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Research Article

Investigations of Some Biomarkers and Nutrients During Malaria Infection in Children in Sinar State-Sudan

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Abstract: Malaria is a distressing health problem that posses a socio-economic burden of considerable magnitude for communities in developing countries. It causes some immunological and hematological changes particularly inflammatory biomarkers and hemoglobin. Research is extensively required in this area to promote health services and to target radical solution to the problem. The study was done to investigate some biomarkers and nutrients during malaria infections in Sudanese children in Sinar State-Sudan and how these biomarkers may relate to factors such as age and hemoglobin which in children may be important in disease progression and control. Thirty-two samples of blood were collected from 16 malaria patient from Sinar hospital and 16 apparently healthy individuals from the same area as control group from which serum was separated. The investigations were carried out to estimate serum C-reactive protein (CRP), tumor necrosis factor alpha (TNF α) and serum magnesium as a nutrient in the two groups. High sensitive and highly specific techniques were used for biomarker determinations. Results showed that children of different age groups with Malaria have significantly high levels of C-reactive protein, mean ± SE 5.93 ±1.52).

Keywords: Investigations, Biomarkers and Nutrients, Malaria Infection, Children

1. Introduction

Malaria is a serious infectious disease spread by mosquitoes and endemic in many developing countries. It is common in tropical climates and is characterized by chills, fevers, and an enlarged spleen. Malaria is caused by protozoan parasites of the genus Plasmodium. In humans malaria is caused by *P. falciparum*, *P. malarae*, *P. ovale*, and *P. vivax*. However, *P. falciparum* is the most important cause of the disease and responsible for about 80% of malaria infections [1].

Over the past few decades, a literature has emerged that argues for most of the pathology seen in all of these infectious diseases being explained by activation of the inflammatory system, with the balance between the pro and anti-inflammatory cytokines being tipped towards the onset of systemic inflammation. Although not often expressed in energy terms, there is, when reduced to biochemical essentials, wide agreement that infection with falciparum malaria is often fatal because mitochondria are unable to generate enough ATP to maintain normal cellular function. Most, however, would contend that this largely occurs because sequestered parasitized red cells prevent sufficient oxygen getting to where it is needed.

There is now remarkably widespread acceptance that cytokines such as TNF and interleukin-1 constitute the essential mechanism of systemic disease caused by infectious agents. Indeed, one would be pressed to find an alternative explanation for the anorexia, tiredness, aching joints and muscles, fever and sleepiness that patients experience in any systemic infection, including both vivax and falciparum malaria [2]. Tumour necrosis factor alpha (TNF-α) is thought to play a role in the development of immunity and pathology in malaria infections in experimental models and in humans

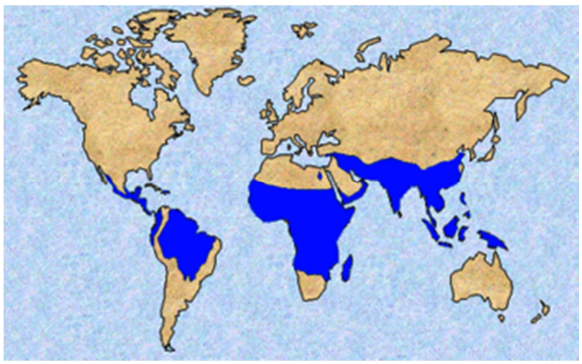
[3]. In fact, the genetic susceptibility to severe forms of falciparum malaria is differentially associated with TNF-α promoter gene polymorphisms. As such TNF can be considered as a biomarker for malaria.

Since red blood cells contain high amounts of magnesium, hemolysis might result in increased serum magnesium. This offers a potential application of this element as a biomarker of acute falciparum malaria infection in adults [4].

