locally process 14 samples failed. Of all patients, 70% were female (245/352) and 93.2% of patients (328/352) had histologic evidence of *H. pylori* infection.

Histologic Diagnosis

Twenty-four out of 352 patients were uninfected by *H. pylori*. From those infected patients, nearly half (48.5%) had non-atrophic chronic gastritis. Atrophic chronic gastritis and metaplasia were seen in nearly one fourth of the patients. In contrast, dysplasia was rarely encountered (Table 1).

Zinc Concentration in Gastric Biopsies

H. pylori-infected patients with non-atrophic chronic gastritis (n = 154) had lower zinc concentrations in gastric mucosa than uninfected patients with similar histologic findings (n = 11) (251.3 ± 225.3 vs. 426.2 ± 279.9 ng/mg of protein; $p = .016$). When all subjects were analyzed (n = 338), those patients with presence of *H. pylori* in their gastric mucosa had relatively lower concentrations of zinc in gastric mucosa in comparison to patients without *H. pylori* infection. This difference was not statistically significant (256.4 ± 214.7 vs. 321.2 ± 269.8 ng/mg of protein; $p = .179$).

Presence of Polymorphonuclear Cells in *H. pylori*-Infected Patients

The correlation between severity of *H. pylori* infection and gastric infiltration by PMN cells was evaluated in all patients. We found that the more severe the *H. pylori* infection, the higher the percentage of subjects with PMN infiltration ($p = .0001$) (Table 2).

Table 2 Comparison between the degree of *H. pylori* infection and presence of polymorphonuclear (PMN) cells in gastric biopsies

Degree of *H. pylori* infection	Presence of PMN cells %[a]
Mild[b]	25% (26/96)
Moderate[c]	59% (82/136)
Severe[d]	74% (72/96)

[a]$X^2 = 46.68$; $p = .0001$.
[b]vs. c = $X^2 = 24.94$; $p = .001$.
[b]vs. d = $X^2 = 44.10$; $p = .0001$.
[c]vs. d = $X^2 = 5.43$; $p = .014$.

Presence of Polymorphonuclear Cells and Gastric Mucosa Zinc Concentration in Patients with *H. pylori* Infection

Lower zinc concentrations were seen in patients with PMN in their gastric mucosa (n = 169) than in patients without PMN infiltration (n = 147) (232.45 ± 192.36 vs. 283.84 ± 235.48 ng/mg of protein; $p = .034$). Furthermore, patients with moderate PMN infiltration (n = 8) had lower zinc concentrations when compared to patients with low PMN infiltration (n = 161) (35.2 ± 20.7 vs. 242.9 ± 191.82 ng/mg of protein; $p = .021$) (Fig. 1).

Discussion

We found that relatively low gastric mucosal zinc concentrations in all *H. pylori*-infected patients were correlated with increased infiltration by PMN cells and, consequently, increased inflammation. Furthermore, *H. pylori* infection was correlated with a significant reduction in the concentration of zinc in gastric mucosa of patients infected with this bacterium and having non-atrophic chronic gastritis.

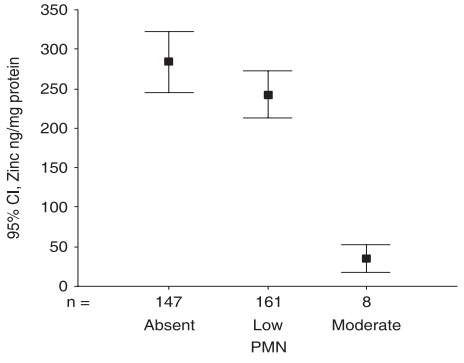

Figure 1 Zinc concentration in gastric mucosa of *H. pylori*-infected patients according to polymorphonuclear (PMN) cell infiltration.

H. pylori-associated inflammation is recognized as an important feature of gastric carcinogenesis [32]. Furthermore, the virulence of infecting *H. pylori* and the stomach environment also play a role in determining the outcome of this infection. One of the mechanisms by which *H. pylori* induces inflammation is by generation of ROS by infiltrating PMN cells [18]. Zinc deficiency induces oxidative stress in mammalian cells with consequent oxidative DNA damage [23,25,26]. These mechanisms suggest an explanation for our findings. *H. pylori* infection together with lower zinc concentration in gastric mucosa would induce increased oxidative stress, which would be associated with increased inflammation. This would explain our finding of statistically significant lower concentrations of zinc in gastric mucosa in patients with non-atrophic chronic gastritis and *H. pylori* infection, compared to those without infection.

In response to *H. pylori* infection, gastric epithelial cells produce the chemokine IL-8 [33], which induces chemotaxis and transepithelial migration of PMN cells. Neutrophils recruited to the site of inflammation generate ROS. Our findings demonstrate a direct correlation between the severity of *H. pylori* infection and the degree of infiltration by PMN. Furthermore, we found that the degree of infiltration by PMN cells is correlated inversely with the zinc concentration in gastric mucosa. This would suggest that patients with low gastric mucosa zinc concentrations have increased inflammation caused by augmented infiltration of PMN cells.

Our hypothesis was that the Ecuadorian population, with high prevalence of zinc deficiency as characterized by plasma zinc concentrations [29,34], may have a higher risk of increased inflammatory damage by *H. pylori* infection. Our study is the first to report zinc concentrations in gastric mucosa. As zinc is essential for the function of numerous intracellular enzymes, zinc concentration in gastric mucosa might be a good indicator of zinc status. However, a potential issue is the absence of plasma zinc determination in our study. Although it is still controversial whether or not plasma zinc concentration is the best marker to evaluate zinc status; plasma zinc concentration is nevertheless widely use as a measure of zinc status. Determination of plasma zinc concentrations in our study would have allowed us to correlate with that of gastric mucosa to have a better idea about zinc status.

An additional limitation to our study is the lack of bacterial culture or urease test to confirm infection by *H. pylori*. However, the Giemsa stain has a sensitivity of 91%, specificity of 100%, and accuracy of 95% [35]. Another limitation was the small number of non-infected subjects.

This is a difficult issue to solve due to the overwhelming amount of *H. pylori*-infected subjects among those with dyspepsia in Ecuador [5].

The observation that *H. pylori* infection in the presence of low zinc concentrations in gastric mucosa results in increased PMN cell infiltration and augmented inflammation suggests a possible explanation for the increased incidence of gastric cancer in developing countries, such as Ecuador, where zinc deficiency and *H. pylori* infection are important public health problems. It has been shown that zinc supplementation inhibits *H. pylori*-induced gastric mucosal oxidative inflammation in gerbils [36,37]. Furthermore, in a small randomized clinical trial, a zinc compound in combination with triple therapy (lansoprazole, amoxicillin, clarithromycin) significantly improved the cure rate for *H. pylori* [38]. However, this study did not evaluate histopathologic changes related to *H. pylori* infection or eradication. Taken together, we propose that zinc deficiency is associated with increased PMN infiltration in gastric mucosa and consequent inflammation. As inflammation is the main mechanism of *H. pylori*-associated gastric carcinogenesis, we suggest that zinc supplementation be considered for future studies as an adjunctive approach to the treatment and eradication of *H. pylori*.

We are thankful with the collaboration of Dr Pelayo Correa, from the Louisiana State University Health Science Center, for the provision of histopathologic protocols, and suggestions to follow those protocols. We also thank Drs José L. Realpe, and Luis E. Bravo, members of Dr Correa staff, for technical training to our endoscopist and pathologist. We recognize the support of Dr Rebecca de los Ríos from the Pan American Health Organization (PAHO).

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Chapter 10

General Conclusions

GENERAL CONCLUSIONS

The body of work as presented in this thesis with regard to zinc deficiency in children in relation to common infectious diseases shows the following:

a) Zinc deficiency is prevalent in Ecuadorian populations, mainly in Andean areas. In random samples of young children who live in different regions of the country the prevalence of zinc deficiency defined as serum concentration values < 65 ug/dL was 31.8%.

b) In children zinc supplementation improves the Delayed Type Hypersensitivity (DTH) response. These data suggest that zinc deficiency results in defective T cell driven immune responses, which can be corrected upon zinc supplementation therapy. Provision of daily zinc supplements
   a. to young children for 6 months reduces the incidence of diarrhea episodes with the lowest efficacious dose ranging from 3 to 7 mg/day.
   b. for 8 weeks (10 mg/day) reduces the incidence of respiratory symptoms in children, which suggests a link between these infections and zinc deficiency. However, the benefit reverses when the supplementation is stopped.
   c. as supplementation (10 mg twice daily) to the standard antimicrobial treatment of severe pneumonia in hospitalized children does not reduce the time to resolution of clinical symptoms. However, a better zinc status at enrollment was found to reduce the time to resolution of clinical symptoms in these children.

