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CONTENTS

In-Vitro Antimalarial Resistance Pattern of *Plasmodium Falciparum* Infection Among Pregnant Women In Northern Nigeria

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Key words:
Antimalarial resistance; pregnant women; Malaria control

Background and study aim: Despite the global priority given to malaria control and prevention, antimalarial resistance is a major factor that encourages persistence of malaria in developing countries. This prospective study sought to determine the antimalarial resistance pattern of *P. falciparum* isolated from infected pregnant women attending antenatal clinics at Kaduna state, Northern Nigeria.

Materials and Methods: Between 16th February and 28th April, 2015, EDTA anticoagulated blood samples were collected from seventy nine pregnant women with plasmodiasis. Antimalarial susceptibility of chloroquine, artesunate, artemether and sulfadoxin-pyrimethamine (SP) against *P falciparum* was done using schizont maturation assay. Multidrug resistant plasmodiasis was defined by resistance to ≥ 3 antimalarial drugs.

Results: Malaria parasites from the pregnant women exhibited the highest resistance against chloroquine, 85 (24.1%) followed by Artemether, 30 (8.5%) then sulfadoxin-pyrimethamine, 29 (8.2%) and least resistant to artesunate, 28 (7.9%). The occurrence rate of multidrug resistance was 40.5%. There was no significant association between occurrence of multidrug resistance and malaria parasitaemia (p=0.092). Seventy five, 94.8% of the *P. falciparum* infected subjects exhibit resistant to at least one antimalarial. Antimalarial resistance was highest in women with severe malaria 20 (80.0%), followed by those with moderate malaria, 15 (62.5%) and least in those with mild malaria, 4 (13.3%). There was significant association between occurrence of antimalarial resistance and densities of malaria parasitaemia (p=0.0125).

Conclusion: Considering the high degree of antimalarial resistance reported from this study, there will be challenges in eradicating malaria in this environment. These findings necessitate the need for regular surveillance for resistant *P. falciparum* and evaluation of more effective alternative drug (s).

INTRODUCTION

Malaria is a major public health challenge in sub-Saharan Africa. In 2015, there were an estimated 214 million cases and 438,000 deaths due to malaria globally with Nigeria accounting for 25% of these **[1]**. Anti-malarial resistance is a major factor that encourages persistence of malaria in developing countries. Despite the public health priority given to malaria, in recent time, the parasite has developed resistance to most of the commonly available prophylactic and therapeutic antimalarial agents **[1,2]**. In view of these, the need for periodic antimalarial sensitivity testing becomes crucial. The main objective of anti-malarial sensitivity testing is to evaluate the parasite (s) sensitivity to increasing antimalarial dosages. This approach allows for complete exclusion of interfering host immunity and metabolism of the compound, thus offering an accurate evaluation of drug impact on parasites **[3]**.

Over the past five decades, antimalarial resistance by *Plasmodium falciparum* has become an issue of utmost concern. *In vitro* assays for assessing antimalarial

drug sensitivity have become indispensable tools for the surveillance of drug resistance and the planning of therapeutic guidelines. These include Schizont Maturation assay, radiolabeled hypoxanthine isotopic assay, malaria histidine-rich protein 2 (HRP2) and lactate dehydrogenase (LDH) quantification by enzyme-linked immunosorbent assay (ELISA) and nucleic acid amplification tests such as polymerase chain reaction (PCR) **[4]**.

The ideal approach for detecting and monitoring antimalarial resistance is the use of polymerase chain reaction. This method detects genes responsible for resistance and results are usually available within 8-12 hours accompanied with high sensitivity and specificity. However, the need for technical training of laboratory personnel (especially in developing countries) and its relatively high cost of procurement and maintenance limits PCR applicability in resource limited settings **[4]**.

A key intervention for controlling malaria and its effects during pregnancy is the administration of intermittent preventive treatment (IPT) **[5]**. This consists of a complete therapeutic course of anti-malarial medicine given to pregnant women at routine prenatal visits, regardless of whether they are infected with malaria or not. IPT reduces incidences of maternal malaria episodes as well as maternal and placental parasitaemia. Therefore, the WHO recommends IPT with SP in areas with stable malaria transmission in the sub-Saharan Africa **[6]**.The efficacy of SP is being compromised in Africa by the emergence of several mutations which has been shown to reduce the efficacy of intermittent preventive treatment **[7-9]**. Importantly, the prevalence of resistance to SP should be determined. Accordingly, IPT-SP should be used in regions with SP resistance prevalence less than 30% **[9]**. This prospective study sought to determine the antimalarial resistance pattern of *P. falciparum* isolated from infected pregnant women attending antenatal clinics at Kaduna state, Northern Nigeria.

MATERIALSAND METHODS

Study Area

This prospective study was conducted on pregnant women receiving antenatal services at General Hospital Kawo, Yusuf Dantsoho Hospital, Gwamna Awon Hospital and Barau Dikko Hospital, in Kaduna state, Northern Nigeria. The selection of health facilities was based on ease of accessibility and number of antenatal attendance, Kaduna State lies at latitude 10°20′ north and longitude 7°45′ east. It has a population of 6,113,503 (2006 census figures) and a population density of 130 people per square kilometer.

Sampling method, Inclusion and Exclusion criteria:

Known pregnant women with malaria parasitaemia at any gestation age who reside within the study area were included; however, those with chronic debilitating diseases were excluded.

Ethical clearance and informed consent:

The study protocol was performed according to the Helsinki declaration and approved by ethical research committee of Kaduna State Ministry of Health, Kaduna State, Nigeria. Informed written consent was obtained from all subjects before recruited for the study.

Sample collection, preparation and precaution

Between 16th February and 28th April, 2015, about 2ml of venous blood samples were collected aseptically from 79 individual subjects and carefully dispensed into EDTA sample containers.

Malaria Microscopy:

The parasite species were identified by preparing thick and thin blood films on microscope slides using the Giemsa staining techniques as was described by Cheesbrough **[10]**. Two glass slides were labeled for each participant. A drop of blood was then placed on the clean, grease free glass slide and allowed to dry. For thick films, a drop of blood was dropped at the center of a clean grease-free microscope slide to cover an area of 15mm in diameter. The smears were allowed to air-dry after which they were covered with 10% Giemsa stain, then allowed to stay for 10 minutes before the stains were washed off with water. The thick films were used to detect the presence of malaria parasites in blood samples. Parasite species and morphology were determined by microscopic examination of the thin films which were also done with the 10% Giemsa stain for 5 minutes. The microscopic examination of the stained slides were done using the oil immersion objective lens (100x objective). At least 100 high power fields were examined before a thick smear was declared negative. Malaria parasites were counted per 500 WBC, which were used to estimate the parasite density per microlitre of blood **[10]**.

Antimalarial drug susceptibility assay:
Schizont maturation inhibition technique was used. Chloroquine, artesunate, artether and sulfadoxin-pyrimethamine were assessed for their *in-vitro* antimicrobial actions on the *P. falciparum* isolated. These drugs were purchased from the shelf (pharmacy store). Stock solutions of the antimalarial drugs will be prepared as follows:

The drugs were made to be of equal concentrations of 100mg/ml by dissolving in the appropriate amount of distilled water. 50mg (1 tablet) of artesunate was dissolved in 0.5 ml of distilled water, 250 mg of chloroquine was dissolved in 2.5ml of distilled water, 250ml of artemeter in 750 ml of distilled water. They were allowed to dissolve and the suspensions were shaken thoroughly to get homogenous solutions. Two-fold serial dilutions of each of the drug suspension was prepared by adding 1ml of the suspension into 1ml of distilled water in the first test tube, from the first tube, 1ml of the solution was taken and added into the second tube already containing 1ml of distilled water. This was done to the fifth tube for all the drugs. Malaria infected blood samples were centrifuged at 2000 revolutions per minute (2000 rpm) for five minutes. After centrifugation, the plasma and buffy coats (leukocyte interface) discarded and 0.2ml of the packed red blood cells was dispensed into thirteen test tubes for each of the samples. The tubes contained different concentrations of the drugs in duplicates. A control (tube without drugs) was set up for each of the samples. 1ml of the physiological saline was dispensed into each of the tubes. After addition, lids were placed over the tubes and the tubes shaken gently to dissolve the drugs.

The samples were then incubated at 37^0C for 42 hours. At the end of incubation, thick and thin blood films were prepared from the samples in each tube including those of the controls. The number of schizonts or gametocytes in the control tubes was compared with that in the other tubes containing different concentrations of the anti-malarial drugs.

Statistical analysis
Results were presented in tabular forms showing the frequencies and percentages of antimalarial resistance in each drug tested. Data were analyzed for statistical association between antimalarial resistance and the densities of malaria parasitaemia in pregnant women using two tailed Chi-square test.

RESULTS

In total, 79 malaria infected pregnant women in their various pregnancy trimesters were recruited for the study, pregnant women ages were between 17 and 44 years and mean age was 22.45± 6.84 (±SD). All the malaria positive cases were that of *Plasmodium falciparum*. Malaria parasites from pregnant women exhibited the highest resistance against chloroquine, 75(94.9%) and least resistant to artesunate, 28 (35.4%). The occurrence rate of multidrug resistance is 40.5%. There was no significant association between occurrence of multidrug resistance and malaria parasitaemia in pregnant women (p=0.092) (Table 1). Seventy five, 94.8% of the *P. falciparum* infected subjects were resistant to at least one antimalarial. Antimalarial resistance was highest in women with severe malaria 25 (100%), followed by those with moderate malaria, 23 (95.8%) and least in those with mild malaria, 27 (90.0%). There was significant association between occurrence of antimalarial resistance and densities of malaria parasitaemia (p=0.0125) (Table 2).