As recently reviewed [5], critical illness associated with an inflammatory response invariably causes multifactorial anaemia. It has often been noted that anaemia could contribute to poor oxygenation of tissues in malaria [6] and there is general acceptance that it can be severe enough to reduce supply of oxygen to mitochondria to dangerously low levels. Thus it can be a major component of malarial pathology. Obviously a high parasite load indicates imminent widespread homlysis, but anaemia does not correlate with parasitaemia, and sometimes is extreme when very few parasites are present

Although it has been suspected that nutrition might influence susceptibility to infection by the malaria parasite or modify the course of disease, there have been relatively few efforts to examine such interactions. Among the studies, some suggest that poor nutritional status or selective nutrient deficiencies may actually be protective; others suggest exacerbative effects of certain deficiencies. Recently, placebo-controlled field trials showed that vitamin A and zinc supplementation may significantly reduce the burden of malarial disease. Although an understanding of the influence of nutrition on malaria is far from complete, it is clear that nutrition strongly influences the disease burden of malaria.

The geographic distribution of malaria within large regions is complex, malarial and malaria-free areas are often found close to each other [7]. In drier areas, outbreaks of malaria can be predicted with reasonable accuracy by mapping rainfall [8]. Malaria is presently endemic in a broad band around the equator, in areas of the Americas, many parts of Asia, and much of Africa as indicated in figure 1.



Source: [9].

Figure 1. *Areas of the world where malaria is endemic (colored black).*

Malaria has been found to cause cognitive impairments, especially in children. The widespread anemia that malaria precipitates during a period of rapid brain development may result in brain damage. This neurologic damage may also result from cerebral malaria to which children are more vulnerable [10].

Malaria is caused by protozoan parasites of the genus *Plasmodium* (phylum Apicomplexa). Parasitic *Plasmodium* species also infect birds, reptiles, monkeys, chimpanzees and rodent [11]. There have been documented human infections with several simian species of malaria [12].

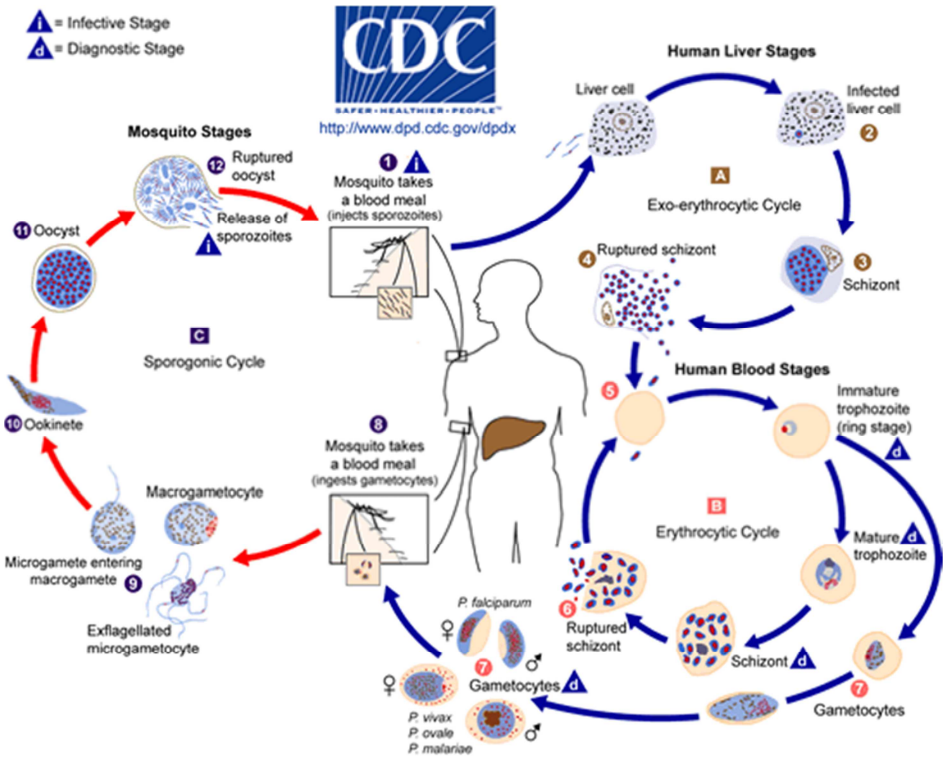
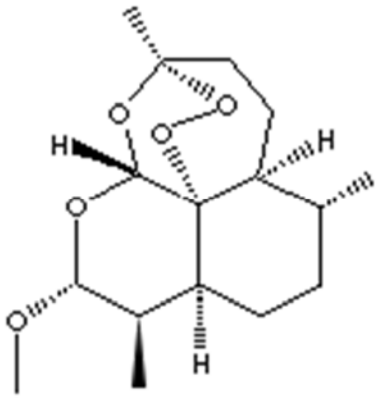