These data imply that an optimal zinc state prophylactically protects against respiratory and intestinal infections by safeguarding an efficient T cell mediated immune responsiveness.

The body of work with regard to the prevalence of micronutrient deficiencies in elderly Ecuadorians (on average over 60 years of age), their immune functioning and the relationship of micronutrient deficiency with the prevalence of infectious disease and the Metabolic Syndrome (MetS) shows the following:

a) Micronutrient deficiencies, infections, and poor DTH responses were common in elderly populations living in a poor urban area of Quito.

b) Zinc deficiency was associated with reduced IL2 and INF-γ production by leukocytes, reinforcing the idea that zinc deficiency results in defective T cell driven immune responses, most notably Th1 driven responses.

c) Vitamin C deficiency was associated with reduced serum IFN-γ levels, also suggesting the requirement of this micronutrient for proper Th1 function.

d) With regard to zinc status the deficiency prevalence (deficiency defined as a serum concentration ≤ 70 ug/dL) was 41% for men and 45% for women. With regard to vitamin C status the deficiency prevalence (defined as <11.4 µmol/L) was 60% for men and 33% for women (statistical significant difference)
e) Metabolic syndrome (MetS) was highly prevalent in an older population of Quito subjects (40%), considerably more among women (80%) than men (19%).
    a. The level of CRP was positively associated with the MetS, reinforcing the view that the MetS is a chronic inflammatory condition.
    b. There was no relationship of the MetS with zinc deficiency.
    c. The MetS was particularly evident in subjects with a deficiency for vitamin C (and E). This observation suggests a role of vitamin C in the regulation of inflammation as an anti-oxidant to counteract inflammation

The body of work in this thesis also shows that a local low zinc concentration in the stomach is associated with a higher number of infiltrated granulocytes in the presence of a *H. pylori* infection. Whether this local zinc deficiency reflects a poor microbicidal defense per granulocyte against the microbe which necessitates a higher influx of the polymorphs, or whether this local zinc deficiency reflects a poor anti-inflammatory action will be discussed.

Chapter 11

Discussion

EFFECTS OF ZINC DEFICIENCY ON THE IMMUNE SYSTEM

Zinc as an essential element for T cell driven immune responses

Zinc deficiency has been found to be associated with thymus atrophy, and with disturbances in the T lymphocyte maturation [1]. The adverse effects of zinc deficiency on both thymus structure and function could be explained through the role of zinc on the activity of one of the thymus hormones, i.e. thymulin, which needs zinc as an activation factor [2]. However, and maybe more importantly, zinc is part of a plethora of biological active compounds, including some thymus epithelial transcription factors, such as Zfp335 of the C2H2 zinc finger family, which modulates T lymphocyte maturation. The activity of these biological active compounds depends on zinc availability [3]. Finally, zinc deficiency induces an increased glucocorticoid production which is able to lead to apoptosis of immature T cells [4], which can clearly interfere with the maturation of a proper T cell system.

Also cytokine production by T cells is affected in zinc deficient subjects. The production of the T cell proliferative factor IL-2 and the Th1-cytokine IFN-γ are decreased, whereas the production of the Th2 related cytokines IL-4, IL-6 and IL-10 is not affected. Reduced Delayed Type Hypersensitivity (DTH) reactions in these subjects suggest a defective Th1-dependent cellular immune response [1].

Our data described in chapters 4, 6 and 7 on a poor DTH reactivity and a poor ex vivo IL-2 and IFN-γ production by blood mononuclear cells in zinc deficiency and improvements after zinc supplementation are in accord with this view.

Zinc as an essential element for an antimicrobial immune activity of macrophages

Triggering of pattern recognition receptors (PRRs) on monocyte-derived macrophages by bacteria has been described as inducing the gene expression of methallothioneins, which increases intracellular zinc and bacterial clearance through cellular autophagy [5]. Moreover, zinc sequestration in the Golgi apparatus has been found after macrophage activation by Granulocyte-Macrophage Colony Stimulation Factor (GM-CSF). This sequestration is associated with an increased production of reactive oxygen species (ROS) and a clearance of fungal infections. This clearance could be related to both the ROS effect and a deprivation of zinc for the pathogens [6]. Since extracellular zinc moves into the cell compartment during acute infections [7], it has been suggested that the zinc deficiency status might reduce the intracellular zinc concentration making phagocytes unable to clear infections.

Our studies described in chapter 5 showing that children with a better zinc status resolved severe pneumonia episodes faster indirectly support the view that zinc is essential for antimicrobial actions of phagocytes.

In chapter 9 we described that a reduced mucosal zinc concentration in atrophic gastritis coincided with *H. pylori* infection and an increased polymorphonuclear infiltration. It can be envisaged that the local mucosal zinc deficiency may have induced a poor antimicrobial activity of local phagocytes and thus a poor eradication of *H. pylori*, necessitating the higher influx of inflammatory cells to counteract the microbial attack. In such a view our data described in chapter 9 would support the role of zinc in the antimicrobial activity of phagocytes.

Zinc as an essential element for myeloid development and a pro-inflammatory immune function of macrophages

Apart from being part of transcription factors important in T cell differentiation or playing a role in antimicrobial defense molecules in phagocytes, zinc is also part of biologically active compounds involved in myeloid differentiation, maturation and inflammatory function. The ZBTB20 zinc-finger protein of the BTB/POZ family is a transcription factor which enhances the activity of Nuclear Factor κB (NF-κB). Myeloid cells of ZBTB20 knockout mice produce reduced amounts of pro-inflammatory cytokines upon Toll-like receptor (TLR) stimulation. ZBTB20 protein inhibits the IκBα gene expression by binding to its promoter, which leads to a decreased production of this NF-κB blocking compound and thus to an increased production of pro-inflammatory cytokines [8] (See figure 1).

Zinc as an essential element for an anti-inflammatory immune state of macrophages

Considering the above described experiments on the pro-inflammatory activity of zinc it is counterintuitive that Prasad has proposed to consider zinc as an anti-inflammatory agent [9]. He found it associated in his set of experiments with a reduced production of pro-inflammatory cytokines, such as IL-1β and TNF-α by monocytes/macrophages. This anti-inflammatory effect was thought to be mediated by inhibition of (NF-κB) signaling via an increased production of the A20 (TNFAIP3) protein [10]. Indeed, further recent evidence using human zinc deficient cultured blood mononuclear cells show an increased production of IL-6 and IL-1β following LPS stimulation. This effect was associated with reduced IL-6 gene promotor methylation [11].

Most likely, a complex dual effect of zinc on the inflammatory activity of monocytes/macrophages exists, with zinc being involved in homoeostatic balanced actions.

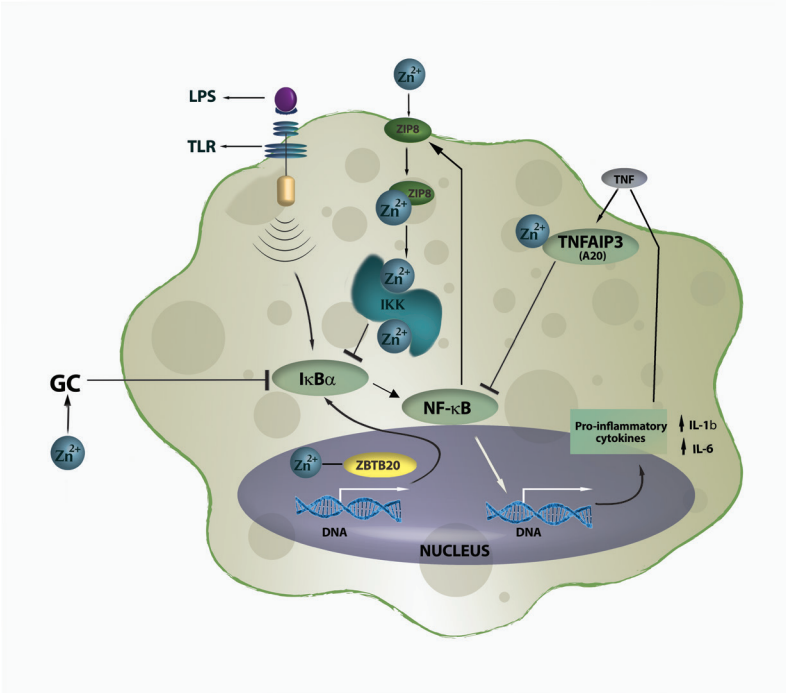

Figure 1. The various and often dual effects of zinc (as ion or integrated in biological compounds) on the inflammatory state of macrophages is depicted.