Table (1): *In-vitro* antimalarial resistance pattern in pregnant women infected with malaria

S/No.	Antimalarial	No. (%) resistant	No. (%) susceptible	*P* –value
1	Artemether	30 (38.0)	49 (62.0)	
2	Chloroquine	75 (94.9)	4 (5.1)	
3	Artesunate	28 (35.4)	51 (64.6)	
4	Sulfadoxine-pyrimethamine	29 (36.7)	50 (63.3)	
5	Resistant to ≥ 3 antimalarial drugs (MDR)	32 (40.5)	47 (59.5)	0.092*

***Significant association determined by Chi-square test**

Table (2): Distribution of antimalarial resistance by malaria densities among pregnant women

	Mild Malaria Parasitaemia (<1000 parasites/uL of Blood) (%)	Moderate Malaria Parasitaemia (1000 -10,000 parasites/uL of Blood) (%)	Severe Malaria Parasitaemia (>10000 parasites/uL of Blood) (%)	Total	P Value
No. of subjects	30	24	25	79	
No. with antimalarial resistance	27 (90.0)	23 (95.8)	25 (100)	75 (94.8)	0.0125*

***Significant association determined by Chi-square test**

DISCUSSION

This study of the blood samples of malaria infected pregnant women showed that 94.8% of them attending antenatal clinics of secondary healthcare facilities had resistance to at least one antimalarial drug tested. Observation from this study also showed 5.2% (4/79) were sensitive *in vitro* to Chloroquine. This observation is similar to Folarin et al.**[11]** report that 85% (71/84) of malaria parasites in Ibadan, southwestern Nigeriawere resistant to Chloroquine. However, the result contradicts Balogun et al.**[12]** who reported that *Plasmodium falciparum* isolates in Northeastern Nigeria were more sensitive to Chloroquine. It was found that 51 (64.6%) of the *Plasmodium falciparum* isolates were sensitive to Artesunate. This is similar to the study by Na-Bangchang et al. **[13]** who reported 36.7% declining in sensitivity to artesunate in Thai-Myanmar border. One of the factors to be considered in the prophylaxis, treatment, and control of *Plasmodium falciparum* malaria is the resistance of parasite strains that may arise against virtually every drug available. Identification of *Pfcrt* is the central determinant of chloroquine-resistant *P. falciparum* malaria that provides molecular marker that can be used for surveillance of resistance and to evaluate drug treatment and prophylaxis policies **[14]**. These findings also agreed with earlier findings where molecular markers of resistance were found in samples that gave *in vivo* resistance/ drug failure **[11,14]**.

The presence of high resistance rate of *P. falciparum* isolates to SP is worrisome. Considering its universal acceptance and usage in the prevention of maternal malaria, in future, the public health benefits of IPT will probably decline due to SP resistance. Intensifying the use of ITNs, research on vaccine development and alternative drugs for IPT become priorities in prevention of malaria. A similar finding was reported by Bouyou-Akotet et al. **[15]**.

In regards to monitoring antimalarial effectiveness in order establishment of the baseline sensitivity of malaria to commonly prescribed antimalarial,*in-vitro* drug sensitivity is important **[15]**. In-vitro resistance against chloroquine, arthether and artesunate was observed among the malaria infected pregnant women in this study. These results conform to earlier findings of Fallet al.**[16]**. Similarly, previous malaria resistance against chloroquine and arthemisin-based antimalarial agents has been reported by in Nigeria**[14,17]**. The host cells response to antimalarial drugs are controlled by the pharmacokinetic properties of drugs, preformed immunity in the patient, as well as the complexity of infections in high transmission areas **[18]**. These factors may contribute to the range of variations in the clinical expression of antimalarial resistance patterns.

CONCLUSION

This study showed the presence of high degree of antimalarial resistance by *P. falciparum* infections among pregnant women in Kaduna state, Nigeria. Based on the findings, there will be challenges in eradicating malaria in this environment. There is a need for periodic surveillance using molecular assays in order to identify the genes responsible for antimalarial resistance. In addition, it's recommended that evaluation of more effective and alternative antimalarial drug (s) be considered.

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Conflict of Interest:
None

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N-acetyl Cysteine Therapy as Adjunctive Therapy for Treatment of Acute Hepatitis A

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Key words: Hepatitis A; N-acetyl cysteine

Background and study aim: Hepatitis A is an acute, usually mild and self-limiting disease affecting the liver. We aim to assess the effect of oral N-acetyl cysteine compared with placebo on length of hospital stay in adult patients who were admitted to the hospital with acute hepatitis A which might cause earlier resolution of hepatitis.

Subjects and Methods: 40 patients were diagnosed as acute hepatitis A and classified into two groups, the first one involved 20 patients who received oral N-acetyl cysteine and supportive treatment, and the second one involved also 20 patients but they received placebo and supportive treatment. We measured complete blood count (CBC), kidney profile (KP), liver function test (LFT), blood glucose, C-reactive protein (CRP) and coagulation profiles on the day of presentation, and every other day till the day of discharge from the hospital. Serological tests were done for HAV Immunoglobulin M (IgM), HEV IgM, HBsAg, HBcIgM, antibody to Hepatitis C virus.

Results: The mean length of hospital stay in the NAC group was 13.2 days compared with 14.3 days in the placebo group. Length of hospital stay differed significantly between groups. The mean time of reliving symptoms at presentation was 3.6 days in the NAC group and 4.4 days in the placebo group. The mean time of reliving symptoms at presentation was significantly lower in NAC group than in placebo group.

Conclusion: use of oral NAC as adjunctive therapy for treatment of acute hepatitis A was safe in these patients and was associated with a shorter length of patient stay in the hospital.

INTRODUCTION

Hepatitis A is an acute, self-limiting disease affecting the liver caused by hepatitis A virus (HAV) **[1]**. The disease varies in clinical severity from a mild illness lasting 1-2 weeks to a severely disabling disease lasting several months. Most patients, who are infected, recover completely with no permanent liver damage **[2]**. Acute hepatitis A does not become chronic and there is no chronic carrier state. On rare occasions the disease may be very severe, with fulminant hepatitis, hepatic coma and death [2]. Severity of illness is strongly age dependent; adult who are infected with acute hepatitis A tend to experience a much more severe form of the disease, whereas young children typically have a milder form of the disease, usually lasting from 1–3 weeks [3]. For adults over 50 years of age, case fatality can reach 2%. The increased risk of death from fulminant hepatitis A can occur in persons with pre-existing chronic liver diseases. Infection with HAV confers life-long immunity **[3]**.

Globally, hepatitis A is more common in regions of the world drinking contaminated water and with poor sanitation **[4]** and around 1.4 million symptomatic cases occur each year **[2, 5]**.The adulthood are immune in the developing world because about 90% of children have been infected by age 10 **[4]**. Outbreaks occur in developed countries where vaccination is not widespread and children are not exposed to the infections when young [4]. In 2010, acute hepatitis A caused 102,000 deaths **[6]**.

N-acetyl cysteine (NAC) can be used as an antidote in paracetamol intoxication and also can be used as a mucolytic **[7, 8]**. N-acetyl cysteine can increase the amount of glutathione within the cell and maintain cell integrity **[9]**. The necessity for a medicine to decrease the duration of acute viral hepatitis is obvious, but it has not been found yet. This problem might be solved with N-acetyl cysteine, which protects the liver cells architecture by increasing the amount of glutathione within liver cells that reacts with reactive oxygen species (ROS) **[10]**. NAC was licensed for use in 1968 **[11]**. It is on the World Health Organization's List of Essential Medicines, it is not very expensive drug, safe and most effective medicines needed in a health system **[12]**.

The objective of this study was to assess the effect of oral N-acetyl cysteine compared with placebo on length of hospital stay in adult patients who were admitted to the hospital with acute hepatitis A which might cause earlier resolution of hepatitis.

SUBJECTS AND METHODS

This study was conducted between February 2014 and July 2015, at the Infectious Disease Hospital (IDH). The patients included in this study were diagnosed as acute hepatitis A. Diagnosis of acute hepatitis A was clinically based on the presence of symptoms, e.g. anorexia, nausea, vomiting, abdominal discomfort, fever, fatigue and jaundice and confirmed serologically by positive of HAV Immunoglobulin M (IgM) antibodies, indicating acute disease.

40 patients were confirmed diagnosis of acute hepatitis A and classified into two groups, the first one involved 20 patients who received oral N-acetyl cysteine and supportive treatment, and the second one involved also 20 patients but they received placebo and supportive treatment. All patients were subjected to history taking and thorough clinical examination. We measured complete blood count (CBC), kidney profile (KP), liver function test (LFT), blood glucose, C-reactive protein (CRP) and coagulation profiles on the day of presentation, and every other day till the day of discharge from the hospital. Also, all patients were investigated for HEV IgM, hepatitis C virus antibody, HBsAg, HBcIgM, and hepatitis delta virus antibody to exclude other causes of acute viral hepatitis.

Patients in the first group (NAC group) were given 600 mg NAC effervescent tablet orally once daily (600 mg/day), NAC was continued as long as required for normalization of laboratory investigations and the patients in the second group were given placebo orally 3 times a day (placebo group). All patients received supportive treatment, according to individual needs.

Statistical Analysis:

The statistical package for social sciences (SPSS) version 8.0 software was used for analysis the data. The t-test was used to evaluate the significance of differences between mean values of the study variables. The significance of differences between proportions was performed using the Chi-square test. Significant differences were expressed at $P<0.05$.

RESULTS

Forty patients were included in the study, they were classified into two groups, N-acetyl cysteine group and placebo group, each of them involved 20 male patients varying in age from 14 years to 29 years. Of these, 11 patients were Indian, 10 Egyptian, 2 Indonesian, 8 Syrian, and 9 Kuwaiti. Symptoms at presentation included fever, anorexia, nausea, vomiting, abdominal discomfort, fatigue, dark urine and jaundice.

At the time of admission, no significant differences were noted between the NAC group and the placebo group as regard to liver enzymes, total bilirubin, direct bilirubin, INR, platelets, white blood cells, C-reactive protein, and serum creatinine (Table I).

After 5 days of admission, we noted a significant decline in liver enzymes (ALT & AST) and total bilirubin in the NAC group than the placebo group (table II). At time of discharge, no significant differences were observed between the two groups regarding total bilirubin, direct bilirubin, liver enzymes, INR, platelets, white blood cells, C-reactive protein, and serum creatinine (Table III).

The mean length of patient stay in the hospital in the NAC group was 13.2 days (±0.67) compared with 14.3 days (±0.75) in the placebo group. Length of hospital stay differed significantly between groups (p-value = 0.03, table III). All patients were started the treatment within one hour of admission to hospital. The mean time of reliving symptoms at presentation was 3.6 days

in the NAC group and 4.4 days in the placebo group. The mean time of reliving symptoms at presentation was significantly lower in NAC group than in the placebo group (p-value = 0.05, Table II).

Table I : Comparison between studied groups at time of admission

	On admission		
	NAC group	**Placebo group**	**P-value**
Age	18.1±4.6	17.5±4.1	0.6
ALT(U/L)	2574.21±157.2	2496.7±149.3	0.4
AST(U/L)	1865.4±103.8	1879.1±113.7	0.83
Total bilirubin (µmol/L)	34.5 ± 4.36	36.1 ± 3.04	0.23
Direct bilirubin (µmol/L)	18.7 ± 3.4	19.3 ± 3.6	0.12
Albumin (g/L)	38.5 ± 5.4	38.8 ± 4.2	0.85
CRP	13.04±3.03	13.05±3.06	1.0
INR	1.43±0.3	1.33±0.4	0.78
Platelet	171.9±4.3	173.5±4.1	0.94
WBCs	6.7±1.3	6.34±1.4	0.83
S. creatinine (µmol/L)	89.32±14.12	88.76±13.23	0.89

NAC = N-acetyl cysteine; ALT= Alanine transaminase; AST= Aspartate aminotransferase; CRP= C-reactive protein; INR= international normalized ratio; WBC= White blood cells; S. creatinine= Serum creatinine.

