The Effect of Retinol Supplement on Blood Cytokine Concentrations in Children with Non-Severe Malaria Vivax

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1. Introduction

Malaria, malnutrition, low concentrations of retinol and intestinal parasitism coexist among the habitants of tropical regions of the world (Nacher, 2002). The Turbo municipality is one of the highly endemic malaria regions of Colombia. During 2006, 5,674 cases of malaria were reported in Turbo, corresponding to annual parasite index >10 (number of malaria cases per 1,000 persons per year). From these, 85% were caused by *Plasmodium vivax* (Eventos de interes en salud Pública, 2006). Also, the Urabá region, where Turbo is a major urban area, is one of the regions of Colombia with more cases of malnutrition in children under 15 years; 53.3% of children under 10 years presented chronic malnutrition risk ($T/E < -1$ Unit Z) and 14.9% acute malnutrition ($P/T < -1$ Unit Z), whereas 33.8% of the adolescents had weight deficit according to the Body Mass Index (Alvarez et al., 2005). Furthermore, 85% of children aged 4 to 10 years with malaria had intestinal parasitism (Carmona et al., 2009).

Previous studies in Colombian children with malaria reported low retinol values during the acute phase, which recovered to normal values > 0.7 mmol/l (20 $\mu\text{g/dL}$) (WHO, 2009) once malaria receded. Within the children with malaria, 85% had anemia, and their haemoglobin values increased after one month of antimalarial treatment, although anemia persisted in 51% of them (Uscátegui & Correa, 2007).

During malaria, TH1 cytokines like interferon gamma ($\text{IFN} - \gamma$) and tumor necrosis factor alpha ($\text{TNF} - \alpha$), are required to control the primary parasitemia. Nevertheless antiinflammatory cytokines or TH2 cytokines, such as interleukin 10 (IL-10) and transforming growth factor beta ($\text{TGF} - \beta$), that modulate the proinflammatory effect, must be present along with those of the TH1 type, in order to prevent emergence of immune pathology (Schofield et al., 2005). Some in vitro studies revealed that retinol had an effect on the TH1/TH2 balance, as evidenced by reduction of $\text{IFN} - \gamma$ and $\text{TNF} - \alpha$ secretion by TH1 cells or by promoting TH2 cells growth and differentiation to produce larger quantities of the IL-10 (Cantorna et al., 1994; Iwata et al., 2003). Vitamin A deficiency has been associated with an increase in TH1 response, intestinal parasitism and malnutrition (Jason et al., 2002; Azevedo et al., 2005). Furthermore, the prevalence of TH1 cytokines in children with malaria has been associated with severe anemia (Kurtzhals et al., 1999).

1.1 Anemia and relationship with cytokines TH1 and TH2, in patients with malaria

Ferritin deficiency is defined basically as the reduction of iron in the body and its diagnosis when not associated with anemia is based on quantification of serum ferritin. However, determinations of serum levels of this protein, which are performed systematically to determine the iron status, ferritin acts as a positive acute phase reactant in the presence of inflammatory/infectious disease clinics and subclinics (Aleo et al., 2004) as happens during malaria.

This explains why this protein is not useful tool for evaluating iron stores, and in contrast, constitutes a good indicator of inflammatory status along with the C reactive protein (CRP). CRP is produced by the liver, is also known as a positive acute phase reactant during malaria and main function is to join the organism, acting as an opsonin, with activation of the classical complement pathway, which is responsible for recruitment of inflammatory cells, opsonization and dead direct of the pathogen (Marsh & Kinyanjui, 2006).

Unlike ferritin, hemoglobin is not considered a reactant acute phase and the low concentrations of blood, result in anemia, which is a public health problem in many regions around the world with a high prevalence in economically dependent countries, especially among children and resulting from the interaction between biological, nutritional and cultural factors (Blair et al., 1999). The Anemia is a common complication of malaria and the mechanisms originally involved have not yet been fully defined. The cause is multifactorial and includes aspects related to the increase destruction of parasitized and non-parasitized cells and other factors causing a decreased production of erythrocytes, by alteration in the maturation of erythroid precursors or lack on response of bone marrow to erythropoietin (EPO). Additionally, there are others conditioning agents of anemia related with the characteristics of the parasite and host as resistance to *Plasmodium* or some disease in the host as well as thalassemia or sickle cell anemia, which enhances the severity of anemia, as well as deficiencies of iron and other micronutrients (Llanos et al., 2004).