1. On the one hand the production of pro-inflammatory cytokines is promoted by ZBTB20 zinc-finger protein, which availability would depend on long provision of dietary zinc.
2. On the other hand the anti-inflammatory effect of zinc is dependent on

a. The provision of extracellular zinc ions transported into the cell via ZIP8 and inhibiting the activity of the enzyme IKK, thus preventing the phosphorylation of IκBα and thus the pro-inflammatory activity of NF-κB.
b. A higher production of glucocorticoids (GC). Zinc deficiency increases GC production.
c. Via A20 (TNFAIP3) zinc-finger deubiquitinase. A feed-back loop via a zinc-finger protein. A high TNF production feeds back on the macrophage and induces A20 which blocks the NF-κB activity.

Although zinc is important as essential element in transcription factors for the NF-κB induced inflammatory response in monocytes/macrophages (such as ZBTB20), in sepsis animal models ex vivo assays show that activated NF-κB induces expression of the zinc transporter SLC39A8 (ZIP8) in leukocytes, which in turns down regulates NF-κB activity [12]. In a closer scrutiny the experiment shows that zinc ions transported into the cell bind directly to the IKK complex preventing the phosphorylation of IκBα and thus the activation of NF-κB. These modes of events illustrate feed-back homoeostatic balanced dual effects of extracellular zinc (See figure 1). It is of note that in the experiments of Prasad et al focus was on extracellular zinc, which might in particular show this anti-inflammatory action.

Also the role of natural T regulatory cells and those of Th2 cells should be considered for the inflammatory state of monocytes/macrophages. Natural T regulatory cells are thought to be able to regulate the pro-inflammatory state of monocytes/macrophages, and zinc is also essential for the development of natural T regulatory cells [13], although very few

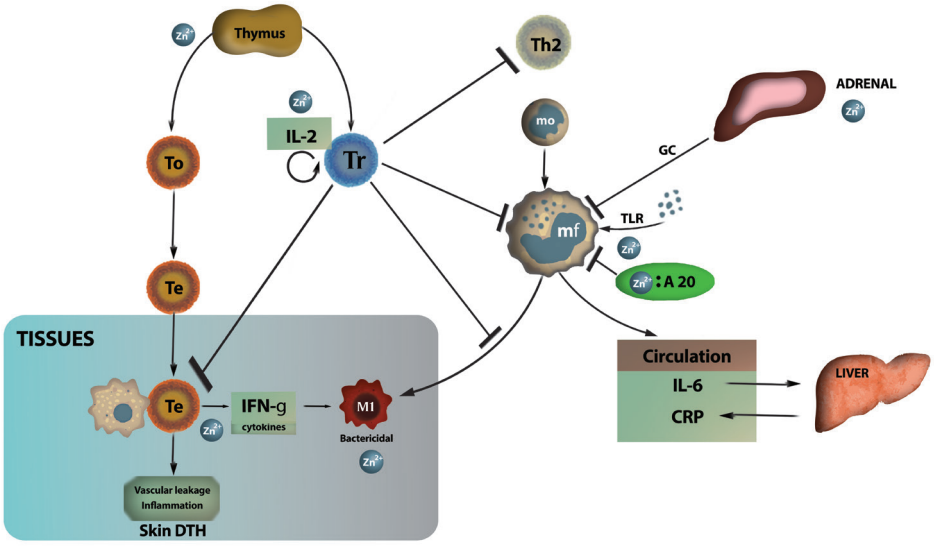

Figure 2. This figure shows a scheme in which the multiple points are shown where zinc can influence either directly (as ions) or indirectly (integrated in biological compounds) the immune response.
Zinc deficiency is in general related to higher infection sensitivity due to a reduced T cell response, poor production of IL-2 and IFN-γ by effector T cells, a poor antimicrobial capacity of phagocytes in the tissues and an increased production of glucocorticoids, further blocking the activity of T cells and macrophages.
Zinc sufficiency is in general related to a low infection sensitivity due to an adequate antimicrobial capacity of phagocytes in the tissues and an adequate T effector response. There is also an adequate natural T regulator response leading to an adequate dampening of the pro-inflammatory actions of macrophages.
The scheme also shows that both anti-inflammatory and pro-inflammatory actions in macrophages are possible due to zinc sufficiency/deficiency, which is depicted in figure 1 in more detail. These dual and often opposing effects of zinc sufficiency/deficiency might be due to specific local effects, preferential mechanisms of action due to genetic or other environmental influences or depending on whether zinc is intracellular available or whether it is freely provided extracellular in culture media.
Te: T effector cells (Th1); **GC**: glucocorticoid; **DTH**: Delayed Type Hypersensitivity; **A20**: Tumor necrosis factor alpha-induced protein 3 (TNFAIP3); **Tr**: T regulatory cells.

experiments have been performed in this respect. Also balances preferring Th2 reactions have been seen in zinc deficient experimental animals developing atopic dermatitis [14].

Furthermore, the increased glucocorticoid production reported by Fraker in zinc deficient subjects promotes an anti-inflammatory state of monocytes/macrophages [4]. Glucocorticoids inhibit NF-κB in macrophages and dendritic cells by inducing IκB [15].

In chapter 9 we described that a reduced mucosal zinc concentration in atrophic gastritis coincided with *H. pylori* infection and an increased polymorphonuclear infiltration and earlier in the discussion we have argued that the local mucosal zinc deficiency may have induced a poor antimicrobial activity of local phagocytes and thus a poor eradication of *H. pylori*, necessitating the higher influx of inflammatory cells to counteract the microbial attack. However our studies in *H. pylori* infected patients may also illustrate the concept that mucosal zinc could act as an anti-inflammatory agent and that its deficiency in atrophic gastritis would lead to more inflammation (and thus higher numbers of polymorphs) in the gastric mucosa. Both explanations are not mutually exclusive.

Clearly, further in vitro, ex vivo and in vivo studies are required to obtain a more accurate picture of the immune and inflammatory state in human subjects with different grades and durations of zinc deficiency. Also various immune compartments as well as immune cells should be investigated.

ZINC IN THE YOUNG: ROLE IN COMBATING INFECTIONS

Zinc deficiency in children who live in developing countries as Ecuador seems to be related to chronic dietary restriction. In zinc deficient children the structure and function of the thymus could be affected as it has been described above. Zinc deficiency affects proper immune functioning, which make these populations more vulnerable to infections caused by certain pathogens mainly viruses and intracellular bacteria.

Respiratory infections, mainly pneumonia, remain the leading cause of mortality in young children in Ecuador [16]. However, the etiology of respiratory infections, mainly pneumonia, remains largely unknown in Ecuador, although in a recent study described in chapter 5 we have found that in children severe pneumonia acquired at the community level is mainly associated with viruses.

As it was referred to in the Introduction, I started my studies on zinc and infection in children in 1992. In the epidemiological context of the nineties, I chose to study the potential relation of zinc deficiency and respiratory infections in malnourished children,

especially in those with low height-for-age Z score. The first study included an intervention with oral zinc as described in chapter 4. A double blind placebo controlled trial was carried out in children with low height-for-age Z score. This study showed that a daily provision of 10 mg of zinc diminishes the incidence of respiratory signs and improves the DTH response. The benefit reversed when the zinc supplementation was stopped.

This finding, reported almost two decades ago, suggested that regular provision of zinc is required to keep the benefit on both the immune response and prevention of respiratory infections because there are not easy available tissue zinc stores. This requirement was subsequently proved in several larger studies [17-19].

Our more recent clinical trial in children consisted in the provision of zinc supplementation to children suffering from severe pneumonia as an adjunct to the standard treatment. The study is described in the chapter 5. A randomized double blind placebo controlled trial was performed. This study included some basal variables to deal with the controversial results of previous studies carried out in Asia. These variables were zinc status at enrollment, pathogens (virus/bacteria) associated with the pneumonia episode, and CRP serum concentration as marker of inflammation. There were no benefits of the zinc supplementation on the duration of clinical signs. However, children with a better zinc status at enrollment resolved the clinical signs shorter. Higher CRP was found associated with longer time to resolution of clinical signs.