Figure 2. *Plasmodium life cycle. Source: [13].*

Severe malaria is commonly misdiagnosed in Africa. In malaria-endemic areas, parasitemia does not ensure a diagnosis of severe malaria because parasitemia can be incidental to other concurrent disease. Recent investigations suggest that malarial retinopathy is better than any other clinical or laboratory feature in distinguishing malarial from non-malarial coma [14].

Artemisinin is a new antimalarial drug of Chinese origin, derived from the herb Artemisia annua L., (sweet wormwood) belonging to the family of Asteraccae.



Artmethinin

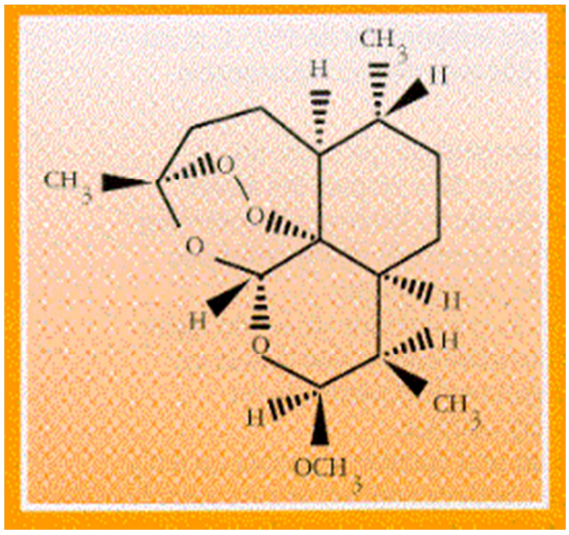


Figure 3. *Artmethinin chemical structure.*

C- reactive protein is currently characterized as a best available inflammatory biomarker and has emerged as a potential marker for cardiovascular risk [15].

Normally there is no CRP in blood serum., "a high or increasing amount of CRP in your blood suggests that you have an acute infection or inflammation. Although a result above 1mg/dl is usually considered high for CRP, most infections and inflammations result in CRP levels above 10 mg/dl.

Currently, no information is available about the biochemical or molecular signatures of severe and complicated malaria or mild and asymptomatic malaria. The detection of biomarkers of severe malaria, along with traditional microscopy, could result in effective management of malaria particularly in more vulnerable groups such as children.

2. Experimental

2.1. Material

2.1.1. Study Population

A total of thirty two school children whose ages ranged between five to sixteen years old were chosen at random from Sinar area. Sixteen of these children were with a medical history of malaria. The remaining sixteen were apparently healthy, and they were selected to serve as a control group.

Consent of children involved was taken from their teachers and parents.

2.1.2. Samples

Three ml of blood sample were collected from each subject by venipuncture. Blood samples were collected in the morning (8-10 a.m.). Samples were allowed to clot and serum was immediately separated by centrifugation for 10 min. Serum was then stored at 4°C and then analyzed.

2.1.3. Equipment

1. Photometer: Bio systems BTS – 310 Photometer.
2. Pipettes:

Manual pipette was used for pipetting samples and standards at the beginning of the assay. Automatic pipettes delivering 200µl 500µl and 1 ml were used for subsequent reagent additions, vortex mixer: GENIE 2, test tubes with round bottomed 12 × 75 mm glass. Magnetic racks and separators compatible with 12 × 75 mm test-tubes. 37°C water bath. Refrigerator. Timer. Various glassware including measuring cylinder beakers and reagent bottles of varying capacities.

2.1.4. Reagents

(i) Reagent for Serum Tumor Necrosis Factor (TNF) Estimation

TNF-alpha standards. Biotinylated TNF-alpha antibody. Streptavidin-peroxidase conjugate (SPconjugate). Mixed diluent concentrate. Wash buffer concentrate. Chromogen substrate. Stop solution.