Table II : Comparison between studied groups after 5 days from admission

	After 5 days from admission		
	NAC group	**Placebo group**	**P-value**
ALT	1057.1±78.2	1409.3±86.2	0.05
AST	503.2±60.1	851.4±49.3	0.04
Total bilirubin (µmol/L)	24.3 ± 3.4	28.2 ± 3.1	0.04
Direct bilirubin (µmol/L)	11.5 ± 2.5	13.3 ± 2.4	0.1
Albumin (g/L)	35.1 ± 4.4	35.9 ± 4.2	0.24
CRP	9.0±3.2	8.0±3.4`	0.62
INR	1.23±0.2	1.26±0.4	0.81
Platelet	184.5±26	183.7±23	0.86
The mean time of reliving symptoms (day)	3.6±0.37	4.4±0.7	0.05

NAC = N-acetyl cysteine; ALT= Alanine transaminase; AST= Aspartate aminotransferase; CRP= C-reactive protein; INR= international normalized ratio; WBC= White blood cells; S. creatinine= Serum creatinine.

Table III: Comparison between studied groups at time of discharge

	On discharge		
	NAC group	**Placebo group**	**P-value**
ALT	250.35±21.97	251.90±26.86	0.92
AST	134.5±12.4	139.9±16.8	0.8
Total bilirubin (µmol/L)	17.3 ± 2.1	18.1 ± 2.2	0.12
Direct bilirubin (µmol/L)	7.5 ± 1.3	8.3 ± 1.4	0.61
Albumin (g/L)	36.2 ± 4.1	35.7 ± 4.1	0.65
CRP	4.7±1.5	4.75±1.3	0.93
INR	0.96±0.2	1.08±0.24	0.29
Platelet	191.74±18.5	189.0±20.6	0.53
WBCs	8.44±1.76	8.41±1.81	0.93
S. creatinine	84.3±11.4	85.5±11.78	0.74
Length of stay (day)	13.2±0.67	14.3±0.75	0.03

NAC = N-acetyl cysteine; ALT= Alanine transaminase; AST= Aspartate aminotransferase; CRP= C-reactive protein; INR= international normalized ratio; WBC= White blood cells; S. creatinine= Serum creatinine.

DISCUSSION

NAC is a specially modified form of the dietary amino acid cysteine. When taken orally, NAC is thought to help the body make the important antioxidant enzyme glutathione. It has shown promise for a number of conditions, particularly chronic bronchitis **[13, 14]**.

NAC has been proposed as supportive therapy for HIV. Despite some intriguing results, overall the evidence is inconsistent at best **[15, 16, 17]**. Recently, some studies have revealed good results and absence of side effects in chronic hepatitis C and hepatitis B patients who treated with NAC **[18, 19, 20]**.

The complications due to acute viral hepatitis were considered to be more frequent in adults than in the pediatrics **[20]**. Recently, reports from hepatic transplant centers suggest that 26% of cases with acute liver failure are caused with acute hepatitis A **[21]**. Hepatitis A is a common infection in the world **[22]**.

When symptoms of acute hepatitis A (AHA) occur, recovery from these symptoms may take several weeks or months. In this study, we noted the mean time of reliving symptoms at presentation was significantly lower in the NAC group than in placebo group. In the first 5 days after admission, we noted that the NAC group showed a much more rapid improvement in liver enzymes and total bilirubin than the placebo group. Also, in this trial, we noted an overall reduction in mean length of hospital stay of more than one day in patients with AHA who were given NAC compared with who were given placebo. These findings support our hypothesis that adjunctive treatment of AHA with oral NAC might change the immune response and thereby reduce morbidity and length of patients stay in hospital.

The optimal duration of administration of NAC is not well known up tell now **[23]**. In this study, the duration of administration of NAC was between 11 days and 14 days, without observation any undesirable side effects, e.g. nausea, vomiting, itching, rash, hypotension, bronchospasm **[17]**. This is in line with Huseyin et al. **[9]** and Hu **[24]**, who have clarified that NAC is a safe drug to the patients with acute viral hepatitis.

The role of NAC, a glutathione precursor, in the treatment of paracetamol-induced acute liver failure (ALF) is well established **[25]**. Also, in small trials, NAC has been used in non-paracetamol-induced ALF with variable results **[26]**. The clinical basis for the use of NAC is based on several mechanisms, of which the most important are: a) to facilitate the synthesis of depleted glutathione in AHA, and replenish hepatic stores of glutathione, b) it has vasodilating effects which improve microcirculatory blood flow and oxygen delivery to vital organs **[27]**, c) increasing the blood flow by increasing the soluble nitric oxide activity in the gultamyl cyclase system, d) it acts as antioxidant that scavenges the free radicals **[27]**, e) blocking oxidative stress and avoiding the accentuation of hepatic damage **[28]**.

The data about using of oral NAC in the treatment of AHA are restricted. To our knowledge, there is no any study assessed the effects of NAC on AHA except one published study evaluating the effect of NAC on AVH (A & B), this article has shown that NAC did not have effect on the length of hospital stay of AVH infection and, on the period in which the ALT value came back to normal and also the prognosis of biochemical parameters **[9]**.

CONCLUSION

This study reported that the use of oral NAC as adjunctive therapy for treatment of acute hepatitis A might be beneficial in decreasing the length of hospital stay. It is also reported that the use of NAC was safe because of absence of its side effects in these patients. There is an undoubted necessity for further research into the treatment of hepatitis A, and this study has identified a promising compound NAC, that may be an integral component of future HAV management.

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Fever of Undetermined Origin in Elderly Patients: Causes and Clinical Characteristics

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Key words:
fever, unknown origin, elderly

Background and study aim: Fever of Undetermined Origin (FUO) continues to be a diagnostic challenge particularly in elderly patients. Reporting local experience is important in guiding clinicians about the epidemiologic pattern in different region. This study aimed to determine causes, clinical presentations and the laboratory findings of FUO among elderly persons ≥ 65 years in comparison with younger patients.

Patients and Methods: This study was conducted on 54 patients during one year duration from the period between January 2015 and January 2016. Patients were divided into two equal groups of 27 patients who were suffering from FUO. The first one (G1) consisted of elderly patients ≥ 65 years and the second group (G2) consisted of patients younger than the age of 65. All patients in this study were subjected to complete history taking adequate physical examinations in addition to routine laboratory investigations and specific investigations (according to case).

Conclusion: Urinary tract infection, chronic calcular cholecystitis and malignancy are important causes for FUO in elderly patients followed by miscellaneous causes as post chemotherapy and drug fever. Non-elderly group showed statistical significant increase in typhoid fever, HIV infection, infective endocarditis, intra-abdominal abscess and auto immune disorders when compared to elderly group.

INTRODUCTION

In 1961, Petersdorf and Beeson introduced the definition of fever of undetermined origin (FUO) that subsequently became standard-namely, fever of more than 3-weeks duration, fever higher than 38.3°C on several occasions, and diagnosis uncertain after 1 week of study in hospital **[1]**.

Because hospital admission is so expensive and thorough diagnostic testing now can be performed in outpatient settings, the definition of classic FUO was modified to remove the requirement that a hospital be the setting for 1 week of evaluation. The revised criteria require an evaluation of at least 3 days in the hospital, three outpatient visits, or 1 week of logical and intensive outpatient testing without determining the fever's cause **[2]**.

Durack and Street have proposed a new system for classification of FUO: 1. classic FUO, 2. nosocomial FUO, 3. Immune deficient FUO, and 4. FUO associated with HIV infection **[3]**.

Febricity in the elderly can be defined as temperature exceeding 37.2°c taken orally or of ear drum, or higher than 37.5°c taken rectally **[4]**.

The diagnostics of FUO in the elderly often differs from the young patients; the manifestation of a disease is often nonspecific in older patients. The physiologic reserves are diminished in the elderly as well as their immunity. In the elderly many other accompanying diseases may affect the diagnosis, treatment, and the outcome of the illness. The symptoms and signs of many illnesses are atypical or less prominent in older patients, which obviously complicate diagnosis. Thus for instance, cognitive function disorders can be the only sign of infection in the elderly **[5]**.

This study aimed to determine causes, clinical presentations and the laboratory findings of FUO among elderly persons ≥65 years in comparison with younger patients.

PATIENTS AND METHODS

This cohort study was conducted on 54 patients admitted to El- Mehalla Fever Hospital, Tanta Fever Hospital and Zagazig University Hospitals during one year duration from the period between January 2015 and January 2016. Patients with FUO of any type with consideration of temperature >37.2°C orally or >37.5°C per rectum for elderly patients ≥ 65 years and >38.3°C orally for younger patients were included.

Patients were divided into two equal groups of 27 patients who were suffering from FUO. The first one (G1) consisted of elderly patients ≥ 65 years and the second group (G2) consisted of patients younger than the age of 65.

After ethical approval of the study, an informed consent was taken from all patients. All cases were followed up in hospital setting.

All patients were subjected to :

Full history taking:

Including: age, sex, residence, occupation, travelling abroad, exposure to animals or vectors, drug history, family history, sexual history, history of special habits, the magnitude of the temperature readings and the patterns of fever.

An adequate physical examinations (general and local) including: vital signs, fever chart, head and neck, extremities, musculoskeletal, lymph nodes, dermatological, cardiac, chest, abdominal and full neurological examinations.

Investigations:

I. Routine Investigations:

a. Urine analysis **[6]**.
b. Complete blood count (HB % - W.B.Cs with differential – platelet count) **[7]**.
c. Liver enzymes tests including ALT, AST **[8]**.
d. Serum urea and creatinine **[9]**.
e. Acute phase reactants: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) **[10]**.
f. Chest X-ray
g. Pelvi-Abdominal ultrasonography: With special emphasis on liver, spleen, kidney and prostate size, echo pattern, focal lesions and abscess , presence of ascites and lymph node enlargement.

II. Specific Investigations: (according to the case)

- Cultures of blood and urine in suspected cases **[11]**.
- Immunological tests as RF, ANA, Anti ds-DNA and ASOT **[12]**.
- Widal agglutination test and Brucella agglutination test **[13]**.
- CMV Ab **[14]**.
- EBV Ab **[15]**.
- HIV Ab **[16]**.
- Tuberculin test **[17]**.
- ECHO: With special emphasis on detection of cardiac size, function and presence of vegetations, mass or abscess.
- Computerized tomography (CT), Magnetic Resonant imaging (MRI) and bone marrow biopsy.

