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Neutrophil Respiratory Burst: an immunological indicator of post stroke infection

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Abstract

Stroke is long known to be followed by a series of immunosuppressive events, and infections might be a cause of death after an acute insult of stroke. The aim of our work was to assess the percentage of neutrophils showing spontaneous oxidative burst in patients with acute ischemic stroke. The study included 30 patients with acute cerebral infarction subjected to the following: magnetic resonance imaging of the brain immediately on admission, and blood sampling on day one of admission (baseline) and after 3 days of admission. Blood samples were used for the assessment of: differential leucocyte count and percentage of neutrophils showing spontaneous oxidative burst, performed by flow cytometry. Thirty age and gender matched controls were also recruited. Neutrophil respiratory burst percentage was significantly lower in stroke patients in comparison to controls (P<0.001), and stroke patients had significantly lower neutrophil respiratory burst percent on day 3 of admission compared to the baseline (P<0.001). Stroke-induced immune alterations including impairment of the first-line defense performed by neutrophils against bacteria. The hypothesis that these changes enhance susceptibility to acquired infections is supported by our observation that oxidative burst in neutrophils was more impaired in patients with stroke who exhibited subsequent stroke-associated infections.

Keywords: Infarction; Neutrophil; Respiratory burst; Stroke.

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Introduction

Infections are common complications after acute ischemic stroke, and studies report up to 65% of stroke patients develop infections, namely chest and urinary tract, in the first few days of onset of stroke.[1] Although complications of large strokes are occasionally the main cause of death, infections like pneumonia represent a

possible cause of death after an acute stroke insult.[2]

Stroke is long known to be followed by a series of immunosuppressive events.[3] Brain ischemia leads to a cascade of local inflammatory and systemic inflammatory response and stroke has been shown to impede innate and adaptive immune cell functions resulting in serious infections.[4] Although the relationship between stroke and adaptive immune system has been investigated, little is known about the relationship between stroke and innate immunity. [5]

Neutrophils, which represent the highest percentage of white blood cell, not only participate in the immune defense against pathogens, but also integrate crucially in inflammatory reaction.[6] Improper activation of neutrophils triggers unwanted results such as autoimmune and exaggerated inflammatory reactions.[7] Neutrophils not only play a crucial role in adaptive immunity, but also stand strongly as a first line of defense in innate immunity. An important function of the neutrophils lies in its plasma membrane that contains nicotinamide adenine dinucleotide (NADPH)-oxidase enzyme, responsible for the important defense mechanism known as oxidative burst which signals microbial and parasitic killing.[8,9] Due to their complex function, immunologists contributed the orchestration of the immune/inflammatory response to neutrophils.[10]

In stroke, neutrophils are the first to infiltrate injured brain tissue. Although their infiltration enhances tissue damage and thrombus formation, they trigger removal of cell debris. However, the role of neutrophils in peripheral circulation after stroke and protection against infection is controversial.[11]

The aim of this cross-sectional, case-control study was to assess the percentage of neutrophils showing spontaneous oxidative burst in patients with acute cerebrovascular infarction in comparison to normal controls.

Subjects and Methods

This case-control study was performed on 30 patients with acute cerebral infarction recruited from the Stroke Unit of Ain Shams University Hospitals. Patients were diagnosed according to the National Institute for Health and Care Excellence (NICE guidelines 2014) and scored according to National Institute of Health Stroke Scale Score (NIHSS) (National Institutes of Neurological Disorders and Stroke, 2014).[12] The NIHSS consists of a list of 11 items; each item represents a specific ability and scored from zero to four. Zero score designates a normal function of that ability, the higher the score the more the disability. A total score of 42 (after summing up all items) is considered the highest level of impairment. Inclusion criteria for the patients were age of 18 years or more, first attack of stroke of 12 hours duration, NIHSS above 6 and C-reactive protein (CRP) less than 15mg/L. History of previous stroke, transient ischemic attack (TIA) or head injury, signs of infection before admission and patients currently on antibiotics or immunosuppressive were excluded from the study. Thirty apparently healthy age and sex matched individuals were included as a control group, recruited from relatives of patients, hospital workers and nursing staff.

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Ain Shams University (approval no. FMASU 1941/2014). An informed consent was taken from all study participants after explaining the aim of the study and ensuring the data confidentiality.