(ii) Reagents for Serum C-Reactive Protein (CRP) Estimation

C-reactive protein antibody. Immage immunochemistry systems wash solution. Buffer. Diluent.

(iii) Reagents for Serum Magnesium Estimation

Methylthymol blue (MTB). Barium salt of (oxyethylenenitrilo). Tetra acetic acid. (Ba. EGTA). Buffer.

2.2. Methods

Estimation of (TNF) is carried out by enzyme linked

immuno-sorbent assay (Elisa). Quantitative determination of human C-reactive protein in serum is done by rate nephelomertry and serum magnesium is measured by a modification of Methylthymol blue complexometric methods.

2.2.1. Tumour Necrosis Factor (TNF) Estimation

The enzyme linked immunosorbent assay (Elisa technique) is used for the measurement of (TNF) in human serum. The time required to complete an assay is approximately 5 hours.

Principle:

A murine monoclonal antibody specific for human TNF-alpha has been recoated onto a microplate. TNF-alpha in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for human TNF.

Assay procedure:

All reagents, working standards and samples were prepared. All reagents were then brought to room temperature before use. The assay was performed at room temperature (20-30°C). Excess microplate strips were removed from the plate frame and returned immediately to the foil pouch with desiccant inside. The pouch was resealed securely to minimize exposure to water vapour and stored in vacuum desiccators.

50 µl of standard or sample were added to each well and then microplates were covered and incubated for two hours. The timer was started after the last sample addition.

Washing was done five times with 200 µl of Wash Buffer. The plate were inverted to decant the contents, and hit 4-5 times on absorbent paper towel to remove liquid at each step.50 µl of Biotinylated TNF-alpha antibody were added to each well and incubated for two hours. Washing was done five times with 200 µl of wash buffer as above.50 µl of chromogen substrate were added to each well and incubated for approximately 15 minutes or till the optimal blue color density developed. The plate was taped gently to ensurethrough mixing and the bubbles in the well were broken with a pipette tip.50 µl of stop solution were added to each well. The color was changed from blue to yellow. The absorbance was read at a wavelength of 450 nm immediately.

2.2.2. C-Reactive Protein (CRP) Estimation

Principle:

CRP levels was measured by nephelomerty method.

Procedure:

After setup, reagents, coded calibrators, controls and samples were loaded onto the system and then analyzed.

2.2.3. Serum Magnesium Estimation

Principle:

Methythymol blue MTB forms a blue complex with magnesium. Calcium interference is minimized by forming a complex between calcium and Ba-EGTA (chelating agent). The amount of MG-MTB complex formed is proportional to the magnesium concentration and is measured using a dichromatic (600 and 510 nm) endpoint technique.

Procedure:

This was performed on the dimension clinical chemistry system after it had been calibrated.

Test steps:

Sampling, reagent delivery, mixing, processing, and printing of results were automatically performed by the dimension system, based on the procedure of spectrophotometery.

2.2.4. Statistical Analysis

Data obtained were statistically analyzed using t-test, Standard error and correlations.

3. Results

3.1. Serum Biomarkers

3.1.1. Serum C-Reactive Protein (CRP) Levels

The levels of C-reactive protein (CRP) in Malaria patients and control group, expressed as means ± standard error (S. E) are shown in table 1, and represented in figure (4). CRP levels of malaria patients at 5.93 ± 1.52 are found to be significantly higher compared to the control group (P<0.003).

Table 1. *C-reactive protein (CRP) in malaria patients and the control group.*

Group	CRP level in mg/dl (mean± SE)
Patients (n=16)	5.93 ± 1.52
Control (n=16)	3.55 ± 0.58

n= number of specimens.