In adults living in Kenya with acute malaria by *P. falciparum* found that TNF- α , interleukin 1 (IL-1) and IL-6, cytokines produced by monocytes, suppresses the synthesis of erythropoietin (Vedovato et al., 1999). Similar results were seen in children in Ghana (Kurtzhals et al., 1999). In Uganda, children 1 to 10 years who had acute uncomplicated malaria by *P. falciparum*, the authors found that age, high concentrations of erythropoietin, low concentrations of α -1 glycoprotein, and IL-10/TNF- α high proportion were associated with significantly increased hemoglobin concentrations. These data indicate that children younger with malaria do not maintain the production of IL-10 in response to inflammatory process, a mechanism that may contribute to the severity of the anemia (Nussenblatt et al., 2001). A study in Kenya in children with malaria revealed that the TNF- α and IL-10 were significantly higher in those subjects with high parasitemia and anemia, compared with control group, the same age and sex, but without malaria (Othoro et al., 1999). In children Colombians living in El Bagre (Colombia), aged from 4 to 9 years old who had acute uncomplicated malaria, 67% *P. vivax*, 29% *P. falciparum* and 4% mixed infection was found average of IL-10 of 266.18 ± 47.9 pg/ml, highly significant and higher than in children with the same age, but not malaria, which was 8.52 ± 1.17 pg/ml ($P < 0.001$); the values of IL-10 in children with malaria correlated with parasitaemia and body temperature. Conversely, TNF- α was only detected in 12% of study subjects, no significant differences between average children malaria and those without the disease. In children with moderate or high parasitemia but not anemia, the proportion was IL-10/TNF- α significantly higher compared with those who did have anemia, indicating that high values of this proportion can prevent

development of anemia with control of excessive inflammatory activity TNF- α (Grencis et al., 1996). The evidence presented shows that high values in the proportion of TH2/TH1 cytokines (IL-10/TNF- α) protect to development severe anemia malaria in children (17). It is proposed that the pathways by which IL-10 exerts its beneficial effects on malaria could be: 1) activating cytotoxic T lymphocytes, with the elimination of cells infected, 2) stimulating the production of antibodies directed against the parasite and 3) inhibiting or blocking the production of cytokines proinflammatory response characteristics TH1 (Blair et al., 1999). According to the studies presented is clear that, in children with acute malaria, the severity of the anemia is determined by the balance in the production of proinflammatory cytokines such as anti-and IL-10 and TNF- α respectively, which therefore are related to the change in hematological and contribute or not to increase hemoglobin and erythropoietin. Although there is no information linking the paper simultaneously TH1/TH2 response modulator by supplements of vitamin A, with hematological values in children with malaria, there may be this interrelationship, it is clear that supplementation with vitamin A improve hemoglobin levels and erythropoietin and favor mobilization of iron deposits, and also is known its immunomodulatory role, as evidenced by the reduction of proinflammatory cytokines, which are also associated with the severity of anemia, and present during acute malaria. For this reason, further research is required to clarify the relationship between the simultaneous retinol, the immune response and iron metabolism in the population children with acute malaria, especially that produced by *P. vivax* that is more prevalent in Colombia.

2. Materials and methods

2.1 Study type and sample

A Pilot study balanced, nonblind, with random allocation of the "exposure factor" (retinol supplement) was carried out. Two groups, each of 25 children with nonsevere *P. vivax* malaria were compared according to WHO criteria (Lopez & Schmunis, 1998) and matched according the sex, age and place of residence. One of the group received retinol supplements (200.000 U.I. retinol palmitate, Retiblan®, Procaps laboratories, Colombia), for one year every 3 months, the final dose was administered between one week and six months before the *P. vivax* episode. The other group did not receive supplement. The "final effect" in each child was the cytokines levels, and nutritional, biochemical and inflammatory indicators.

This project was approved by the committee of ethics of the Centro de Investigaciones Médicas de la Facultad de Medicina, Universidad de Antioquia. A written informed consent was obtained from each patient. The resolution N° 008430 of 1993 of the Ministerio de Salud de Colombia was considered.

2.2 Inclusion criteria

It required the following requirements: a) Reside on a regular basis in El Tres, Antioquia, Colombia b) Have not chronic (diabetes) or infectious disease (such as tuberculosis or leprosy) at the time of admission, c) Be free from trauma, accident or poisoning, known and judged as serious by the medical examination; d) A number of 25 children must have participated in research in which they received supplementation with retinol and 25 should not have received such a supplement; e) Have uncomplicated malaria by *P. vivax*; f) Agreeing to participate in the study by signing for his guardian, written informed consent.

2.3 Exclusion criteria

Participants were excluded if: a) Occurre done of those events in subparagraphs b) and c) inclusion criteria; b) Withdrawal of informed consent or for any reason of the study.