Statistical analysis :

Data collected throughout history, basic clinical examination, laboratory investigations and outcome measures coded, entered and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) (Statistical Package for the Social Sciences) software for analysis. According to the type of data qualitative represent as number and percentage, quantitative continues group represent by mean ± SD. The following tests were used to test differences for significance;. Differences between frequencies (qualitative variables) and percentages in groups were compared by Chi-square test. Differences between parametric quantitative independent groups by t test. P value was set at <0.05 for significant results & <0.001 for high significant result.

RESULTS

Table (1): Fever Pattern of elderly group versus non elderly group

Parameter	Group I ≥ 65 n=27	Group II <65 n=27	Test	P. Value
Intermittent	8(29.6%)	8(29.6%)	8.87	0.03*
Continuous	14(51.9%)	7(25.9%)		
Remittent	1(3.7%)	9(33.3%)		
Undulant	4(14.8%)	3(11.1%)		
Association:				
Rigor	5(18.5%)	5(18.5%)	0.00	1.00
Sweating	1(3.7%)	7(25.9%)	5.28	0.022*

NB: * Significant. ** Highly significant**.**

Fever in elderly group showed statistically significant increased percentage of the continuous pattern, on the other hand sweating was significantly prominent in non-elderly group.

Table (2) : Laboratory investigation of elderly group versus non elderly group

Parameter		Group I ≥ 65 n=27	Group II <65 n=27	Test	P. Value
Total leucocytes count		9485.18 ± 3315	8922.22 ± 2720	0.534	0.595
Neutrophils		68.11% ± 16.4	69.55% ± 14.3	-0.344	0.732
Lymphocytes		28.66% ± 14.8	28.00% ± 14.3	0.168	0.867
HB (gm%)		10.73 ± 1.9	10.59 ± 2.2	0.270	0.782
Platelets $(10)^3$		174.000 ± 96.1	234.518 ± 124.5	-1.999	0.051
ESR	1st hour mm/ hr	72.29 ± 26.5	59.81 ± 19.4	1.309	0.196
	2nd hour mm/ hr	100.18 ± 38.8	86.22 ± 26.5	1.359	0.180
CRP mg/dl		18.44 ± 11.0	14.66 ± 9.6	0.920	0.362
ALT U/L		57.66 ± 37.1	68.85 ± 60.3	-0.820	0.416
AST U/L		64.40 ± 53.6	89.59 ± 96.4	-1.186	0.241
Serum creatinine mg/dl		1.1259 ± 0.51	0.8889 ±0.25	2.159	0.035*
Serum urea mg/dl		40.22 ± 26.5	37.74 ± 31.6	0.321	0.756
Urine analysis	Normal	6(22.2%)	11(40.7%)	2.14	0.14
	Abnormal	21(77.8%)	16(59.3%)		
Widal test titre 1/160		0(0.0%)	3(11.1%)	3.17	0.075
Brucella test titre 1/160		4(14.8%)	3(11.1%)	0.16	0.68

NB: * Significant. ** Highly significant**.**

Serum creatinine was statistically significant increased in elderly group

Table (3) : FUO categories of elderly group versus non elderly group

Parameter	Group I ≥ 65 n=27	Group II <65 n=27	Test	P. Value
Classic FUO	23 (85.2 %)	22 (81.5 %)	6.3	0.09
Nosocomial FUO	3 (11.1 %)	0 (0.0 %)		
Immune deficient FUO	1 (3.7 %)	2 (7.4 %)		
HIV related FUO	0 (0.0 %)	3 (11.1 %)		
Total	27 (100%)	27 (100 %)		

NB: * Significant. ** Highly significant.

There was no statistical significant difference in FUO categories between both groups.

Table (4): Causes of FUO in both groups

Parameter	Group I ≥ 65 n=27	Group II <65 n=27	Test	P. Value
Infectious causes	15(55.5%)	18(66.6%)	6.8	0.14
Auto immune causes	0(0.0%)	3(11.1%)		
Malignant causes	3(11.2%)	2(7.4%)		
Miscellaneous causes	3(11.1%)	0(0.0%)		
Undiagnosed causes	6(22.2%)	4(14.8%)		
Total	27(100%)	27(100%)		

NB: * Significant. ** Highly significant**.**

There was no statistical significant difference in causes of FUO between both groups.

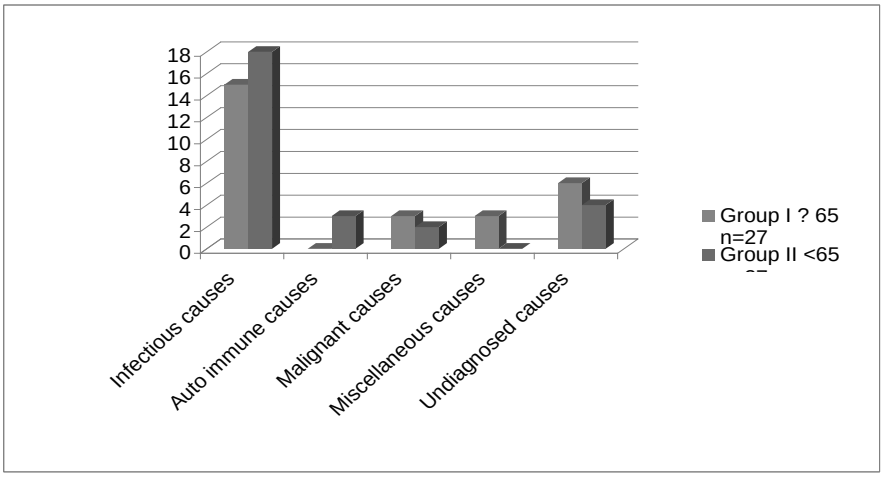

Figure (1): Causes of FUO in both groups

Table (5) : Infectious causes of FUO in both groups

Parameter	Group I ≥ 65 n=15	Group II <65 n=18	X^2	P. Value
UTI	7(46.7%)	1(5.5%)	32.2	0.00**
Brucellosis	4(26.7%)	3(16.7%)	2.3	0.12
chronic calcular cholecystitis	2(13.3%)	0(0.0%)	11.07	0.0003**
Typhoid fever	0(0.0%)	3(16.7%)	14.7	0.0001**
CMV	0(0.0%)	1(5.5%)	3.6	0.055
EBV	0(0.0%)	1(5.5%)	3.6	0.055
HIV	0(0.0%)	3(16.7%)	14.7	0.0001**
Malaria	0(0.0%)	1(5.5%)	3.6	0.055
Infective endocarditis	0(0.0%)	2(11.1%)	9.1	0.002*
Intra-abdominal abscess	0(0.0%)	2(11.1%)	9.1	0.002*
T.B	2(13.3%)	1(5.5%)	3.23	0.07
Infectious causes	**15(100%)**	**18(100%)**		

NB: * Significant. ** Highly significant**.**

Elderly group showed statistical significant increase in urinary tract infection (UTI) and chronic calcular cholecystitis, while non-elderly group showed statistical significant increase in typhoid fever, HIV infection, infective endocarditis and intra-abdominal abscess when compared to elderly group.

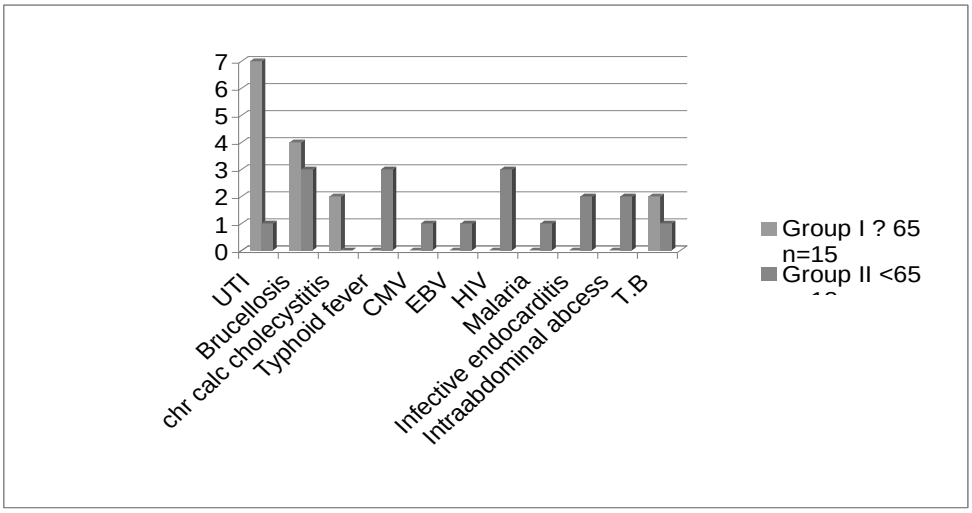

Figure (2): infectious causes of FUO in both groups

Table (6): Non infectious causes of FUO in both groups

Parameter	Group I ≥ 65 n=6	Group II <65 n=5	Test	P. Value
Auto immune thyroiditis	0(0.0%)	1(20%)	18.05	0.00**
SLE	0(0.0%)	2(40%)	38.02	0.00**
HCC	2(33.2%)	0(0.0%)	31.2	0.00**
Bone marrow carcinoma	1(16.7%)	2(40%)	9.5	0.001**
Post chemotherapy	1(16.7%)	0(0.0%)	14.7	0.0001**
Drug fever	1(16.7%)	0(0.0%)	14.7	0.0001**
Septic shock &multi organ failure	1(16.7%)	0(0.0%)	14.7	0.0001**
Total	6(100)	5(100)	----	-----

NB: * Significant. ** Highly significant**.**

Elderly group showed statistical significant increase in hepatocellular carcinoma, post chemotherapy, drug fever and septicemia while non-elderly group showed statistical significant increase in auto immune disorder when compared to elderly group.

DISCUSSION

Fever of undetermined origin constitutes one of the greatest challenges of clinical practice; it has been categorized into four categories: classic, nosocomial, Immune deficient and HIV associated FUO **[3]**. The diagnostics of FUO in the elderly often differs from the young patients, the manifestation of a disease is often non specific in older patients, physiologic reserves are diminished in the elderly as well as their immunity **[5]**.

This study was conducted to determine causes, clinical presentations and the laboratory findings of FUO among elderly persons ≥65 years in comparison with younger patients and define the most common causes responsible for FUO in elderly.

In this study continuous fever pattern formed 51.9% of cases in elderly group ≥65 and 25.9% of cases in group <65. The higher incidence of continuous pattern in elderly group ≥65 is due to the increase of UTI in this group. It was reported by John Marx **[18]** that, UTI is accompanied with continuous fever pattern. Sweating was less prominent in elderly group ≥65 due to disturbance of autonomic nervous system by chronic illness as diabetic neuropathy, uremia and excessive drugs intake **[19]**.

In this study serum creatinine showed statistically significant increase in elderly group, although it was still in the normal range. It may remain within the reference range despite marked renal impairment in patients with low muscle mass, so the sensitivity of serum creatinine for the early detection of kidney disease is poor **[20]**.