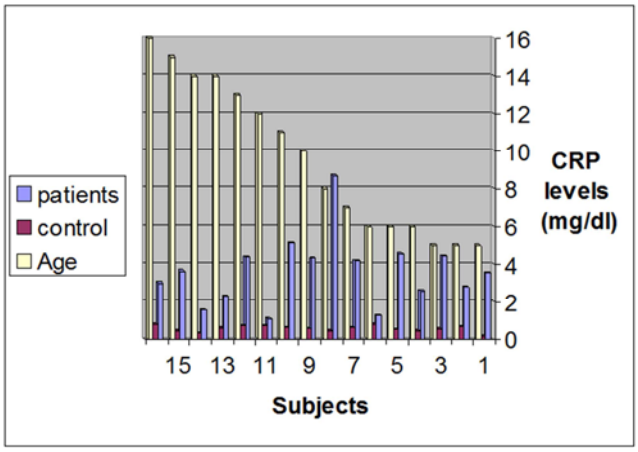


Figure 4. *The C-reactive protein profile in malaria patients and the control group.*

3.1.2. Serum Magnesium Levels

The serum levels of magnesium in the patients and the control group are shown in table 2 and profiles illustrated in figure 5. The mean value of serum magnesium in patients was compared with mean value of control group and was not high among patients.(reference value of serum magnesium is 1.8 – 3 mg/dl).

Table 2. *Serum magnesium in malaria patients and the control group.*

Group	Serum magnesium in mg/dl (mean± SE)
Patients (n=16)	2.04 ± 0.68
Control (n=16)	2.27 ± 0.79

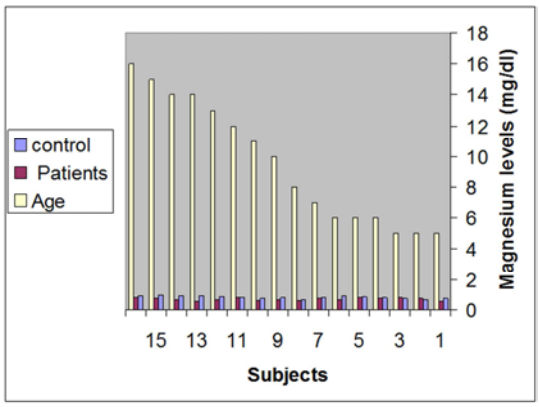


Figure 5. *Serum magnesium profiles (mg/dl) in malaria patients and the control Group.*

3.1.3. Tumor Necrosis Factor (TNF–Alpha) Levels

Table 3 shows the serum levels of tumor necrosis factor – alpha, figure 6 illustrated serum TNF – alpha in patients and the control group was found to be low but it was within the normal levels, for patients it was slightly higher compared to control group, mean ± SE of 1.50. ± 0.18 and no significance was found.

Table 3. *Tumor necrosis factor (TNF – alpha) levels in malaria patients and control group.*

Group	TNF level (pg/dl) (mean±SE)
Patients	2.69±0.74
Control	1.50±0.18

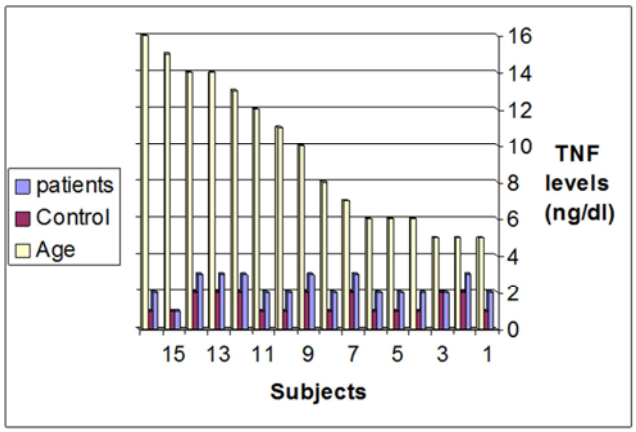


Figure 6. *TNF profiles in malaria patients and the control Group.*

3.1.4. Hemoglobin Levels

Levels of hemoglobin in patients and the control group are shown in table 4 and in figure 7. The level of hemoglobin in patients was found to be low when compared with that of control group, mean ± SE 2.28 ± 1.79 and P value < 0.001 which is significant. (reference value of hemoglobin is 12 – 14 mg/dl in females, 14- 16 mg/dl in males).