2.4 Diagnosis and treatment of malaria

The parasitological diagnosis of malaria was carried out as recommended by PAHO/WHO (López & Schmunis, 1988) with respect to sampling, processing and reading it. Antimalarial treatment was carried out orally, according to the schemes of the Ministry of Health of Colombia and the Regional Health Direction of Antioquia (drug and dose by age), the drugs are accompanied with water and food as well:

- a. Chloroquine: total dose of 25 mg/kg body weight, which is split into three days: day 1 was given 10 mg/kg, days 2 and 3 are supplied 7.5 mg/kg in each.
- b. Primaquine at 0.25 mg/kg/day for 14 days, given from day 4 (after completion of chloroquine).

The treatments were obtained in the DSSA-Ministry of Health and were delivered as monitored by the researchers observing the patient during the first half hour, in case of vomiting; the full dose was repeated, with new supervision for 30 minutes. If the patient vomited again, it was excluded from the study and transferred to the municipal hospital.

2.5 Anthropometric evaluation

Weight was measured on the children in an standing position, few clothing and without shoes; with an electronic scale of 100 kg capacity and 0.01 kg sensitivity. Height was measured with a flexible estadiometer fixed on the wall, of 2 m capacity and 1 mm sensitivity. Each measurement was evaluated and registered. The mean reading was recorded. The age was calculated as the difference between date of birth and date of evaluation. The height for age index (T/E) was constructed and those who had values <-1 of Z unit were classified as with chronic malnutrition risk and those with equal or greater values to -1 of Z unit as without risk. The population of the National Center of Statistics of Health of the United States (NCHS) was used as reference as accepted by the WHO for international comparisons (WHO, 1995).

Since reference values for weight and height are not available for men higher than 145 cm and children higher than 137 cm, it was not possible to evaluate acute malnutrition with the indicator P/T, hence Body Mass Index (BMI, $\text{weight} / \text{height}^2$) was used, which is accepted by the WHO for evaluation of children up to 15 years, using as reference values the proposed by the same organization. Those children below percentile 15 ($p < 15$) were classified as low weight.

2.6 Laboratory examinations

The procedure followed for each of the laboratory tests was as follows:

2.6.1 Testing for malaria

The parasitological diagnosis of malaria to detect the presence of *Plasmodium* parasites by thick film was confirmed in the extended, in the manner provided by the OMS (López & Schmunis, 1988). The spread thin and thick were stained with Field and Giemsa, respectively. The thick smear was observed with 100X magnification and the search for parasites was done in 200 consecutive microscopic fields. The parasitemia was calculated based on 200 leukocytes and a

standard of 8,000 cells /mL and expressed in rings/ μ L. A thick smear was diagnosed as negative when there was no asexually in 200 microscopic fields.

2.6.2 Stool

The stool test was conducted on one single sample, once the patient was admitted to the study.

We established the presence of helminths, the eggs were quantified and trophozoites and cysts of protozoa were identified. To do this we proceeded as follows: 3 g of feces was added formaldehyde 10% to cover the sample, which was stored in 4-7 days, as was reviewed, consisting of "direct examination" and, if the parasites were not passed to "concentration examination". Treatment with 10% formalin well preserved helminths eggs and protozoan cysts. Direct stool examination with saline-iodine and examination by formalin-ether concentration as Ritchie were made according to the usual procedure only when the second evaluation was negative as declared such to the sample (Botero & Restrepo, 2003). Stool analysis was performed by professional staff of the Laboratory of Intestinal Parasites of the Faculty of Medicine of the University of Antioquia.

2.6.3 Determination of biochemical parameters

2.6.3.1 C-reactive protein

Serum was measured by a kit BioSystems (CRP) Latex. C-reactive protein serum causes agglutination of latex particles coated with anti-human C-reactive protein. The agglutination of latex particles is proportional to the concentration of CRP and can be measured by turbidimetry (Rice et al., 1987). Inflammation was considered when the concentration of CRP was 8 mg/L or higher, recommended by the Clinical Laboratory of the IPS at the University of Antioquia, where they processed these samples.

2.6.3.2 Ferritin

The ferritin was measured in serum with Abbott AxSYM kit ® Sistem (reference 7A58-20B7A583 56-4324/R12, Abbott Laboratories, USA). The AxSYM Ferritin assay was based on microparticle enzyme immunoassay technology (MEIA). The determinations were made in Clinical Laboratory of the IPS at the University of Antioquia.