In the present study, there was no statistical significant difference in FUO categories (classic,

nosocomial, Immune deficient and FUO associated with HIV infection) between both groups.

In this study 55.5% of cases in elderly group were due to infection versus 66.6% of cases in the other group. 11.1% of cases were in group <65 due to auto immune causes. In the elderly group malignant causes represent 11.2% of cases versus 7.4% of cases in group <65. Miscellaneous causes as post chemotherapy, drug fever and septicemia represent 11.1% in elderly group ≥65 and not present in group <65 and undiagnosed causes were 22.2% in elderly group ≥65 while 14.8% in group <65. Ankunda et al. **[22]** reported that high incidence of HIV in young adults due to sexual activity and IV drug abuse

In studied infectious causes; elderly group showed statistical significant increase in urinary tract infection. The higher incidence of UTI in in group ≥65 was due to risk factors including uncontrolled diabetes, stones, urinary catheter, increase size of prostate and uterine prolapse **[21]**. The non-elderly group showed statistical significant increase in typhoid fever, HIV infection, infective endocarditis and intra-abdominal abscess. Ankunda et al. **[22]** reported that high incidence of HIV in young adults due to sexual activity and IV drug abuse. Infective endocarditis was due to rheumatic cardiac valvular lesions.

This study agreed with previous studies where infections are the commonest cause of FUO. Ammari **[23]** and MIR et al. **[24]** mentioned that infections were ranged from 41.3% to 53% of cause of FUO. On the other hand the current results disagreed with the results of studies of Knockaert et al. **[25]** and Naito et al. **[26]** who reported that infections to be responsible for 25.5% and 23.1%, of cases of FUO. This may be due to difference in geographical distribution of infectious diseases.

In this studied groups, 11.1% of cases were due to auto immune causes that were prominent in group <65 and not present in elderly group ≥65, where SLE formed 40% of noninfectious causes while auto immune thyroiditis formed 20% of noninfectious causes. This agreed with MIR et al. **[24]** and Ammari **[23]** who reported auto immune causes to be responsible for 12% of cases of FUO. However disagreed with Stamatis et al. **[27]** and Naito et al. **[26]** who reported auto immune causes to be responsible for 33% and 30.6% of cases of FUO. This may be explained by genetic difference and exposure for provocative factors.

In this study 11.2% of cases were due to malignant causes in elderly group ≥ 65, while 7.4% of cases were due to malignant causes in group <65, where hepatocellular carcinoma formed 33.3% of non-infectious causes in elderly group ≥65. Bone marrow carcinoma formed 16.7% of noninfectious causes in elderly group ≥65 and 40% of noninfectious causes in group <65.

The high incidence of hepatocellular carcinoma in our study because there is number of patients had chronic hepatitis C virus infection which predispose to hepatocellular carcinoma. This study agreed with Knockaert et al. **[25]** and Hu et al. **[28]** who reported malignant causes to be responsible for 12% and12.7% of cases of FUO. However disagreed with Esposito and Gleckman **[29]** and Ali-Eldin et al. **[30]** who reported malignant causes to be responsible for 23.4% and 30.1% of cases of FUO.

In current study miscellaneous causes as post chemotherapy, drug fever and septicemia represent 11.1% in elderly group ≥ 65 and not present in group <65. This agreed with Knockaert et al. **[25]** and Naito et al. **[26]** who reported miscellaneous causes to be responsible for 10.6% and 12.4% of cases of FUO. However disagreed with MIR et al. **[24]**, Kejariwal et al. **[31]** and Ammari **[23]** who reported miscellaneous causes to be responsible for 4.3%, 5% and 23% of cases of FUO. This discrepancy may be due to different study population.

In our study, undiagnosed causes were 22.2% in elderly group ≥65 versus 14.8% in group <65. This agreed with Ali-Eldin et al. **[30]**, Hu et al. **[28]** Kejariwal et al. **[31]** who reported undiagnosed causes to be responsible for 12.9%, 14.1% and 14% of cases of FUO in group <65. Also agreed with Naito et al. **[26]** MIR et al. **[24]** and Stamatis et al. **[27]** who reported undiagnosed causes to be responsible for 23.1%, 23% and 20.5% of cases of FUO in elderly group ≥ 65.

CONCLUSION

We concluded that, Urinary tract infection, chronic calcular cholecystitis, malignant causes (including hepatocellular carcinoma) and miscellaneous causes (as post chemotherapy, drug fever and septicemia) are important causes for FUO in elderly patients. Non-elderly group showed statistical significant increase in auto immune disorders when compared to elderly group. Also we found that categories

of FUO (classic, nosocomial, Immune deficient, and FUO associated with HIV infection) in elderly patients did not differ from non-elderly patients.

Limitation of the study: Further studies with larger sample size are needed to obtain more accurate statistical analysis of the causal categories or studying of the individual causal categories alone.

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Conflicts of interest: None.

Ethical Approval: Approved

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Digital Clubbing may be a Clinical Sign of Gastrointestinal Kaposi Sarcoma in a HIV-Positive Patient

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Key words: *Digital clubbing , HIV*

A 48-year-old human immunodeficiency virus (HIV) positive male patient presented with two months history of intermittent left iliac fossa pain, tenesmus, and gradual enlargement of fingertips. He denied diarrhea, cough, fever and weight loss. Home medications included recent initiation of highly active antiretroviral therapy (HAART).

Seven months before, he had been hospitalized for two months with cutaneous Kaposi sarcoma diagnosed by skin biopsy, with no other AIDS-related illness. His cutaneous lesions were healed with chemotherapy. After the HAART had been started, the absolute CD4 cell count increased to 550 cells/μL, and HIV-RNA became undetectable. As he had episodes of abdominal pain and diarrhea, colonoscopy and endoscopy were performed during hospitalization. These endoscopic exams revealed inflammatory lesions, and ulcered nodules in gastric, ileal, colonic and retal regions. Typical volcano-like reddish and bleeding masses with central umbilication and ulceration were seen[1]. Biopsy specimens obtained from these lesions showed eosinophils and lymphoplasmocytic cell infiltrates. *Cryptosporidium* oocyst was not found and a culture for bacteria, mycobacteria and fungi were negative.

A recent digital clubbing and edema in lower limbs were observed. Biochemical markers were normal, including amylase, lipase, albumin, and liver function panel. Evaluation with computed tomography imaging of the thorax/abdomen/pelvis was unremarkable. After five months of the first endoscopic examination performed during his hospitalization, a new endoscopy and colonoscopy with biopsy were ordered. The biopsy specimens revealed whorls of spindle-shaped cells and neovascularization with small-vessel proliferation suggestive of Kaposi sarcoma (KS). Immunohistochemical testing for human herpes virus 8 (HHV-8) was positive, supporting the KS diagnosis. The differential diagnosis of this case could have been Crohn Disease, induced by an immune reconstitution inflammatory syndrome (IRIS), after HAART start; or a chronic viral, bacterial or parasitary colitis.

Kaposi Sarcoma (KS) physiopathology includes HHV-8 mutagenic potential to create an angiogenic tumor and hypervascularity **[1]**. About 50% of gastrointestinal (GI) Kaposi Sarcoma cases are reported in HIV patients with cutaneous manifestations of KS **[1,2]**. Digital clubbing (DC) is characterized by a focal bulbous enlargement of the terminal segments of the fingers and toes due to the proliferation of connective tissue between the nail matrix and the distal phalanx. Although clubbed fingers are mostly asymptomatic, it often predicts the presence of some severe underlying diseases **[3]**. There are only a few reports linking HIV infection and clubbing **[4]**. However, direct linking of HIV to

clubbing is still a matter of controversy. Clubbing in HIV-infected patients has been attributed to concomitant pulmonary, neoplasic and hepatic illness **[5,6]**. However, in the present patient the arterial blood gas, computed tomography and magnetic resonance imaging of the thorax/abdomen/pelvis, echocardiogram, pulmonary function test and specific laboratory tests were normal. As in the patient the only disease detected was GI KS, his digital clubbing may be related to gastrointestinal KS. The patient had no Intestinal Inflammatory Disease history before AIDS diagnosis, so that IRIS occurrence would be improbable. Increase of pro-inflammatory cytokines and Vascular Endothelial Growth Factor in HIV can lead to DC and KS development **[6]**. The majority of GI KS cases remain underdiagnosed; these data indicate the importance of digital clubbing among the large range of HIV-positive patients nail manifestations **[4]**, now including patients with gastrointestinal disorders, as our case of Kaposi Sarcoma disease.