Table 4. *Hemoglobin levels in malaria patients and the control group.*

Group	Serum Hemoglobin in mg/dl (mean± SE)
Patients (n=16)	8.69 ± 0.24
Control (n=16)	10.6 ± 0.37

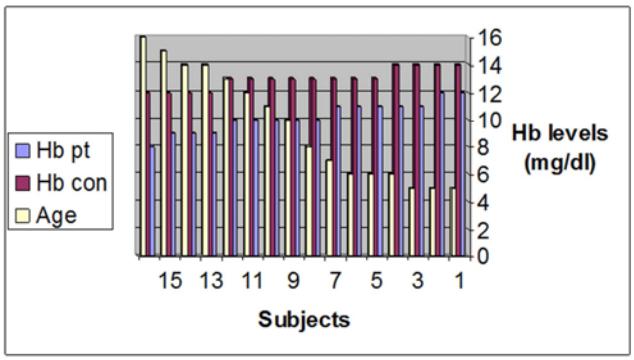


Figure 7. *Hemoglobin (mg/dl) profiles in patients and the control group in mg/dl.*

3.2. Correlations Between Biomarkers and Other Factors (Age and Hemoglobin) Among Patient Group

3.2.1. Correlations Between C-Reactive Protein Concentration and Age

Table 5 shows the serum levels of CRP correlated with age in patient group. (illustrated in figure (8) statistically there in no correlation between CRP concentration and age.

Table 5. *Correlation between C-reactive protein concentration and age in malaria patients.*

Parameter	Correlation	(Mean±SE)
CRP	0.65	7.88±1.18
Age		11.6±0.86

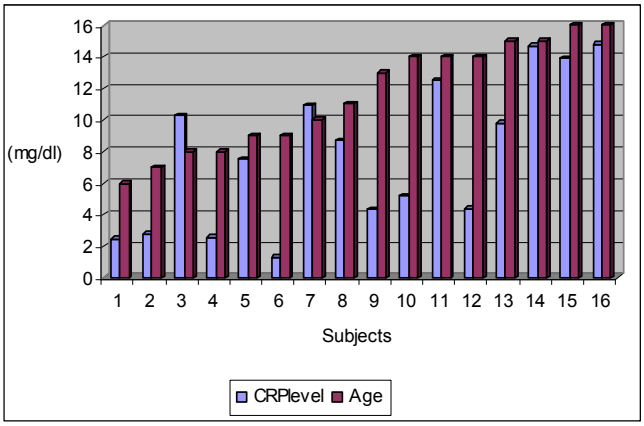


Figure 8. *correlation between CRP concentration and age.*

3.2.2. Correlation Between Serum Magnesium and Hemoglobin (Hb)

Table 6 shows the relation between serum magnesium and hemoglobin levels in patient group illustrated in figure 9. Although serum magnesium was found to be slightly high and hemoglobin was low in malaria patient, there is no correlation between two groups.

Table 6. *Correlations between serum magnesium (mg/dl) and hemoglobin levels among patients.*

Parameter	correlation	(mean ± SE)
Serum magnesium	0.06	0.69±0.04
Hb		10.3±0.29

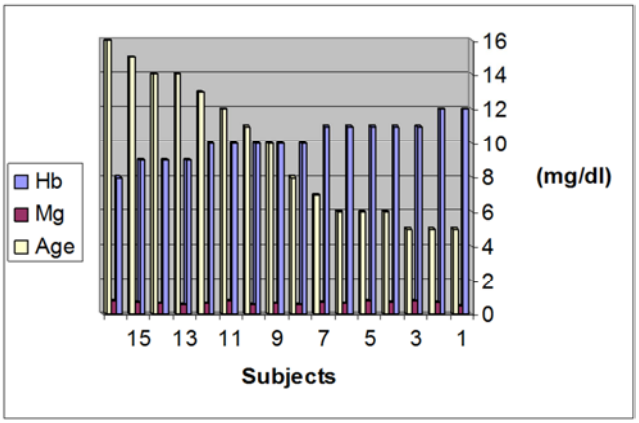


Figure 9. *Correlation, between Serum magnesium and hemoglobin (Hb) in malaria patients.*

4. Discussion

4.1. Serum C-Reactive Protein

Human C-reactive protein (CRP) is a clinically important classical acute phase response (APR).

Multivariate analysis showed that CRP levels were significantly associated with splenomegaly, fever, hemoglobin, and age. CRP levels also increased with increasing parasitemia but remained <3.5 µg/ml [16].