2.6.3.3 Plasma retinol

Chemical analysis was done by affinity high performance liquid chromatography (HPLC) (Talwar et al., 1998), with a team scores Waters, using a manual injection system Reodyne 77251, a solvent delivery system 660E, a UV-VIS detector 400 and a spine C-18 Simetry, using the Millennium software for management. 0.5 mL of plasma were measured and denatured with 0.5 mL of absolute ethanol with BHT (1.0 g/L) as antioxidant and 0.5 mg/mL of ethyl- β -apo-8-carotenoato and 0.5 mg/mL retinol acetate as internal standards.

The mixture was extracted five times with 5 mL of hexane.

The separated hexane was evaporated with N₂ and the residue was reconstituted with 200 mL of methanol, an aliquot of 50 mL was injected into the HPLC system, the mobile phase used was methanol, acetonitrile in a proportion 70: 30. There were three chromatograms for each sample to evaluate. It was considered subclinical deficiency of vitamin A, when plasma levels were <20 μ g/dL. Measurements were made at the Laboratory of the National Institute of Health in Bogotá.

2.6.4 Hemoleucogram (cyanmethaemoglobin (g/dL) and Leucogram)

Hemoleucogram became type III-V in automated equipment in the clinical laboratory of hospital Francisco Valderrama of Turbo, Antioquia, Colombia, when it was not possible, was performed manually. The method used was that of the cyanmethaemoglobin recommended by WHO. The reference values for classifying anemia were those recommended by WHO as the concentration of hemoglobin (Hb) as follows: for children 2-4 years <11.0g/dL and the 5 years and older <12 g/dL (WHO, 2001).

Hemoglobin values were not corrected for altitude because Turbo is located less than 200 meters above sea level.

2.6.5 Determination of serum cytokines

The cytokines IFN γ , TNF α , IL-10 and TGF- β 1 were determined by sandwich ELISA using the Duo Set kit developed by ELISA (R&D systems), the basis and procedure are described below:

2.6.5.1 Fundament

This assay used an ELISA (Enzyme Linked Immuno sorbent Assay) quantitative sandwich, which was based on the detection of cytokines that bind to an immobilized antibody (capture antibody) on a solid phase antibody which directly or indirectly produced a reaction whose product could be measured by spectrophotometry.

2.6.5.2 Reagents and samples required

Were used: a) Polystyrene microplates with 96 wells previously sensitized or attached to a polyclonal antibody capture (From mouse)-specific cytokine (IL-10, IFN- γ , TNF- α and TGF- β 1). b) Standard, 1 vial of lyophilized cytokine (IL-10, IFN- γ , TNF- α and TGF- β 1) recombinant in a buffered protein base. c) Sample from human serum (approximately aliquotes of 600 μ L) and stored in liquid nitrogen or at -70° C. d) 21 mL of concentrated buffer solution mixed with condoms (wash buffer solution). e) Conjugate is 21 mL of polyclonal antibody specific for each cytokine detection coupled with horseradish peroxidase with preservatives. f) Substrate solution de12.5 made mL stabilized hydrogen peroxide (reagent color A) and 12.5 mL of stabilized chromogen (color reagent B). g) 6 mL of 2 N sulfuric acid (stop solution) h) Diluent of standard and diluents of sample RD1-51.

2.6.5.3 Procedure

In the commercial kits used in this procedure microplates were previously coated with a capture antibody polyclonal-specific cytokine (IL-10, IFN- γ , TNF- α and TGF- β 1). The standards and samples were added to the wells contained each plate (96 wells per plate) in the amount stated and corresponding cytokine (contained in the standard and samples) are joined to the capture antibody. After the corresponding washes to remove nonspecific binding, was added the respective conjugate (polyclonal antibody specific for each cytokine, together with an enzyme). Subsequently was it washed to remove nonspecific binding to conjugate. Substrate solution was added to the plates, and development color (corresponding to the product) was proportional to the amount of cytokine bound in the initial step of the procedure. After time estimated to measure the color reaction, the development of this stopped with stop solution and the color intensity was measured by a spectrophotometer at the wavelength indicated.

2.7 Statistical analysis

For variables exhibiting a normal distribution, the mean values between the groups were compared using the T test for matched groups. For variables lacking a normal distribution, median values were compared using the Mann Whitney test. To explore intragroup relations was applied spearman correlation coefficient. The comparison between the groups of the categorical variables was made using Chi square. The programs Prism, Epi info version 6.4D and SPSS version 15.0 were used and unilateral values of $P < 0.05$ were set up as significative.

3. Results

In total 17 boys and 8 girls in each treatment group were included, they were aged 2.8 -15.7 years old in the supplemented group, and 3.2 - 15.7 years old in the non-supplemented group. The weight, height and parasitemia values were similar in both groups (Table 1).