The cellular uptake of zinc provided by supplements depends on available efficient zinc transporters molecules. Zinc transporter (ZnT, and Zip) gene expression assessed by messenger RNA (mRNA) in leucocytes of obese women were found by Noh et al to inversely correlate with serum inflammatory markers, such as CRP, and TNF-α [20]. This finding suggests that zinc transporters gene expression is not only dependent on intracellular NF-κB activity as shown in the previous mentioned sepsis animal models [12], but also by extracellular inflammatory compounds. The study by Noh et al., found in particular a negative correlation between CRP and mRNA levels of ZnT4, Zip1, and Zip6, while mRNA levels of ZnT4 and ZnT5 were inversely correlated with TNF-α. Intracellular NF-κB selectively induced gene expression of the more efficient zinc influx transporters Zip8. It can thus be envisaged that very short short-term zinc supplementation could not have reached the inflammatory cells, provided the cells or their environment had been seriously inflammatory changed. This might have played a role in the fact that zinc supplementation did not have an acute effect on pneumonia signs in our study, while the initial zinc state did.

In any case, our findings suggest that the impaired immune function due to chronic zinc deficiency cannot be reversed in very short periods of supplementation as that of the intervention during an acute pneumonia episode. As a consequence, regular provision of enough dietary zinc or supplements seems to be the best approach to deal with this micronutrient deficiency at community level. Precisely, a major challenge for public health policy makers is finding a good approach to provide daily zinc to populations suffering from chronic dietary zinc deficiency. Some strategies have been suggested by an international team of experts of which I was a member [21].

EFFECTS OF ZINC DEFICIENCY IN THE ELDERLY

In older subjects who live in areas of chronic dietary zinc deficiency, the impaired immune response could be related to both the immune senescence and the altered immune mechanisms due to the insufficiency of zinc itself. At ageing the thymus involutes, which is associated with reduced numbers of naïve T cells [22], defective anti-apoptotic mechanisms due to a reduced expression of BCL2 and p53 [23], and a reduced T cell proliferation in response to mitogen or T cell receptor (anti-CD3) stimulation [24]. Similar effects on the immune response are related to zinc deficiency, so that zinc-deficiency might reinforce the adverse effects of ageing on immune mechanisms.

The profile of cytokines at ageing shows lower production of IL-2 and IFN-γ whereas that of IL-6, IL-4, and IL-10 is increased [25]. We have found that zinc deficient older subjects in Ecuador show lower ex vivo production of IL-2 and IFN- γ than zinc sufficient older subjects [26]. These findings suggest a synergy between ageing and zinc deficiency on the immune senescence. Indeed the zinc deficient status at ageing in Ecuador could be worsened due to age-related factors which have been described in several studies: poor mastication, affected taste, inadequate intestinal absorption, concurrent diseases, drug interaction, and competition with other divalent minerals [27]. From these factors, inadequate intestinal absorption seems to be most relevant since the mechanisms of impaired uptake have been found associated with defective gene expression of zinc transporters at ageing [28].

Since the MetS is associated with inflammation, it is plausible to hypothesize that deficiency of antioxidant micronutrients, such as vitamins C, and E, and zinc, would have a role on the mechanisms associated with the MetS, mainly on tissue inflammatory injury. In fact, the extended deficiency of these micronutrients found in our preliminary study in older subjects added to the plausibility of this hypothesis. However, in our larger study, described in chapter 8, although high CRP serum concentration (> 3mg/L) was

positively associated with the MetS in older subjects (OR= 1.79, 95% CI 1.04, 3.06), low serum zinc concentration was not associated with the MetS. Low serum vitamin C, and E were associated with MetS. These findings suggest that oxidant mechanisms such as ROS production are associated with the MetS as has been reported in other studies [29].

EFFECTS OF ZINC DEFICIENCY IN THE METS?

The antioxidant role of zinc is mediated by the copper-zinc superoxide dismutase (Cu/ZnSOD). Expression of Cu/ZnSOD intracellular genes (sod1, sod2,) is dependent on the activity of NF-κB transcription factor [30]. Therefore, according to the studies showing and inhibition of NF-κB activity by zinc [12], it would have been logical to find an association between zinc deficiency and the MetS in our subjects, since more than 40% were zinc deficient. Given the cross sectional characteristic of this study it is not possible to draw definite conclusions, but a possible explanation for the non-association of the MetS with zinc concentration could be the mild zinc deficient status of these subjects as suggested by the median and the 10th and 90th percentiles of serum zinc concentration (75 (48–106), 71 (47–108) ug/dl, for men and women, respectively). The other possible explanation could be related to the dual findings on the association of chronic human zinc deficiency with inflammation.

More than 48% of our studied older Ecuadorian subjects had high CRP concentration (> 3mg/L) and CRP values correlated positively with the MetS. We did not perform an analysis of the correlation between zinc status and CRP. Studies in obese women show increased inflammatory markers such as CRP and IL-6, which are reduced by short-term zinc supplementation [31]. This finding suggests a benefit of zinc supplements on amelioration of inflammation in obese women. The reduction of inflammatory markers by zinc supplementation suggests that this mineral inhibits NF-κB activity as it was effectively found in another study [12]. However, since data of baseline zinc are not provided it is not possible to know if the inflammatory markers were associated with zinc deficiency before the intervention in these subjects.

Fasting hyperglycemia is one of the components of MetS definition. Since zinc is included in insulin molecules stored in pancreatic β cells [32], after insulin secretion due to glucose stimulation zinc ions are released from the molecule and return to the pancreatic cells via ZnT8-dependent reuptake mechanism [33]. We did not find a correlation between zinc serum concentration and glycemia in our older subjects (data not published), which could be due as describe above to the mild chronic zinc deficiency of this population.

Some studies have suggested that polymorphisms of the ZnT8 gen (SLC30a8) are related to different risks for type 2 diabetes (T2D). Specifically, there are two isoforms associated with substitution of Arg by Trp amino acid at 325 position due to single-nucleotide polymorphisms (SNPs) at ZnT8 gene (rs13266634 allele), both have been found associated with a low risk for T2D [34]. The protection could be related to the traffic and intracellular localization of zinc ions. In fact, Nicolson and colleagues found higher cytoplasmic zinc in the presence of Trp than Arg isoform [35]. Based on this finding, Davidson and colleagues have speculated that the Trp isoform is active at the plasma membrane in the reuptake of zinc. The Arg isoform instead would reduce the rate of uptake of cytoplasmic zinc into the insulin secretion granules (ISGs) [33]. Intracellularly the protective role of zinc could be related to anti-oxidant mechanisms since glucose stimulation of β cells is associated with an increase of ROS and reactive nitrogen species (RNS) [36]. Then, chronic zinc deficiency could be associated with a persistent deleterious toxic effect of ROS and NOS on pancreatic β cells, contributing to the high fasting glucose levels in a low percent of our elderly population.

VITAMIN C DEFICIENCY AND METS

We found an inverse correlation between serum vitamin C and MetS (OR=0.16,95% CI 0.03, 0.81), and the prevalence of vitamin C deficiency was high (60% and 33 %, for men and women, respectively). Vitamin C has been found negatively associated with leptin serum concentration in obese women [41], which adds to its potential benefit on amelioration of inflammation in obese subjects [42]. It is indeed tempting to speculate that vitamin C sufficiency prevents the MetS via anti-oxidant and anti-inflammatory properties, interfering with low grade chronic inflammation of the adipose tissue, stopping a vicious circle leading to more severe signs and progression of the MetS.

Since central obesity is the primary criterion to define MetS according to the International Diabetes Federation (IDF), one can speculate that vitamin C prevents development of central obesity, although given the transversal feature of our study it is not possible to draw definitive conclusions. Ascorbic Acid (AA) inhibits the expression of genes associated with differentiation of cultured mesenchymal cells (3T3-L1) into adipocytes as well as the lipids accumulation in adipocytes [37]. However, another study found that AA provided with adipogenic cocktails is highly efficient in differentiating mouse embryonic stem cells into adipocytes [38]. These controversial findings could be related to the AA dose and the type of cells used.

A recent study in vitamin C deficient animal models shows impaired glucose tolerance and diminished insulin secretion. The study also shows low ATP production in pancreatic islet cells, which suggests that vitamin C is implicated in mitochondrial oxidative phosphorylation [39]. This mechanism might explain the high fasting glycemia we found in some subjects with MetS. In a study reported by Franke and colleagues vitamin C intake correlated negatively with necrosis and apoptosis of peripheral blood cells of pre-diabetic subjects, which suggests that improvement of vitamin C intake would benefit tissues from oxidative damage associated with high blood glucose [40].