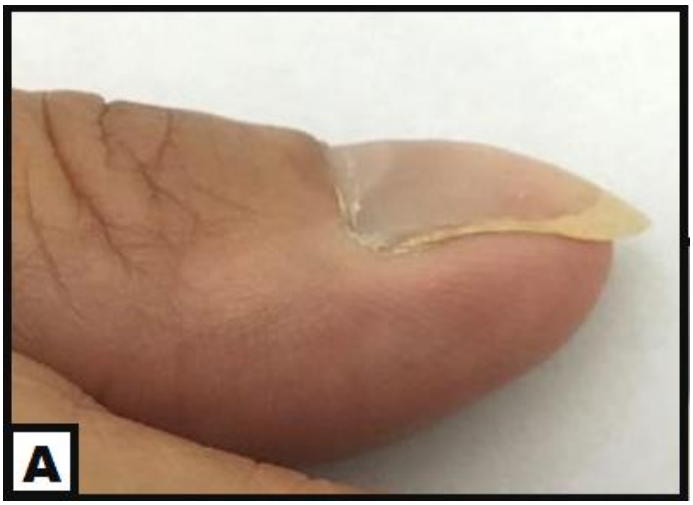

Figure A: Digital clubbing

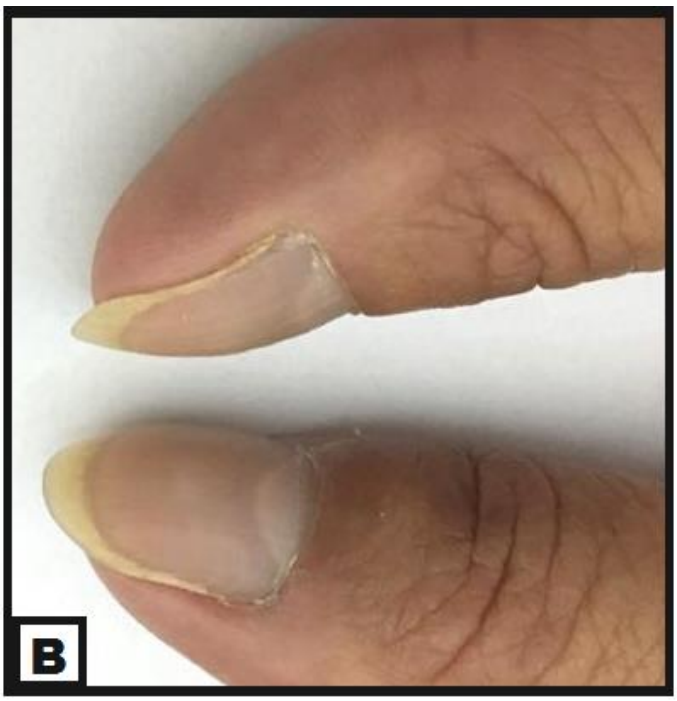

Figure B: Bulbous enlargement of soft parts of the terminal phalanges

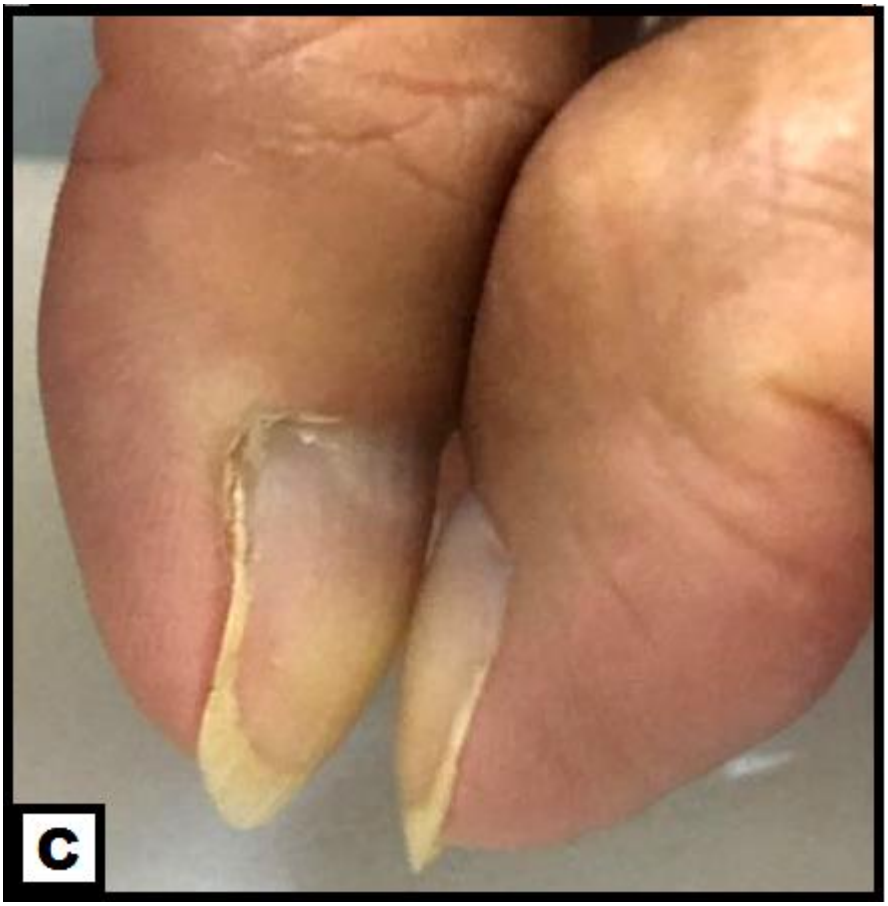

Figure C: Schamroth's sign

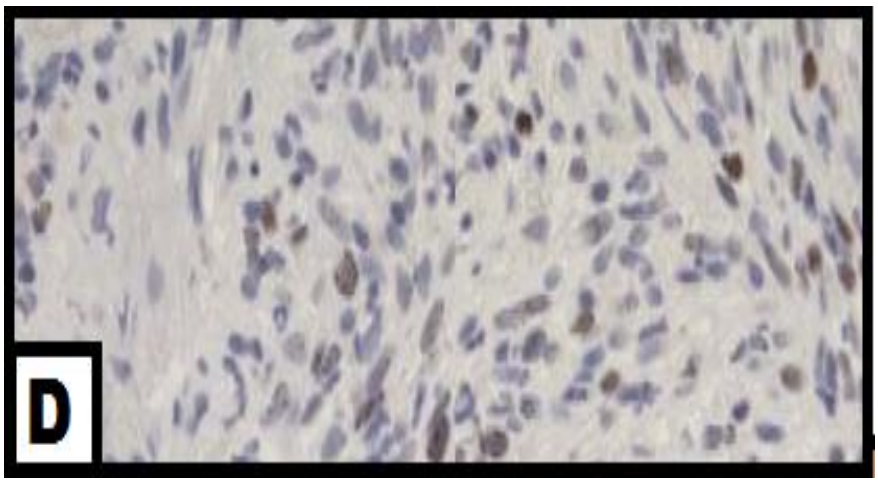

Figure D: Immunohistochemical stain of the stomach mucosa cells for Human Herpes Virus 8

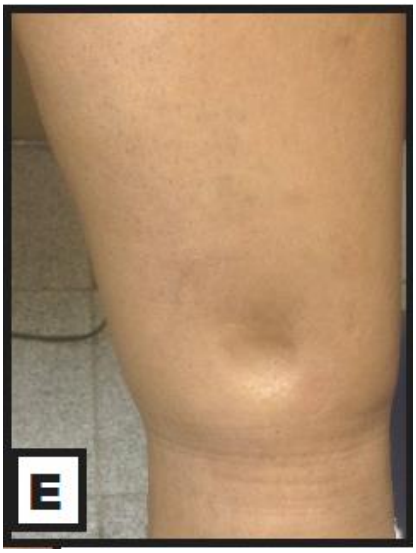

Figure E: Leg Edema

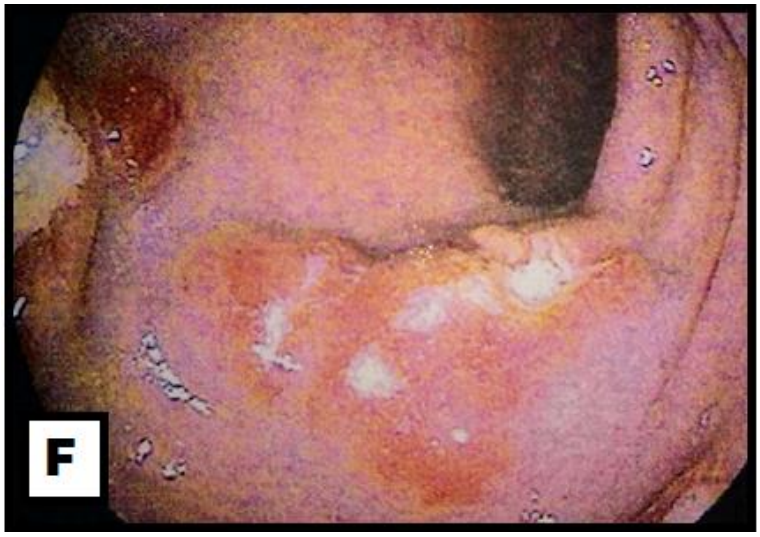

Figure F: Inflammatory sections of the ileum.