Variable	Group with retinol	Group without retinol	p
Weight in kg (X±SD)	28±10	30±13	0.621 ^a
Height in cm (X±SD)	127±20	131±20	0.567 ^a
Parasitemia (P/μl)	5557±4350	5830±3901	0.574 ^a
With chronic under nutrition risk (T/E) (yes/no)	17/8	15/10	0.384 ^b
Low weight for BMI (yes/no)	6/19	4/21	0.363 ^b
Coprologic positive (yes/no)	19/4	20/4	0.625 ^b
Helminths (yes/no)	10/13	17/7	0.054 ^b
Protozoa (yes/no)	17/6	17/7	0.536 ^b

X= average, SD= Standard deviation, P/μl= parasites/microlitre, T/E= indicator height for age, BMI= Body Mass Index

^a U de Mann-Whitney test $p < 0.05$.

^b Chi square $p < 0.05$.

Table 1. Characteristics of the children according to treatment group.

All children were tested for parasitaemia, ferritin, retinol and C reactive protein, 40 of them were tested for cytokines and 47 for haemoglobin and stool tests.

The risk of chronic malnutrition (T/E) in the group with retinol supplement was 68% in contrast to 60% in the non-supplemented group. Prevalence of low weight (IMC) was 24% in the supplemented children versus 16% in the non-supplemented.

Prevalence of intestinal parasites in children was high in both groups; overall 83% of the children had a positive stool test. The group supplemented with retinol exhibited infection in 74% and 43% with protozoa and helminths, respectively; while 71% of the non-supplemented group had protozoa and helminths (Table 1). There was no significant

difference in the presence of intestinal parasitism among the groups. With exception of the haemoglobin and retinol, the remaining variables did not exhibit a normal distribution. The cytokines, C reactive protein, ferritin, haemoglobin and retinol, were similar among the groups and only the C reactive protein and haemoglobin values showed significance with the lower concentrations. The TNF- α median values were zero in the non-supplemented group, which means that at least in 50% of the children, this cytokine was not detected (Table 2). For IL-10, the highest concentration for both groups was 677pg/ml, and only 25% of the children from the group supplemented with retinol and 15% of the children from the non-supplemented group reached that value. With the technique used, no TGF- β 1 values were detected in any sample tested.

The frequency of inflammation, anemia and subclinic deficiency of vitamin A was similar in both groups (Table 3).

Variable	With retinol			Without retinol			P
	n	X $\pm$ SD	Median	n	X $\pm$ SD	Median	
IL-10 (pg/ml)	20	275 $\pm$ 283	112	20	233 $\pm$ 253	125	0.989
TNF- α (pg/ml)	20	32.2 $\pm$ 66.3	5.5	20	16.2 $\pm$ 49.2	0.0	0.162
IFN- γ (pg/ml)	20	49.1 $\pm$ 60.2	29.3	20	68.5 $\pm$ 80.3	31.1	0.473
C reactive protein (mg/l)	25	29 $\pm$ 25	24	25	48 $\pm$ 39	36	0.070
Haemoglobin (g/dl)	23	10.5 $\pm$ 1.5	10.3	24	11.2 $\pm$ 1.8	11.1	0.054
Ferritin (μ g/l)	25	143 $\pm$ 191	105	25	154 $\pm$ 108	113	0.160
Retinol (mmol/l)	25	0.59 $\pm$ 0.06	0.57	25	0.61 $\pm$ 0.08	0.59	0.786

IL-10=interleukyne 10, TNF- α =tumor necrosis factor alpha, IFN- γ =interferon gamma, SD= Standard desviation.

U de Mann-Whitney test, except haemoglobin and retinol that one became for T pared test $p < 0.05$.

Table 2. Comparison of the concentrations of cytokines and nutritional biochemical indicators in children according to treatment group.

Category	With retinol	Without retinol	p
Inflamation (yes/no)	21/4	23/2	0,334
Anemia (yes/no)	22/1	19/5	0,104
Subclinic deficiency of vitamin A	23/2	23/2	1

Inflamation = PCR values ≥ 8 mg/L, Subclinic deficiency of vitamin A = retinol values < 20 μ g/dL, anemia= haemoglobin in children 2-4 years old < 11 g/dL and in children ≥ 5 years old < 12 g/dL Chi square $p < 0.05$.

Table 3. Comparison frequency of Inflamation, anemia and subclinic deficiency of vitamin A according to treatment group

3.1 Stratification by nutritional state and presence of intestinal parasitism

Because intestinal parasitosis and malnutrition affect the variables of our interest, results were analyzed according to: 1) absence of malnutrition risk and parasites, 2) at malnutrition risk and without parasites, 3) absence of malnutrition risk and presence of parasites and 4) malnutrition risk and presence of parasites. From these groups, only the group 4 was adequate for statistical analysis and this included, 13 children in the retinol supplemented group and 14 in the group without retinol supplement.