Recently, in animal models, hypo-ascorbemia has been found associated with increased Lipoprotein (a) (Lp (a)), an important risk factor of cardiovascular disease. The study showed that transgenic mouse with Lp(a) expression and lack of ascorbate production fed a diet without ascorbate showed a higher Lp(a)serum concentration than controls fed enough ascorbate. Serum concentration of LDL was also higher in ascorbate deficient mouse [43]. In human adults with high basal total and LDL-cholesterol long term provision of orange juice with improvement of serum vitamin C concentration was found associated with lower LDL, apolipoprotein B (apo B), and HDL/LDL ratio than controls [44]. These findings suggest a role of vitamin C on the prevention of vascular damage due to lipid deposition.

VITAMIN E AND METS

Our study in older subjects showed an inverse correlation between serum vitamin E: TAG ratio with MetS (OR=0.78, 95% CI 0.71, 0.86). A recent study in murine macrophages has shown that γ-tocotrienol inhibits NF-κB by up regulation of A20 protein [48]. In cultured mice macrophages challenged with LPS the addition of α-tocopherol showed a significant reduction of TNF-α comparable to that of controls [49]. Splenic lymphocytes of LPS-challenged mice treated with antioxidants (α-tocopherol, co-enzyme Q, and β-carotene) showed a significant decreased IκB phosphorylation thus inhibition of NF-κB [49]. Therefore, one can speculate that older Ecuadorian subjects with MetS suffer from low grade inflammation due to a regular dietary intake of different isoforms of vitamin E.

We also found the prevalence of vitamin E deficiency assessed by serum concentration to be low in our older subjects, which might be explained by the extended consumption of palm oil in Ecuador. Chromatographic analyses of palm oil detects six isoforms (tocopherols and tocotrienols) α-T, α-T1, γ-T, α-T3, γ-T3, and δ-T3 [45]. The antioxidant mechanisms of tocopherols and tocotrienols have been described extensively [46]. Those mechanisms

are linked to vitamin C availability. Tocotrienols seems to be more efficient antioxidants than tocopherols [46]. In fact, in vitro studies in rat aorta have shown a beneficial effect of rich palm oil extract on reducing oxidative stress and improving endothelium-dependent relaxation , although some concern remains on its fat saturated acid contain [47].

We could not find studies on central obesity and vitamin E status. However, some studies suggest that body mass index (BMI) and leptin are positively associated with γ-tocopherol [50]. Since there is not a direct association between serum γ-tocopherol level and dietary intake the variation of serum γ-tocopherol might indicate a metabolic balance with other micronutrients such as vitamin D [51]. In fact, the above referred study found that vitamin D status is negatively associated with BMI and leptin [50]. Since in our study severe vitamin D deficiency assessed by serum concentration (< 10ng/ml) affects <15% of elderly population, it is possible to speculate that if low grade inflammation affects the Ecuadorian older subjects with MetS, it could also be explained by the role of vitamin D. Furthermore, it is possible to hypothesize that our population has low serum level of γ-tocopherol. Although currently the cutoff point for vitamin D deficiency is higher (20 ng/ml) and in this case a higher percent of the Ecuadorian elderly population would suffer from moderate deficiency. Still this situation would be associated with low grade inflammation .

Observational and intervention studies with vitamin E in populations have offered controversial findings. One cross-sectional study in subjects with MetS did not find association of dietary vitamin E intake and MetS [52]. A study in adolescents with MetS showed that vitamin E supplementation did not have effect on the serum level of vascular endothelial growth factor (VEGF) nor on HDL serum concentration [53]. A larger study in US adolescents did not find consistent association between serum vitamin E and MetS [54]. A recent meta-analysis of randomized trials found that vitamin E supplementation improves endothelial function [55].

Clearly, further studies are required in populations with MetS where vitamin E deficiency is prevalent.

LIMITATIONS OF OUR STUDIES FUTURE APPROACHES

Immune tests. It is clear that more advanced immune assays should be done in future studies to unravel the complex effects of zinc deficiency and supplementation in the various components of the immune response. Preferably serum and circulating leukocytes should be studied. With regard to the assays a spectrum of cytokines and growth factors

can now be studied (using multiplex arrays), circulating lymphocyte populations better identified (certainly including natural T regulator cells and the various T effector cells, including Th17 cells) and monocytes tested for their phagocytic and microbicidal capacity and their capacity to produce inflammatory and anti-inflammatory cytokines and growth factors (gene expression, protein production). Only then a better picture will emerge on the protective capability of pro- and anti-inflammatory state of not-treated and treated patients. However this requires up to date laboratory facilities in the Quito area, which are costly and not immediately available.

Mechanistic studies. To better understand the role of zinc, zinc deficiency and zinc supplementation mechanistic studies are essential on animal models and cell lines. Our studies only approached the problem from an epidemiological point of view, with a strong focus on what is possible in a country like Ecuador and what might lead to direct changes in health care. The intriguing dual role of zinc on the immune system (pro- and anti-inflammatory) can probably only successfully be approached via mechanistic studies.

Patient groups. In future studies we ideally should stratify patients on the basis of zinc status before treatment. Also their immune state before treatment should be taken into consideration. For older patients more in depth studies are required before we are able to link the zinc state to signs of the MetS or the effects of zinc supplementation on improvement of the MetS. Clearly studies before and after zinc supplementation taking into account inflammatory aspects of obesity (circulating cytokines, leptin and adiponectin) and hyperglycemia are warranted.

Sample sizes. Larger intervention studies are warranted to draw robust conclusions. In Ecuador, and particularly in Andean areas where the prevalence of zinc deficiency is higher than in the coastal areas, some communities will be required both urban and rural.

Reproducibility. Doses to be provided should be within the range of most of published studies. Field and laboratory methods should be those providing the update information related to both clinical and mechanistic effects.

Confounding factors. Prospective studies are threatened by confounding factors such as consumption of micronutrients, other concurrent treatments, basal undetected pathologies especially in older subjects. The living conditions are also relevant, especially in large studies. Then the study design will require different approaches to deal with these issues. Randomized controlled trials should be advisable to deal with cofounders. Furthermore, cluster randomized design would allow us to control the variability of living conditions across different settings.

FUTURE APPROACHES

Recommendations for supplementation.

In the light of the micronutrient deficiencies found in older Ecuadorian people, especially those of vitamins C, E, and zinc, and their potential association with MetS, more research is needed to define causal association. Dietary interventions aimed at improving the micronutrient status are warranted to test the effect on weight gain, central obesity, glycemia, insulin resistance, lipids profile and inflammation in older subjects with MetS. In fact, currently we are completing a randomized placebo-controlled trial in older Ecuadorian subjects to test the effect of daily provision of vitamins C, D, and zinc and iron on infection, immune function, and MetS markers. The study includes 320 older Ecuadorian subjects (> 65y) who live in Quito urban slums. During one year subjects were given daily a tablet with those micronutrients or placebo. A weekly visit at home was carried out to identify respiratory infections. At enrollment and once the supplementation was completed we measured some anthropometrics: height (knee-height in subjects unable to stand up), weight, BMI, and abdominal circumference. Serum cathelicidine, and DTH response were included to test the innate and cellular immune response. Serum lipids (total cholesterol, LDL-cholesterol), and glycemia were also measured. CRP was included as marker of inflammation.

Since zinc deficiency is extended at both children and elderly Ecuadorian populations different approaches could be developed. In areas where severe deficiency exists providing supplements (7-10 mg/day) seems to be a priority, although difficult given the low compliance. Then, other strategies are relevant, especially in rural Andean areas, such as legume production, and refined cereal consumption with low phytate content .

Experiments to fill in the gaps in the present literature.

Clearly more studies are required to deepen the knowledge of both molecular mechanisms and clinical outcomes of micronutrient intervention especially vitamins C, D, E, and zinc. Regarding the molecular mechanisms the association with adipogenesis as well as with inflammatory markers, including gene expression and regulation through mRNAs are warranted. Interactions of micronutrients at molecular level are needed. Clinical intervention trials are warranted and should include the tests of molecular mechanisms through ex vivo assays. For Ecuador the scope of these studies will demand sustained collaboration with academic research centers such as Erasmus MC.

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Addendum

ENGLISH SUMMARY

This thesis explores the association between zinc deficiency with immune function and infection in both young children and older subjects in Ecuador. Furthermore, the potential link between zinc deficiency and that of vitamins C and E with the metabolic syndrome (MetS) in Ecuadorian elderly population has been studied. Research studies published in peer reviewed journals, carried out over the last two decades at both the community level and at hospitals are the ground to exam the proposed associations.