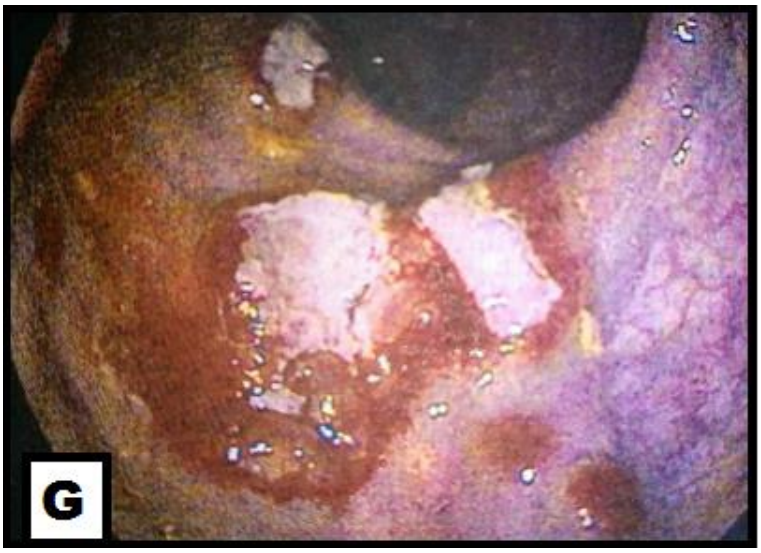

Figure G: Ulcerated and bleeding colon regions covered by fibrin

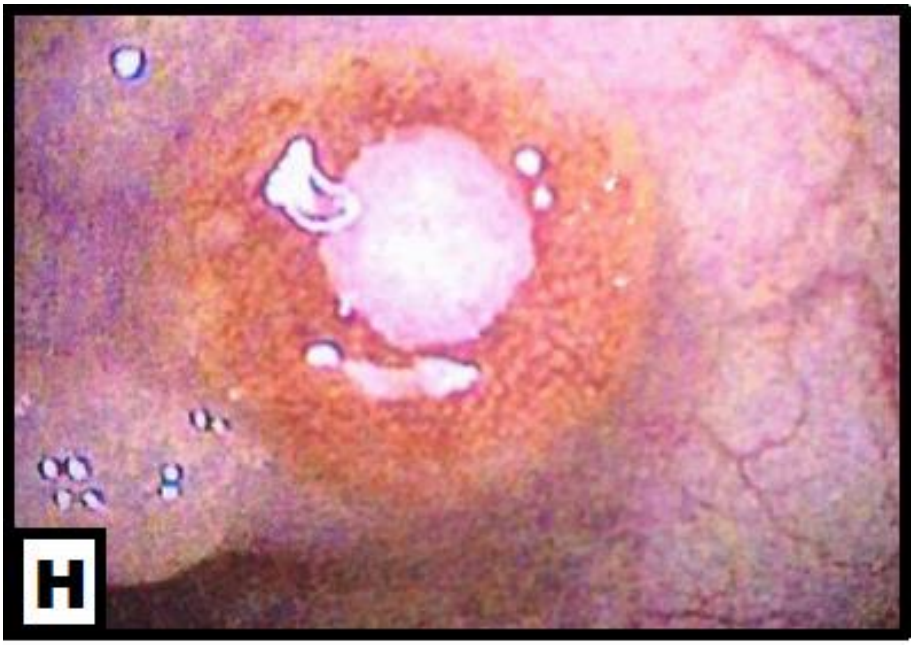

Figure H: Umbilicated rectal nodule

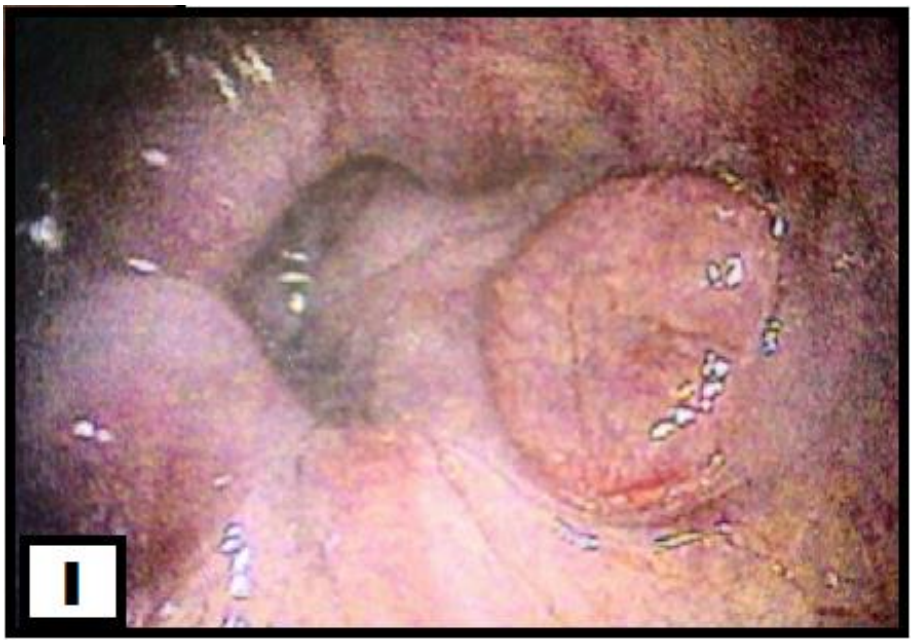

Figure I: Endoscopic image of the gastric antrum with Kaposi's sarcoma umbilicated lesions.

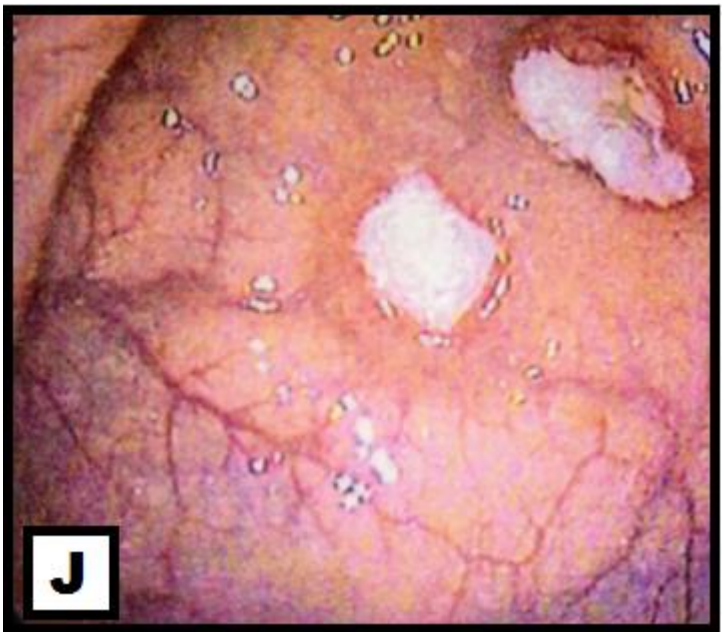

Figure J: Sigmoid colon ulcers.

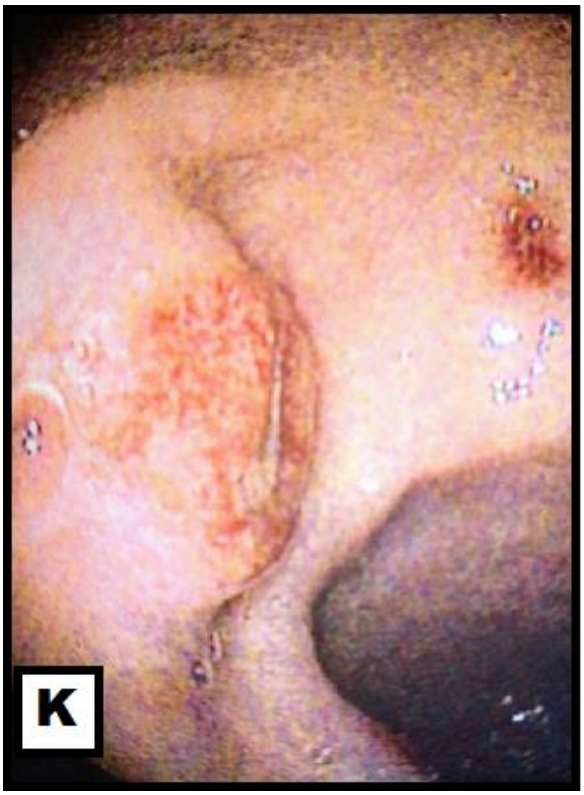

Figure K: Ulcerated duodenal nodule

Figure 1: A,B- Digital clubbing of the thumb. **C-** Schamroth's sign. **D-** Immunohistochemical stain of the stomach mucosa cells for human herpes virus 8. **E-** Leg edema. **F-** Inflammatory sections of the ileum. **G-** Ulcerated and bleeding colon regions covered by fibrin. **H-** Umbilicated rectal nodule. **I-** Endoscopic image of the gastric antrum with Kaposi's sarcoma umbilicated lesions. **J-** Sigmoid colon ulcers. **K-** Ulcerated gastric nodule.

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Role of Serum Midkine Level as a Diagnostic Biomarker for Very Early and Early Hepatocellular Carcinoma

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Background and study aim: AFP is the most commonly utilized biomarker for HCC although it has a low sensitivity and specificity for the disease. New biomarkers with better sensitivity and specificity are to be studied. The aim of this work is to evaluate the role of midkine as a biomarker for early detection of HCC and for detection of HCC in patients negative for AFP.

Patients and Methods: This study included 46 HCC patients on top of Child Pugh class A cirrhosis (group A), 46 patients with Child Pugh class A cirrhosis without liver focal lesion(s) (group B) and 46 apparently healthy controls (group C). Demographics, clinical data, radiologic findings, biochemical profile including serum AFP and midkine assessment using specific ELISA tests of the study participants were entered in the study.

Results: Serum midkine had a statistically significant better sensitivity, specificity, positive predictive value, negative predictive value and accuracy over serum AFP for diagnosing very early and early HCC from liver cirrhosis and for diagnosing AFP negative HCC from liver cirrhosis.

Conclusion: Serum midkine is to be considered for diagnosis of very early and early HCC especially in AFP negative cases.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of malignancy related mortality all over the world with incidence rising both in the USA and abroad **[1]**. Globally, there are nearly 700,000 new cases of HCC reported each year. Studies show that the incidence rate continues to approximate the death rate indicating that most of the patients who develop HCC die from it. Five-year survival rates in the USA have modestly improved to approximately 26%. This improvement can be attributed to the advanced surveillance in identifiable high-risk groups (eg, patients of chronic infection with hepatitis B and C viruses) and to the improvement in surgical intervention (resection or transplantation) for patients with early stage disease **[2]**.

According to the revised version of Barcelona Clinic for Liver Cancer (BCLC) system released by the American Association for the Study of Liver Diseases (AASLD) **[3]**, HCC is defined as very early when one nodule smaller than 2 cm is present in a cirrhotic liver Child-Pugh class A with no symptoms and no change in the performance status. Early HCC is defined when one nodule smaller than 5 cm or up to 3 nodules smaller than 3 cm each is/are present in a cirrhotic liver Child-Pugh class A or B with no symptoms and no change in the performance status.

HCC incidence is increasing in Egypt. The current increase in incidence of HCC among Egyptians may be due to the HCV epidemic in the last 3 decades in Egypt. HCV is regarded as a primary risk factor for HCC among Egyptians **[4]**. HCC is the second most frequent cause of malignancy incidence and mortality among Egyptian men after bronchogenic carcinoma **[5]**.

Although histopathologic evaluation of a tumor biopsy is considered the gold standard for establishing the diagnosis of HCC, it is considered an invasive technique with a high risk of seedling of tumor cells along the biopsy track **[6]**. As regard serologic screening, AFP still represents the most commonly utilized test for HCC although it has a sensitivity of 39 to 65% and there is a high rate of false negative and false positive results **[7]**. Many studies showed that des-γ-carboxyprothrombin and AFP-L3 were not of better sensitivity nor specificity for diagnosis of early HCC **[8]**. This highlights the need for new more reliable non-invasive biomarkers with better sensitivity and specificity for the early diagnosis of HCC and for diagnosis of AFP negative HCC.

Midkine (MDK) - also known as neurite growth promoting factor 2 (NEGF2) - is a basic heparin binding growth factor of low molecular weight. In humans, it is encoded by the MDK gene on chromosome 11 **[9]**. It is a developmentally important retinoic acid responsive gene product strongly induced during the mid gestation; hence the name midkine. Expression of the MDK gene in human adult tissues is extremely low and restricted. Different studies showed that MDK plays a significant role in carcinogenesis-related activities including proliferation, migration, antiapoptosis, mitogenesis, transformation and angiogenesis in many types of solid tumors including hepatocellular carcinoma **[10]**. The clinical utility of serum MDK evaluation has been previously studied in pancreatic cancer **[11]**, neuroblastoma **[12]**, oral squamous cell carcinoma **[13]**, esophageal squamous cell carcinoma **[14]** and breast cancer **[15]**.

In the present study, we aimed at investigating the diagnostic utility of serum MDK evaluation as a biomarker for very early and early HCC and especially for AFP negative cases.

PATIENTS AND METHODS

This is a case-control study that included 46 patients diagnosed with HCC (37 males and 9 females with age in the range of 45-88 years) as the case group and 46 cirrhotic patients (34 males and 12 females with age in the range of 45-80 years) as the disease control group. All these patients were admitted to Tropical Medicine Department affiliated to Zagazig university hospitals in the period from June, 2016 to February, 2017. Also, 46 healthy subjects selected from patients' relatives (32 males and 14 females with age in the range of 44-87 years) were included and represented the healthy control group.

All study participants exhibited good compliance and provided a written consent to be included. Patients with HCC and naïve to treatment (diagnosed by triphasic CT criteria and / or by histopathology according to AASLD guidelines) were included in group (A). Cirrhotic patients with no evidence of hepatic focal mass(es) in ultrasound evaluation were included in group (B). Diagnosis of liver cirrhosis was based on clinical, laboratory and imaging studies. All patients included in groups A and B were Child-Pugh class A. Healthy controls included in group (C) were selected from patients' relatives and were free from any clinical, laboratory and sonographic abnormalities.

Patients who had any malignancy other than HCC, those who had a history of intravenous administration of heparin prior to evaluation of their serum midkine by 48 hours, those who had rheumatoid arthritis, Child-Pugh classes B and C cirrhotic patients and those having end stage major organ disease were excluded from the study.