Subjects with T/E <-1 Unit Z o BMI $p < 15$ were classified as chronic malnutrition risk or with low weight. Since all children who had chronic malnutrition risk simultaneously presented low weight, the number of children with chronic malnutrition risk or low weight, was identical to that of children with chronic malnutrition risk.

In these children, the concentrations of TNF- α , IFN- γ and IL-10 were similar between both groups. Nevertheless, the group that received retinol exhibited a tendency to have lower values of ferritin and C reactive protein ($p=0.058$ vs 0.089) (Table 4). The parasite blood count was 6.111 ± 3.801 P/ μ l in the group receiving retinol versus 7.160 ± 4.046 P/ μ l in the other ($p=0.332$).

Variable	With retinol			Without retinol			P
	n	X $\pm$ SD	Median	n	X $\pm$ SD	Median	
IL-10 (pg/ml)	13	283 $\pm$ 289	112	8	186 $\pm$ 219	119	0.827
TNF- α (pg/ml)	13	43.7 $\pm$ 82.7	0.0	8	31.9 $\pm$ 74.3	0.5	0.876
IFN- γ (pg/ml)	13	34.5 $\pm$ 25.1	24.5	8	85.4 $\pm$ 79.7	45.0	0.218
C reactive protein (mg/l)	14	30 $\pm$ 25	25	13	47 $\pm$ 31	38	0.089
Haemoglobin (g/dl)	12	10.5 $\pm$ 1.1	10.7	13	11.6 $\pm$ 2.2	11.5	0.120
Ferritin (μ g/l)	14	117 $\pm$ 72	110	13	184 $\pm$ 127	145	0.058
Retinol (mmol/l)	14	0.59 $\pm$ 0.07	0.56	13	0.61 $\pm$ 0.09	0.59	0.698

X= average, SD= Standard desviation, IL-10=interleukyne 10, TNF- α = tumoral necrosis factor alpha, IFN- γ =interferon gamma.

U de Mann-Whitney test $p < 0.05$.

Table 4. Nutritional comparison of the concentrations of cytokines and biochemical indicators in the stratum of children with chronic malnutrition risk and parasites, according to treatment group.

Among children with chronic malnutrition risk and presence of parasites, no differences were observed when the proportions of inflammation, anemia and subclinical deficiency of retinol were compared, regardless of the group (Table 5). Inflammation was detected in 13 out of 14 children from the group administered retinol versus 13 out of 13 children from the group without retinol. Anemia was detected in 11 out of 12 children from the group that receiving retinol and in 9 out of 13 children from the group without retinol. Finally, 12 out of

14 children supplemented with retinol had subclinical deficiency of retinol, while this was evident in 12 out of 13 from the group without retinol.

Category	With retinol	Without retinol	p
Inflammation (yes/no)	13/1	13/0	0,519
Anemia (yes/no)	11/1	9/4	0,186
Subclinic deficiency of vitamin A	12/2	12/1	0,529

Inflammation = values of PCR ≥ 8 mg/L, Subclinic deficiency of vitamin A = values of retinol <20 $\mu\text{g/dL}$, anemia= haemoglobin in children of 4 years old $<11,0$ g/dL and children of 5–10 years old <12 g/dL U Mann-Whitney test, except for hemoglobin and retinol was made by paired t test, $p < 0.05$.

Table 5. Comparison of intensity of inflammation, anemia and deficiency subclinical vitamin A, in the stratum of chronically malnourished children parasites, according to treatment group.

The immunological and biochemical variables studied, which showed correlation among themselves and with parasitemia in one of the two groups of treatment are shown in Table 6. In the group with retinol it is noted that as the parasitemia increased the values of IL-10 and TNF- α were also increased and this was not observed in children of group without retinol. Similarly, in the group receiving retinol, when ferritin increased so did CRP, IL-10, TNF- α and IFN- γ , variables with ferritin which showed positive correlation behavior was not observed in the group without retinol, in which only one ferritin correlated with parasitemia.

The variables that are similarly associated in both groups were IL-10 and TNF- α , which correlated positively with each other and moreover, were those that showed the highest ratios of all the correlations shown in Table 7, with a Rho = 0.786 in group with retinol and 0.751, which received no retinol. This indicated that as it raised one of the two variables, so did the other with the same strength, regardless of having received or not retinol. This same behavior was observed in the supplemented group, between IL-10 and IFN- γ . In the group without supplement there was no correlation of hemoglobin with IFN- γ , which, though unexpected, was one of the highest in this group (Rho = 0.738) (Table 6).