Zinc deficiency is highly prevalent in Ecuador particularly in the Andean region. Rural areas are more affected than urban ones due to the limited access to zinc rich animal food. There, the diet is based mainly on cereals which do not provide enough zinc to meet the daily requirements, especially due to the traditional practice of consuming unrefined cereals with phytic acid content. Public health policy aimed at improving dietary zinc is pending. Difficulties arise from the required daily provision of either food or supplements since body zinc stores are not available.

In young Ecuadorian children population anemia due to chronic iron deficiency has been another relevant problem. However, provision of iron supplements at health centers has reduced the prevalence of anemia, especially at urban areas. This approach faces a problem at rural areas due to the low compliance of populations to the supplements. However sustained progress is being reached. Competition between iron and zinc uptake mechanisms at the intestine could worsen the zinc status.

Since zinc is related to both the innate and adaptive immunity, zinc deficient populations are vulnerable to diarrheal and respiratory infections. Currently there is enough evidence to show this association. In fact, it has become clear that zinc deficiency affects the macrophage killing capacity as well as the Th1-dependent immune response. However at the year 1992, when we started studies on zinc, these cellular mechanisms were just beginning to be revealed. On 1996 we published a study which tested the effect of daily zinc supplements or placebo on respiratory infection and Delayed Type Hypersensitivity (DTH) in young children with low height-for-age. The study showed a reduction of respiratory signs and improved DTH response in the intervention group. The benefit reversed when the supplements were stopped. These findings were ancillary evidence on the association between zinc deficiency with infection and impaired immune function.

Later, we participated in a collaborative study with the University of California, at Davis, which demonstrated that 3-7 mgs of daily zinc are enough to reduce significantly the

incidence of diarrhea in young children with low height-for-age. At those doses the copper status was not affected.

On the year 2008, we published a study on the relationship between zinc in gastric tissue and local inflammation in *H. pylori* infected adults who lived in Quito. Low zinc tissue concentration was found associated with higher infiltration with mononuclear and polymorphonuclear (PMN) cells.

Recently, in another published study, we found that in hospitalized children zinc supplements provided as an adjunct to the standard treatment of an acute severe pneumonia episode does not shorten the clinical evolution of the episode. However, children with better basal zinc status have a shortened duration of the clinical signs of the severe pneumonia.

Based on those findings we conclude that chronic zinc deficiency affects both the innate and adaptive immune response. There is growing evidence to understand the mechanisms associated with these effects. In fact, zinc is required for optimal maturation of T cells in the thymus and then for efficient support of macrophage-dependent killing of intracellular pathogens. Zinc deficiency is, therefore, associated with low production of Th-1 pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. However, zinc deficiency could be related with increased inflammation as suggested by the PMN cells infiltration in *H. pylori* infected subjects with low zinc tissue concentration.

The prevalent theory links zinc deficiency with increased inflammation which seems to be in contradiction with our previous findings and with those from others who have found an increment of glucocorticoid production in zinc deficient children. The prevalent theory emerges from experimental studies in cultured activated macrophages treated with zinc ions. These studies show that zinc ions bind to IKK enzyme and then prevent NF-κB activation. However, others studies show that zinc-finger proteins such as ZBTB20 promotes NF-κB activation. Furthermore, and to add to the complex picture of the association between zinc and inflammation, T regulatory cells depend of zinc availability. Therefore, a dual effect of zinc deficiency on inflammation is proposed in this thesis which would depend on the grade and duration of this deficiency.

Over the last decade we have studied the nutritional, immunological and health status of elderly Ecuadorians who live in Quito urban slums. In fact, our studies have been the first in Ecuador aimed at describing the health status of older Ecuadorian subjects. The first study was preliminary and based on findings in 145 subjects of over 65 years old. We

found a high prevalence of micronutrient deficiencies, and respiratory infections, as well as low DTH responses, and high caloric intake and overweight. These findings led us to deepen the knowledge of immune function, infection, and nutrition, and metabolic health in those subjects.

In a larger study in 352 older subjects we found low plasma concentration of some micronutrients, especially of vitamin C and zinc. Plasma vitamin C was associated with interferon-γ (IFNγ) and zinc with IFNγ and interleukin-2 production in ex vivo cultured peripheral blood cells. Respiratory infection history was associated with any micronutrient deficiency. DTH testing revealed that a minority of the study population mounted responses to tetanus toxoid and *Trichophyton*, whereas more than one-half responded to tuberculin and *Candida* test antigens. In sum, Th1-dependent immune response seems to be affected by the most prevalent micronutrient deficiencies in these older subjects. These deficiencies worsen the impaired immune function associated with ageing.

Metabolic syndrome (MetS) was highly prevalent in this elderly Ecuadorian population (40%), more in women (81%) than in man (19%) as assessed by the International Diabetes Federation (IDF) criteria. Plasma vitamin C and vitamin E concentrations were inversely associated with MetS. C-reactive protein (CRP) was positively associated with MetS. Plasma zinc concentration was not associated with MetS. The lack of association between zinc and MetS was unexpected due to its anti-oxidant roles through the superoxide dismutase (SOD), a zinc-dependent enzyme. This finding would depend on the mild zinc deficiency of these subjects as suggested by their zinc plasma concentration. Unfortunately, we did not test the correlation between plasma zinc and CRP which had provided us a clue on the potential dual effect of zinc deficiency on inflammation suggested above.

The prevalence of vitamin C deficiency was high in our study (60% and 33 %, for men and women, respectively). Vitamin C was found associated negatively with MetS. This finding is consistent with the antioxidant role of this vitamin. Furthermore, vitamin C has been found associated with low adipogenesis in some studies, although others have found the opposite effect. These conflicting results would be related to the dose of vitamin C and the type of cells used. However, vitamin C has been found negatively associated with leptin serum concentration in obese women, which adds to its potential benefit on amelioration of inflammation in obese subjects.

The prevalence of vitamin E deficiency was low in our study (<15%). However, an inverse correlation between vitamin E:TAG ratio and MetS was found (OR=0.78, 95% CI 0.71, 0.86). Recent studies suggest that γ-tocotrienol and α-tocopherol inhibit NF-κB.

Therefore, one can speculate that older Ecuadorian subjects with MetS suffer from low grade inflammation due to an irregular dietary intake of different isoforms of vitamin E. In fact, in Ecuador there is an extended consumption of palm oil which contains six isoforms of vitamin E. In vitro studies in rat aorta have shown a beneficial effect of rich palm oil extract on reducing oxidative stress and improving endothelium-dependent relaxation, although some concern remains on its fat saturated acid contain.

We could not find studies on central obesity and vitamin E status. However, some studies suggest that body mass index (BMI) and leptin are positively associated with γ-tocopherol. Since there is not a direct association between serum γ-tocopherol level and dietary intake the variation of serum γ-tocopherol might indicate a metabolic balance with other micronutrients such as vitamin D. In fact, the above referred study found that vitamin D status is negatively associated with BMI and leptin . Since in our study severe vitamin D deficiency assessed by serum concentration (< 10ng/ml) affects <15% of elderly population it is possible to speculate that if low grade inflammation affects the Ecuadorian older subjects with MetS, it could also be explained by the role of vitamin D. Even if the new cut-off point for vitamin D deficiency is considered (20 ng/ml) a low grade inflammation would be expected in Ecuadorian older subjects with MetS. Furthermore, it is possible to hypothesize that our population has low serum level of γ-tocopherol.

In the light of the micronutrient deficiencies found in older Ecuadorian people, especially those of vitamins C, E, and zinc, and their potential association with MetS, more research is needed to define causal association. Dietary interventions aimed at improving the micronutrient status are warranted to test the effect on weight gain, central obesity, glycemia, insulin resistant, lipid profile and inflammation in older subjects with MetS. Clearly more studies are required to deepen the knowledge of both molecular mechanisms and clinical outcomes of micronutrient intervention especially vitamins C, D, E, and zinc. Regarding the molecular mechanisms the association with adipogenesis , as well as with inflammatory markers, including gene expression and regulation through mRNAs and microRNAs are warranted. Studies on the interactions of micronutrients at the molecular level are also needed.

SPANISH SUMMARY

Resumen

Esta tesis examina la asociación entre deficiencia de zinc, función inmunitaria e infecciones en niños pre-escolares y en adultos mayores del Ecuador. Además explora la potencial relación entre la deficiencia de zinc y de las vitaminas C y E con el síndrome metabólico (MetS) en la población de tercera edad en el Ecuador. Nuestros estudios científicos publicados en revistas indexadas, realizados en las dos últimas décadas tanto en comunidades como en hospitales son el fundamento de las asociaciones propuestas.