All participants were subjected to history taking, thorough clinical examination and laboratory investigations in the form of complete blood count, liver function tests, kidney function tests, coagulation profile, viral markers (HCV Ab and HBsAg) and alpha fetoprotein assessment. These investigations were done by the conventional methods used.

Midkine assessment was done for all participants. The assay was performed using enzyme-linked immunosorbent assay (ELISA) kits supplied by Glory Science (Glory Science Co., Ltd, 2400 Veterans Blvd. Suite 16- 101, Del Rio, TX 78840, USA).

Imaging studies were performed for all patients. Pelviabdominal ultrasonography using sonoscape c11 was done. The liver was examined for its size, surface, echogenicity and hepatic viens. The portal vein diameter and splenic axis were measured. A special emphasis regarding the HCC masses was done including their number, site, size, echogenicity and any special character.

Triphasic computed tomography (CT) with contrast of the liver was performed for patients with hepatic focal lesion(s) in ultrasonography.The

diagnostic character of HCC on contrast-enhanced CT is arterial hypervascularity with washout of intra-lesional contrast in portal venous and delayed phase images **[16]**.

Data were checked, entered and analyzed through Epi-Info (2000) for data processing and statistics. Data were expressed as numbers and percentages for qualitative variables and mean, standard deviation, median and interquartile range for non parametric quantitative data. Comparison of means was done using standard (t) test, Mann-Whitney test, one way ANOVA (f test), Kruskal Wallis test and Chi-square test (X^2). Validity of a screening test was expressed as sensitivity, specificity, positive predictive value, negative predictive value and accuracy. For all these statistical tests used, the threshold of significance was fixed at 5% level (P-value). The smaller the P-value obtained, the more significant were the results.

RESULTS

Table (1): Criteria of hepatic focal lesion(s) in group A

Criteria of hepatic focal lesions	Group A (HCC patients) (n=46)	
	No	%
Number of focal lesion		
Single	39	84.78
Multiple	7	15.22
Size of focal lesion		
≤2 cm	17	36.95
2-5 cm	29	63.05
Portal vein thrombosis		
No	46	100
Yes	0	0

Table (2): Serum AFP and midkine levels among the studied groups

Tumor markers	Group A (HCC patients) (n=46)	Group B (Liver cirrhosis) (n=46)	Group C (Control group) (n=46)	Test	P1	P2	P3
AFP (ng/ml)							
Median	91	16.4	1.9	Kruskall Wallis 73.4	0.02	0.00	0.00
Range	1.1 – 761	9.4 – 23	1.6 – 2.2				
Q1-Q2	7.3-404	12.5-18.5	1.8-2.08				
Midkine (ng/dl)							
Mean ± SD	62.6 ± 26	25.8 ± 9.9	19.1 ± 4.2	F=93.5	0.00	0.00	0.00
Range	22 – 165	15 – 75	0 – 0.6				

P1 denotes p value of significance test comparing between groups A and B.
P2 denotes p value of significance test comparing between groups A and C.
P3 denotes p value of significance test comparing between groups B and C.

Table (3): Diagnostic performance of the best cut off values of midkine and AFP in detecting HCC versus non hepatocellular carcinoma controls

	Cut off	AUC 95% CI	Sensitivity	Specificity	+ve predictive value	-ve predictive value	Accuracy	P
Midkine (ng/dL)	34	0.94 (0.90-0.99)	91%	90%	89.3%		91.1%	0.00
AFP (ng/mL)	21.5	0.63 (0.5-0.7)	56 %	90%	81.2%	66.6%	71	0.02

Table (4): Diagnostic performance of the best cut off values of midkine and AFP in detecting very early HCC (≤2cm) versus non hepatocellular carcinoma controls

	Cut off	AUC 95% CI	Sensitivity	Specificity	+ve predictive value	-ve predictive value	Accuracy	P
Midkine (ng/dL)	32	0.8 (0.7-0.9)	94%	91.3%	66.6%	98.8%	91	0.00
AFP (ng/mL)	18.5	0.7 (0.5-0.8)	70%	86%	50%	94%	84	0.00

Table (5): Diagnostic performance of the best cut off values of midkine and AFP in detecting early HCC (2-5cm) versus non hepatocellular carcinoma controls

	Cut off	AUC 95% CI	Sensitivity	Specificity	+ve predictive value	-ve predictive value	Accuracy	P
Midkine (ng/dL)	27.5	0.9 (0.8-0.9)	96%	82.6%	73.3%	98.7%	87.6	0.00
AFP (ng/mL)	19.5	0.6 (0.5-0.8)	50%	80%	76.4%	94.2%	89.8	0.00

Table (6): Diagnostic performance of the best cut off values of midkine in HCC with AFP-negative (<20ng/mL) from non hepatocellular carcinoma controls

	Cut off	AUC 95% CI	Sensitivity	Specificity	+ve predictive value	-ve predictive value	Accuracy	P
Midkine (ng/dL)	36.5	0.934 (0.8-0.9)	85%	88%	77%	92%	78.7%	0.00

Table (7): Comparison between HCC patients negative for AFP (< 20 ng/mL) and HCC patients positive for AFP as regard to midkine level

	HCC (low or negative AFP) N=20	HCC(high AFP) N=26	T	P
Midkine (ng/dL)				
Mean ± SD	46 ± 10.9	75 ± 28	4.7	0.000
Q1-Q3	38 – 56	62.5 – 83.5		

DISCUSSION

AFP is the only serologic biomarker commonly utilized for diagnosis of HCC. However, its sensitivity is still limited (39-65%); especially in small well-differentiated HCC. In addition, false positive levels of AFP were as high as 40% **[17]**. The normal range for serum AFP is 10–20 ng/ml and a value more than 200 ng/ml is usually regarded as of diagnostic index. However, up to 65% of HCC patients with a nodule smaller than 5 cm in diameter have serum AFP less than 200 ng/ml and up to 20% of HCC masses do not secrete AFP **[18]**. Therefore, the lack of AFP sensitivity and specificity has promoted research for new tumor biomarkers for differentiating HCC from benign hepatic lesions **[19]**.

The aim of this study was to assess the role of serum midkine measurement as a non-invasive biomarker for diagnosis of very early, early and AFP negative HCC.

The present study was conducted on 138 subjects divided into 3 groups; 46 patients with HCC as group A, 46 cirrhotic patients as group B and 46 apparently healthy control subjects as group C. All patients were matched as regard age, gender and residence. Child Pugh class A patients were included in the study while Child Pugh classes B and C were excluded. Patients with HCC were of very early and early stages of the disease. Advanced stage of HCC and cases with portal vein thrombosis were excluded from the study.

Our results revealed a significantly higher level of AFP in HCC group (median =91 ng/mL) and chronic liver disease group (median= 16.4 ng/mL) compared to the control group (median= 1.9 ng/mL). In addition, AFP values were significantly higher in HCC patients compared to chronic liver disease patients. This was in agreement with Wei et al. **[20]** and Othman et al. **[21]** who found that AFP increases in chronic liver disease patients and also proved that AFP increases significantly in HCC patients than in chronic liver disease patients.

In this study, there was a highly significant statistical difference between the mean value of serum MDK levels in patients with HCC compared to patients with liver cirrhosis and the healthy controls with mean ± SD values of 62.6 ± 26, 25.8± 9.9 and 19.1 ±4.2 ng/dL respectively ($p<0.001$). These findings are in agreement with those of Zhu et al. **[22]** who found that serum MDK was significantly elevated among HCC patients when compared with chronic liver disease patients and healthy individuals.

In the present study, MDK had sensitivity of 91% and specificity of 90% for detection of HCC at the optimal cut off value of 34 ng/dL with AUC of 0.94 when compared with liver cirrhosis while AFP had sensitivity of 56% and specificity of 90% at the optimal cut off value of 21.5 ng/mL with AUC of 0.63 when compared with liver cirrhosis. These results are similar to those of Karim et al. **[23]** who found that MDK and AFP had sensitivities of (92.5% versus 40%), specificities of (83.3% versus 96.7%) respectively. Through the analysis of the ROC curve, they found that the AUC (0.941) for serum MDK was larger than that of serum AFP (0.671).

On the contrary of the previous results, Hung et al. **[24]** found that at cut off value of 50 ng/dL, MDK had sensitivity of 51% and specificity of 60%. This difference in results can be attributed to difference in the studied population and the number of patients included in both studies. Patients included in that study had HCC complicating HBV induced liver cirrhosis and were of advanced stage of HCC while most of the patients in this study were HCC complicating HCV induced liver cirrhosis and all of them were of very early and early stages of the disease.

The best cutoff values for MDK and AFP to discriminate very early HCC ≤2 cm from non-hepatocellular carcinoma controls (liver cirrhosis patients and healthy controls) were 32 ng/dL and 18.5ng/mL respectively; with sensitivities of 94% versus 70% and specificities of 91.3% versus 86%. The AUC was 0.8 for serum MDK

and was found to be larger than that of serum AFP (0.7) with a highly significant statistical difference (P<0.001). While the best cutoff values for MDK and AFP to discriminate early HCC 2-5 cm in diameter from non-hepatocellular carcinoma controls (liver cirrhosis patients and the healthy controls) were 27.5 ng/dL and 19.5 ng/mL respectively; with sensitivities of 96% versus 50% and specificities of 82.6% versus 80%. The AUC was 0.9 for MDK and was found to be much larger than that of AFP (0.6) with highly significant statistical difference (P <0.001). This means that the overall diagnostic performance of serum MDK for diagnosis of very early HCC ≤2 cm and early HCC 2-5cm in diameter is much better than that of serum AFP. This is in agreement with Zhu et al. **[22]** who reported that serum MDK had a better performance compared with AFP for distinguishing very early hepatocellular carcinoma as well as early hepatocellular carcinoma from liver cirrhosis and healthy controls.

In this study, there was highly significant statistical difference between mean value of serum MDK in HCC patients negative for AFP (values < 20 ng/mL) compared to HCC patients positive for AFP (values >20 ng/mL) with mean ± SD values of 46 ± 10.9 and 75± 28 ng/dL respectively (p<0.001). Diagnostic performance of serum MDK in discriminating HCC negative for AFP from liver cirrhosis and healthy controls revealed that serum MDK at a cut off value of 36.5 ng/dL showed sensitivity of 85%, specificity of 88%, PPV of 77%, NPV of 92% and AUC of 0.934 (p<0.001). These results agree with results obtained by Zhu et al. **[16]** who reported that serum MDK had a good performance for distinguishing AFP-negative hepatocellular carcinomas from non-hepatocellular carcinoma controls with AUC 0.926.

Finally, this study revealed that assessment of serum MDK is to be used as a non-invasive marker for detection of HCC patients complicating chronic hepatitis C with very early and early disease stages and/or negative for AFP.

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