4. Discussion and conclusion

This pilot study answered the need to obtain primary data on the effect of retinol supplements of, on blood concentrations of IL-10, TNF- α , IFN- γ , TGF- $\beta 1$, C reactive protein, haemoglobin and ferritin, in Colombian children with vivax malaria; as well as the relationships between these variables; aspects rarely addressed.

The prevalence of chronic malnutrition risk (T/E < -1 unit Z) was high (64%), in children with malaria from 6 to 10 years of the same municipality (58.2%) (Uscátegui & Correa, 2007). Similarly, the proportion of children with low weight according to BMI (20%) was higher than in other studies of the region (Alvarez et al., 2005). A common finding within malaria endemic areas is the presence of intestinal parasitism (Nacher, 2002); 83% of our children had a positive stool test, 57.4% with helminths and 73.3% with protozoa, which is similar to previous reports in malaria infected children from the same region (Turbo) (Uscátegui et al., 2008).

Pairs of variables	Measures (1)	With retinol	Without retinol
Parasitemia (P/ μ L) -IL-10 (pg/mL)	r	0,574	0,643
	p	0,040	0,086
Parasitemia (P/ μ L)-TNF- α (pg/mL)	r	0,693	0,621
	p	0,009	0,100
Parasitemia (P/ μ L)-Ferritin (μ g/L)	r	0,330	0,731
	p	0,108	0,005
Haemoglobin (g/dL)-IFN- γ (pg/mL)	r	-0,013	0,738
	p	0,954	0,037
Ferritin (μ g/L)- CRP (mg/L)	r	0,574	0,330
	p	0,032	0,271
Ferritin (μ g/L)-IL-10 (pg/mL)	r	0,696	0,381
	p	0,008	0,108
Ferritin (μ g/L)-TNF- α (pg/mL)	r	0,575	0,436
	p	0,006	0,062
Ferritin (μ g/L)-IFN- γ (pg/mL)	r	0,580	0,219
	p	0,006	0,367
IL-10 (pg/mL)-TNF- α (pg/mL)	r	0,751	0,786
	p	0,003	0,021
IL-10 (pg/mL)-IFN- γ (pg/mL)	r	0,569	0,068
	p	0,042	0,782

(1) r: Rho spearman coefficient, p probability associated with "r".

It is clear that in our study, the small size sample and the great variability in the data are explained partly because we did not found differences between the groups. In our children the age range was wide, 2-16 years. The age might have contributed to variations in the studied parameters. It is known that when children under 5 years are in contact with a pathogen, they produce a very pronounced TH1 cytokine response but as age increases, they shift towards a TH2 response, as a result of the maturation of the immune system (Kovaiou & Grubeck-Loebenstein, 2006).

In spite of the limited scope of our results, we reached some interesting findings. In the supplemented group, C reactive protein values were lower than in the group not supplemented, this was confirmed in children with chronic malnutrition risk and parasitism. In the later group, lower values of ferritin in the group with retinol (117 ± 72 μ g/l) versus the group without retinol were also observed (184 ± 127 μ g/l). Since C reactive protein and ferritin are acute phase reactants increasing during infections (Gruys et al., 2005), our findings suggest that children that received retinol had less intensity on inflammation, which could be of clinical importance, since a exaggerated inflammatory response during malaria, has been associated with development of complications and death (Riley et al., 2006).

Nevertheless, that tendency to present lower intensity of inflammation associated with the retinol supplement was not reflected in the concentrations of cytokines. We expected that in the group with retinol, concentrations of IL-10 would be higher and TNF- α lower, as it has been found *in vitro* murine models of chronic inflammatory processes (Xu & Drew, 2006), but this effect was not seen in our children, which emphasizes the need to be cautious when extrapolating the results of studies from animal models to humans. In addition, it is important to consider that the dose of retinol supplemented to the cultures did not correlate to blood concentrations reached in humans, even after vitamin A administration (Hamzah et al., 2004).