La deficiencia de zinc es de alta prevalencia en el Ecuador y en la región Andina. Las áreas rurales andinas son más afectadas que las urbanas debido al limitado acceso a los alimentos animales ricos en zinc biodisponible. En estas áreas la dieta se basa principalmente en cereales que no proveen suficiente zinc para los requerimientos diarios, lo cual se agrava por la práctica tradicional de consumir cereales no refinados con alto contenido de ácido fítico que interfiere la absorción de zinc y de hierro. Hacen falta políticas públicas orientadas a mejorar la provisión de zinc en la dieta. El zinc debe ser proporcionado diariamente ya que no existe almacenamiento tisular de fácil disponibilidad, lo que complica la instauración de estrategias eficientes como el acceso a alimentos o la administración de suplementos con las cuotas suficientes de este mineral.

En niños ecuatorianos pre-escolares la anemia por deficiencia crónica de hierro ha sido otro problema relevante. Sin embargo, la provisión de suplementos de hierro en los centros de salud ha reducido la prevalencia de anemia, especialmente en las zonas urbanas. Esta estrategia enfrenta problemas de aplicación en áreas rurales debido a la baja adhesión de las poblaciones a los suplementos. Sin embargo, se está logrando progreso sostenido. La competencia entre zinc y hierro por los mecanismos de absorción intestinal podría agravar el estatus de zinc.

Puesto que el zinc se relaciona tanto con la inmunidad innata como con la inmunidad adaptativa, las poblaciones que sufren deficiencia crónica de este mineral son vulnerables a infecciones respiratorias y diarreicas. Actualmente hay suficientes evidencias sobre esta asociación. De hecho, se ha demostrado que la deficiencia de zinc afecta la capacidad bactericida de los macrófagos y la inmunidad adaptativa dependiente de células Th1. Sin embargo, en el año 1992, cuando nosotros empezamos los estudios de este mineral, los mecanismos celulares apenas empezaban a develarse. En el año 1996 publicamos un estudio que evaluó el efecto de suplementos diarios de zinc o placebo en la infección respiratoria y en la hipersensibilidad retardada (DTH) en niños con retraso de talla

para la edad. La hipersensibilidad retardada se evalúa mediante la reacción local a la administración cutánea de un antígeno. El estudio mostró reducción de la incidencia de signos respiratorios y mejoramiento de la respuesta DTH en el grupo que recibió zinc. El beneficio revirtió cuando se suspendió la administración del suplemento. Estos hallazgos fueron una de las evidencias de fundamento original al conocimiento de la relación entre deficiencia de zinc, afectación de la respuesta inmunitaria e infecciones.

Más tarde, participamos en un estudio colaborativo con la Universidad de California, Davis, que demostró que la cuota diaria de 3-7 miligramos de zinc es suficiente para reducir la incidencia de diarrea de manera significativa (en términos estadísticos) en niños con baja talla para la edad. Con esas dosis de zinc no se afectó el estatus de cobre evaluado mediante el examen de una batería de enzimas dependientes de cobre.

En el año 2008 publicamos un estudio realizado en adultos residentes en Quito infectados con *H. pylori,* sobre la asociación entre el zinc de la mucosa gástrica y la severidad de la inflamación local. Bajas concentraciones de zinc en la mucosa se encontraron asociadas con alta infiltración de células mononucleares y polimorfonucleares (PMN).

Recientemente, en otro estudio publicado, encontramos que la administración de suplementos diarios de zinc como adyuvantes del tratamiento estándar de neumonía severa en niños hospitalizados, no disminuyó la duración clínica del episodio neumónico. Sin embargo, los niños que ingresaron al estudio con mejor estatus de zinc previo a la suplementación sí tuvieron reducción significativa de la duración de los signos clínicos de neumonía severa.

Basados en los hallazgos descritos, concluimos que la deficiencia crónica de zinc afecta tanto la inmunidad innata como la inmunidad adaptativa. Creciente evidencia permite comprender los mecanismos asociados con dicha afectación. El zinc es requerido para la maduración óptima de los linfocitos T en el timo y para el apoyo eficiente de estos linfocitos a la capacidad bactericida del macrófago para combatir a los patógenos intracelulares. La deficiencia de zinc, por lo tanto, se asocia con baja producción de citocinas inflamatorias dependientes de células Th1, tales como TNF-α, IL-1 e IL-6. Paradójicamente, la deficiencia de zinc podría estar relacionada con aumentada inflamación como sugiere la infiltración de la mucosa gástrica por células PMN en adultos infectados con *H. pylori* cuando la concentración de zinc es baja en la mucosa.

La teoría prevalente relaciona la deficiencia de zinc con incrementada inflamación, teoría que parece estar en contradicción con nuestros hallazgos citados y con los de

otros autores, por ejemplo con quienes han encontrado incremento de producción de glucocorticoides en niños con deficiencia de zinc. La teoría prevalente emerge de estudios experimentales en macrófagos activados cultivados tratados con iones de zinc. Estos estudios muestran que los iones de zinc se unen a la enzima IKK y, por lo tanto, impiden la activación del factor NF-κB. Sin embargo, otros estudios muestran que proteínas-zinc, tal como la proteína ZBTB20, promueven la activación del factor NF-κB. Más aún, y para hacer más complejo el escenario de la asociación entre zinc e inflamación, las células T reguladoras dependen de la disponibilidad de zinc. En consecuencia, proponemos un efecto dual de la deficiencia de zinc en la inflamación, efecto que dependería de la duración y grado de esta deficiencia.

En la última década hemos estudiado el estado nutricional, inmunológico y de salud de ancianos ecuatorianos que viven en barrios pobres de Quito. Nuestros estudios han sido los primeros en Ecuador orientados a describir el estado de salud de este grupo de población. El primer estudio fue preliminar en 145 sujetos mayores de 65 años de edad. En ese estudio encontramos alta prevalencia de deficiencia de micronutrientes, infecciones respiratorias, baja respuesta DTH, alto ingreso calórico y sobrepeso. Hallazgos que nos condujeron a profundizar el conocimiento de la función inmune, el estado nutricional, la alimentación y la salud metabólica en los adultos mayores.

En un estudio más grande, con mayor potencia estadística, que incluyó 352 adultos mayores, encontramos bajas concentraciones plasmáticas de micronutrientes, especialmente vitamina C y zinc. La concentración plasmática de vitamina C se asoció positivamente con interferón-γ (IFNγ), mientras la concentración plasmática de zinc se asoció positivamente con IFNγ e inter leucina-2 (IL-2). Estas citocinas fueron medidas en el sobrenadante de cultivos de células sanguíneas de adultos mayores. Historia de infección respiratoria se asoció con la deficiencia de cualquier micronutrientes. La prueba DTH demostró que una minoría de sujetos respondió al toxoide tetánico y al Trichophyton, mientras más de la mitad respondió a la tuberculina y a Candida. En suma, la respuesta inmune dependiente de células Th-1 parece estar afectada por las deficiencias de micronutrientes más prevalentes en esta población de adultos mayores. Estas deficiencias agravarían la afectada respuesta inmune asociada al envejecimiento (inmune senescencia).

El síndrome metabólico (MetS) fue altamente prevalente en esta población de adultos mayores del Ecuador: 40%, más en mujeres (81%,), que en varones (19%), definido conforme a los criterios de la Federación Internacional de Diabetes (IDF). Las concentraciones plasmáticas de vitaminas C y E se asociaron inversamente con MetS. La proteína C reactiva (CRP) se asoció positivamente con MetS. Sorprendentemente, la concentración plasmática

de zinc no se asoció con MetS. Este falta de asociación entre zinc y MetS fue inesperada dado el rol anti-oxidante de este mineral a través de la enzima superóxido dismutasa, dependiente de zinc. Este hallazgo podría depender de la moderada deficiencia de zinc de estos sujetos como sugiere su concentración promedio de zinc y el desvío estándar. Lamentablemente, nosotros no evaluamos la correlación entre zinc plasmático y CRP que nos habría proporcionado una clave sobre el potencial efecto dual de la deficiencia de zinc en la inflamación, como hemos sugerido en líneas anteriores.