The present study shows that there was a higher significance between malarial patients and control group in the levels of serum C-reactive protein because of malaria infection as it is known as an inflammatory biomarker.

4.2. Serum Magnesium Level

Serum magnesium concentration was measured in other study [17] in 4 adult patient (age range: 18–40 yr) presenting with acute, uncomplicated *falciparum* malaria infection and a control group. Magnesium concentration in the patients was 1950.0 ± 10.0 μg/dl. The control serum magnesium was 640.0 ± 40.0 μg /dl. This represents an over threefold increase in serum magnesium levels above normal value ($P<0.01$). The key pathogenic event of acute *falciparum* malaria infection is the hemolysis of both infected and uninfected red blood cells. Therefore, the increase serum magnesium concentration might occur because of the hemolysis because red blood cells contain high amounts of magnesium. The increased serum magnesium has potential application as a biomarker of acute *falciparum* malaria infection in adults [4].

The present study shows no significant change in serum Mg levels in malarial patients and control group.

4.3. Serum Tumour Necrosis Factor

Tumour necrosis factor alpha (TNFα) is thought to play a role in the development of immunity in malaria infectious.

Some malaria disease severity is attributed to the induction of the pro-inflammatory cytokines TNF-alpha. Susceptibility/resistance to plasmodium falciparum malaria has been correlated with polymorphisms in more than 30 human genes with most association analyses having been carried out on patients from Africa and Southeast Asia. Significantly higher TNF levels were observed in patients with severe malaria [18].

Plasma concentration of TNF-alpha and IFN-gamma were higher in parasitaemic than aparasitaemic individuals and donors who had clinical malaria had higher levels of TNF-alpha, IFN-gamma and IL-6 than asymptomatic parasitaemic donors. There was a negative correlation between age of the individual and the concentration of plasma TNF-alpha and IFN-gamma suggesting that the production of these cytokines could be modulated by repeated malarial infections [19].

The findings in the present study is in agreement with the findings of other workers, where peripheral levels of TNF, and ferritin were found to be elevated during Placental malaria. [20].

In the present study TNFα level was significantly elevated in children, but remained within the normal range.. Whether this is an indication of different mechanisms of pathogenesis or reflects differences in immunological responses at different stages of development of the host (during fetal life, at a very early age and in adolescences) remains to be investigated. The pathogenesis of severe malaria is multi-factorial and appears to involve cytokine and chemokine homeostasis, inflammation and vascular injury/repair [21].

It has been suggested that IL-10, with a sensitivity of 79.5% and a specificity of 84.3% may have utility as a biomarker for inflammatory malaria but that additional biomarkers may be required to improve clinical diagnosis and management of malaria during pregnancy. Following this argument one is tempted to investigate the levels of the two inflammatory biomarkers in the age groups 5-10 and 11-15 simultaneously and assess their diagnostic value as well as their role in the course of malaria infection.

High level of serum TNFα correlate with resistance to malaria infection in contrast to this finding this study shows no significance between malaria patients and control group.

4.4. Hemoglobin Levels

Anemia or low hemoglobin commonly occurs in chronic infection inflammation and malignancy, in the present study hemoglobin levels in malaria patients were significantly low (8.69 ± 0.24) in most of the children and some of them were suffering from anemia compared to their counterpart control group because of poor nutritional status. When these children encounter the malaria their hemoglobin drop slightly and probably not to the extent of affecting serum magnesium levels this argues is due to hemolysis and release of magnesium which leaves the findings of this study of no significance in serum Mg concentrations open for further investigations. In this situation one is temped to question the sensitivity and specificity of quantifying method used for estimation, or perhaps if the mineral chelated during the process.

5. Conclusion

It's concluded from the present investigation that the

malaria can affect some inflammatory biomarkers such as c -reactive concentrations which increased in malaria patient and of high incidence among children.

In accordance with other studies there is no clear difference in serum magnesium concentration in the tow groups either malaria patient or healthy individuals which was within the normal range. TNF level is increased among patients slightly but serum magnesium levels is not affected by malaria in this study, hemoglobin was low also there is no clear relationship between these some factors such as age, and hemoglobin and c-reactive protein and serum magnesium respectively.

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