Although no differences were detected in the concentrations of IL-10 neither among the groups with/without retinol in the children of the study, or in the children with chronic under nutrition risk and parasitism, the values of this cytokine were high; 25% of the children with retinol and 15% without retinol, had values exceeding the maximum limit of detection of the kit (677 pg/ml), concentrations very higher than those found in Turkish subjects with *P. vivax* malaria (Yildiz et al., 2006). These findings led us to think that our subjects had more ability to modulate the immune response, protecting themselves from later complications, since IL-10 is considered very important in the process of malarial immunopathogenesis due to its anti-inflammatory role, and high serum concentrations of this cytokine have been associated with better prognosis of the disease (Shofield et al., 2005). Haemoglobin concentrations were lower in the group with retinol in comparison to the group without retinol, when the analysis was unstratified. An increase in the destruction of infected red blood cells, which also increases the anemia might be associated to this result as other authors showed that the main receptor that mediates the phagocytosis of the infected erythrocyte by macrophages is CD36 and that the 9-cis-retinoic acid derived from retinol, stimulated the expression of CD36 and increased phagocytosis of erythrocytes infected with *P. falciparum* (Serghides & Kain, 2001).

Nor was there any difference in the prevalence of deficiency subclinical vitamin A, or retinol values between groups. However, we must take into account that concentrations retinol below 20 mg / dL in most children in this study have not really mean deficiency of vitamin A, due to the retinol binding protein is a reactant negative acute phase (Rosales et al., 2000), which decreases their concentrations during malaria and other infections (Ahmed et al., 1993; Rosales et al., 2000).

As for the correlations seen during the malaria episode, in children with and without retinol in the stratified group, most of these corresponded to what was expected. Correlations between parasitemia, CRP, ferritin, proinflammatory cytokines such as TNF- α and IFN- γ and anti-inflammatory such as IL-10, are adjusted as described during the inflammatory process that causes the early phase *Plasmodium* infection as a strategy to control initial parasitemia, limiting the spread of the parasite, with the subsequent removal of circulating forms (Ansar et al., 2006; Torre et al., 2002; Marsh & Kinyanjui, 2006). Additionally, these results allow us to verify has been seen in other previous studies, including: 1) the important role that TNF- α against asexual stages of *Plasmodium* erythrocytic phase early malaria, with a key role in the immune response protective cell, limiting the rate and contributing to death of *Plasmodium* (Maestre et al, 2002), 2) the behavior of ferritin as acute phase protein positive for malaria, which increases their concentrations in the presence of inflammatory/infectious subclinics clinics and, therefore in this research, and similar to observed in other study (Beard et al., 2006) this protein is not considered an adequate indicator of iron stores during

the acute phase infection, and 3) regulation that makes the IL-10 on proinflammatory cytokines, increasing their concentrations simultaneously in to maintain the TH1/TH2 balance, which as mentioned before, important to avoid all the complications associated with immunopathogenesis in malaria (Moormann et al., 2006).

However, an unexpected finding in children in the present study, was the positive correlation between IFN- γ hemoglobin, result which contrasts with other studies that found no association between anemia and iron deficiency in malaria, with high IFN- γ , IL-6, TNF- α and IL-1 (Jason et al., 2002; Kanjaksha et al., 2007; Feelders et al., 1998) situation, although can not be explained, is an interesting finding for further studies.

The undetectable concentrations of TGF- β 1 that were found in all subjects might be due to the use of different among the studies (Esmail et al., 2003).

This study is the first one exploring the effect of a supplement of retinol on some immunological parameters in children with *P. vivax* malaria and simultaneous infection with intestinal parasites and at chronic malnutrition risk. Although the results are limited by the small number of the sample and the variability in the data, studies of this type with higher number of subjects and narrower ranges of age, are worthwhile performing to clarify the effect of this supplement on malaria infection. We concluded that: 1) The tendency to have higher values of C reactive protein in the group without retinol and of ferritin in children with chronic malnutrition risk and parasitism of the group without supplement, suggests a possible anti-inflammatory effect of retinol during the acute phase of malaria and 2) A tendency to present lower concentrations of haemoglobin in children of the group that received retinol supplement and 3) The observed positive correlation between IFN- γ and hemoglobin, was a completely unexpected result. These findings could contribute to clarify the issue about the effect of supplemental retinol in children with malaria and relevance of supplementation population, as part of the strategy aimed to control malaria.

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This book provides an up-to-date summary of many advances in our understanding of anemia, including its causes and pathogenesis, methods of diagnosis, and the morbidity and mortality associated with it. Special attention is paid to the anemia of chronic disease. Nutritional causes of anemia, especially in developing countries, are discussed. Also presented are anemias related to pregnancy, the fetus and the newborn infant. Two common infections that cause anemia in developing countries, malaria and trypanosomiasis are discussed. The genetic diseases sickle cell disease and thalassemia are reviewed as are Paroxysmal Nocturnal Hemoglobinuria, Fanconi anemia and some anemias caused by toxins. Thus this book provides a wide coverage of anemia which should be useful to those involved in many fields of anemia from basic researchers to epidemiologists to clinical practitioners.

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