La prevalencia de deficiencia de vitamina C fue alta en nuestra población de adultos mayores (60% y 33%, para hombres y mujeres, respectivamente). La vitamina C estuvo negativamente asociada con MetS. Este hallazgo es consistente con el rol anti-oxidante de esta vitamina. La vitamina C se asocia con baja adipogénesis según algunos estudios, aunque otros han encontrado el efecto opuesto. Estos resultados conflictivos podrían estar relacionados con las dosis de vitamina C utilizadas en los diferentes estudios o con el tipo de células utilizado. Sin embargo, la vitamina C se ha encontrado asociada con menor inflamación en sujetos obesos.

La prevalencia de deficiencia de vitamina E fue baja en la población de adultos mayores que estudiamos en Quito (<15%). Sin embargo, encontramos una relación inversa entre la razón de vitamina E:TAG y MetS (OR=0.78, 95% CI 0.71, 0.86). Estudios recientes sugieren que el γ-tocotrienol y el α-tocopherol inhiben la activación del factor NK-κB. Por lo tanto, uno puede especular que individuos adultos mayores de Ecuador con MetS sufren de inflamación moderada gracias a la regular ingesta dietética de diferentes isoformas de vitamina E. De hecho, en Ecuador hay extenso consumo de aceite de palma que contiene seis isoformas de vitamina E. Precisamente, estudios en aorta de ratas han mostrado el efecto beneficioso de extractos de aceite de palma en la reducción del estrés oxidativo y en el mejoramiento de la relajación vascular dependiente del endotelio, aunque hay controversia por su contenido de ácidos grasos saturados.

No pudimos encontrar estudios sobre obesidad central y el estatus de vitamina E. Sin embargo, algunos estudios sugieren que el índice de masa corporal (IBM) y la concentración plasmática de leptina, un marcador de inflamación en MetS, se asocian positivamente con la concentración plasmática de γ-tocopherol. Puesto que no hay asociación directa entre el γ-tocopherol plasmático y el ingreso dietético, las variaciones plasmáticas podrían indicar un balance metabólico con otros micronutrientes, como la vitamina D. Precisamente, los estudios mencionados encontraron que la vitamina D se asocia negativamente con leptina e IBM. Puesto que en nuestro estudio la deficiencia severa de vitamina D (10 ng/ml) afecta < 15% de la población de adultos mayores es posible especular que inflamación moderada

afecta a la población ecuatoriana de adultos mayores con MetS, debido al rol de la vitamina D. Inclusive, si aplicamos el nuevo punto de corte para deficiencia de vitamina D (20 ng/ml), los adultos mayores de Quito con MetS tendrían moderada inflamación. Más aún, es posible proponer como hipótesis que nuestra población tiene bajas concentraciones plasmáticas de γ-tocopherol.

A la luz de las deficiencias de micronutrientes encontradas en nuestros estudios en adultos mayores que residen en Quito, especialmente las deficiencias de vitaminas C, E y zinc, así como su potencial asociación con MetS, más investigación es necesaria para definir asociaciones causales. Son de alta prioridad las intervenciones en la dieta para mejorar el estatus de micronutrientes y examinar su efecto en la ganancia de peso, la obesidad central, la glicemia en ayunas, la resistencia a la insulina, el perfil sérico de lípidos y la inflamación en individuos adultos mayores con MetS. Claramente, se necesitan más estudios para profundizar el conocimiento de los mecanismos moleculares y de los efectos clínicos de intervenciones con micronutrientes, especialmente vitaminas C, D, E y zinc. En relación con los mecanismos moleculares son prioritarios los estudios para evaluar los efectos de la intervención en la adipogénesis, en marcadores de inflamación y en la regulación de la expresión genética a través de micro RNAs (mRNAs). También son cruciales los estudios sobre interacciones moleculares de los micronutrientes.

ACKNOWLEDGEMENTS

Special recognition is due to:

Prof. Moselio Schaechter, former director of Microbiology and Molecular Biology at Tufts University, for his mentorship and encouragement to continue my research career over almost the last three decades.

I am grateful to:

Prof. Eric J.G. Sijbrands, of Internal Medicine of Erasmus Medical Center Rotterdam for extra reading the manuscript for suitability of thesis defense.

Prof. Gerald T. Keusch, former director of both the Geographic Medicine Division at Tufts University, and the Fogarty International Center, for entrusting me the initial grants.

My colleagues at Tufts University, professors Jefffrey K. Griffiths, Davidson H. Hamer, Simin N. Meydani, Elena Naumova, for enrolling me as adjunct professor, and for their continued collaboration over the last two decades.

Prof. Hemmo A. Drexhage, of Erasmus Medical Center, University Medical Center Rotterdam, for trusting me as well as his support and guidance to my thesis.

Prof. Kenneth Brown, of University of California, Davis, and his former student, Sara Wuehler, for their collaboration in some research studies carried out jointly in Ecuador.

Dr. Plutarco Naranjo, former Minister of Public Health of Ecuador, for his support to my initial studies on zinc.

Dr. Manuel E. Patarroyo, former Director of Institute of Immunology at Universidad Nacional de Colombia, for entrusting me the evaluation of his malaria vaccine.

Dr. Laura Arcos Terán, former Director of the Ethical Committee of Corporacion Ecuatoriana de Biotecnologia.

Special gratitude is due to:

The members of my Ecuadorian team of Central University of Ecuador and of the Corporacion Ecuatoriana de Biotecnología: Bertha Estrella, Josefina Egas, Mercy Sempertegui, Alicia Rodriguez, Marcia Racines, Ximena Narvaez, Oswaldo Rodriguez-Mora, Lucy Baldeon.

Collaborators of some hospitals: : Myriam Diaz, Ricardo Mejia, Carlos Guarderas, Edgar Renteria, Juan Egas, Lenis Ortiz, Cecilia Cruz, Miriam Cabezas, Dinor Gomez, Gilda Salgado, Monica Paladines, Martha Almeida, Guadalupe Segura.

Children and elderly populations of Quito Urban Communities and of some rural areas who permited us to study their health status.

CURRICULUM VITAE

Dr. Sempértegui got his Medical Doctor degree at the Faculty of Medical Sciences of the Central University of Ecuador in Quito. He got a postdoctoral training on Molecular Biology and Microbiology at Tufts University in Boston. Furthermore, he attended the one year training on Pediatrics at Postgraduate Institute of the Faculty of Medical Sciences of Central University of Ecuador. He has a diploma on International Relations obtained at Latin-American Faculty of Social Sciences in Quito.

Dr. Sempértegui is professor of Immunology at the Medical School of Central University of Ecuador where he was a former Dean. Currently he is Rector (President) of this university. He is also Adjunct Professor at Tufts University in Boston.

Dr. Sempértegui has published 28 papers in peer reviewed journals and presented 16 Abstracts in international scientific meetings. He started his scientific career on 1982 with a study on intrauterine growth in Quito. Later, he was principal investigator of the clinical trial carried out in Ecuador to evaluate a malaria vaccine developed at the Institute of Immunology of Bogota, Colombia, under the leadership of Dr. Manuel Patarroyo. After these initial experiences, Dr. Sempértegui concentrated his research career on the association between micronutrients, mainly zinc and vitamin A, with immune function and infection in children. During the last ten years he has focused his research work on the nutritional status, immunity and metabolic syndrome in elderly Ecuadorians who live in Quito. In fact, his studies in elderly have been the first in Ecuador. Recently he has completed some studies on the effect of urban air pollution on health.

Dr. Sempértegui has got grants from the World Health Organization (WHO), the Pan American Health Organization (PAHO), the United Nations Children´s Fund (UNICEF), the US National Institutes of Health (NIH) through a collaborative agreement with Tufts University, the Applied Diarrheal Disease Research Project (ADDR) of the Harvard Institute for International Development, the Applied Research on Child Health Program (ARCH Program) of Boston University, the Fogarty International Center through a collaborative agreement with the Jean Mayer Human Nutritional Research Center on Aging (JM-HNRC) of Tufts University, the University of California at Davis, the Thrasher Foundation.

Dr. Sempértegui has received awards from the Central University of Ecuador, the Ecuadorian Academy of Medicine, the Municipality of Quito. Special recognition was awarded to him by the Department of Family Medicine at Tufts University, for his research contributions on the Epidemiology of Malnutrition.

Dr. Sempértegui has been peer reviewer of five international journals and been invited as commentator of the International Journal of Epidemiology. He has authored and co-authored 10 books published locally on medical science and health policy. He has been invited speaker in some different scientific meetings in Brazil, Colombia, El Salvador, United States, Guatemala, Peru, and Venezuela.

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