Methods

All patients were subjected to the following: Magnetic resonance imaging (MRI) of the brain was immediately performed on admission, at the Radiology Unit of Ain Shams University Hospital. The examination was done on 1.5 Tesla MRI scanner (Achieva, Philips, Healthcare, Best, the Netherlands). Routine MRI of the brain was done that included: axial DWI (B=O, 1000), axial T2WI, T2*WI, T1WI and FLAIR and sagittal T1WI. Recent infarcts were identified on the diffusion sequences by as areas of diffusion restriction. Lacunar infarcts were assigned to the small infarcts of less than 15 mm in the diameter. Larger infarcts were assigned according to the known vascular arterial territories, anterior cerebral artery (ACA),

middle cerebral artery (MCA) and posterior cerebral artery (PCA).

Collection of blood samples

Blood samples were collected from each patient and control under complete aseptic conditions at baseline (day 1 of admission) and on day 3 of admission. Two mL venous blood samples were obtained using vacutainer tube (Becton Dickinson, Oxford, UK), containing K2-EDTA (ethylenediamine tetra-acetic acid, dipotassium salt) for complete blood count (CBC) using a Coulter counter (T660, Beckman Coulter, California, USA), preparation of Leishman-stained peripheral blood smears, and flow cytometric assays. Also, 2 mL of venous blood were collected into a gel vacutainer tube (Becton Dickinson, Oxford, UK) for isolation of serum samples, for measurement of CRP level. CRP (mg/dl) was assessed with the nephelometric method using a commercial kit (Minineph Human C-reactive protein kit, supplied by Binding Site, Birmingham, UK), according to the manufacturer's instructions.

Flow cytometric assay of oxidative burst using Dihydrorhodamine (DHR) 123

The procedure used in this study was basically that described by O'Gorma and Corrochano in 1995[13] with few modifications. Stock solutions, of DHR 123 and phorbol-12-myristate-13-acetate (PMA), were made in dimethyl sulfoxide (DMSO) and stored at −20°C. Further dilutions were made with phosphate buffer saline (PBS), 0.2% Bovine serum albumin and 0.2% sodium azide. Three preparations were used per sample of patient or control. The first one with blood only, the second had blood and DHR 123 and the third had blood, DHR 123 and PMA (supplied by Sigma-Aldrich, St Louis, MO). The procedure involved pre-dilution of blood with PBA (PBS with azide) and pre-incubation with the DHR 123 for 15 minutes prior to addition of PMA. Samples were analyzed using the 488nm laser and the FITC filter set up using flow cytometer (Coulter Epics XL flow cytometer, Coulter, Electronics, Hialeah, FL, USA). Neutrophils were gated in the forward scatter/side scatterplot. After excluding doublets in a forward scatter width/forward scatter height (FSC-W/FSC-H) scatterplot, the fluorescence signal of rhodamine 123-positive cells was gated in a histogram. Fluorescence is quantitated by mean peak channel fluorescence (MPC-FL). Results were expressed as percent of neutrophils showing oxidative burst after stimulation (Normal range was 90%-100%).

Statistical methods

Data was analyzed using IBM© SPSS© Statistics version 23 (IBM© Corp., Armonk, NY, USA), and MedCalc© version 15 (MedCalc© Software bvba, Ostend, Belgium). Normality of numerical data distribution was examined using the D'Agostino-Pearson test. Normally distributed numerical variables were presented as mean ± SD and intergroup differences were compared using the unpaired *t* test (for two-group comparison) or one-way analysis of variance (ANOVA) (for multiple-group comparison). The Tukey-Kramer *post hoc* test was used for pairwise comparison whenever the ANOVA test revealed a statistically significant difference among the groups. Paired numerical data were compared using the paired *t* test. Non-normally distributed numerical variables were presented as median and interquartile range and between-group differences were compared using the Mann-Whitney test. Categorical variables were presented as ratio or number and percentage and intergroup differences were compared using fisher's exact test. Correlations among numerical variables were tested using the Pearson product- moment correlation or Spearman rank correlation as appropriate. Backward multivariable regression analysis was used to identify determinants of neutrophil respiratory burst. Numerical variables not fulfilling the assumption of normality were subjected to logarithmic transformation and were entered in the regression model as log (values). A *P*-value of <0.05 was considered statistically significant. The receiver-operating characteristic (ROC) curve analysis was used to examine the value of neutrophil respiratory burst for discrimination between cases and controls, and for prediction of the occurrence of infection of any type in cases. The area under the various ROC curves (AUC) was compared

with that of random prediction using the DeLong method.

Results

Thirty patients with acute cerebrovascular infarction and 30 age and gender matched controls were included in the study. There was no significant difference in CRP and white blood cell (WBC) count between cases and controls at baseline. Neutrophil respiratory burst percentage was significantly lower (P <0.001) in cases than controls. Demographic and laboratory data are shown in Table 1.

Table 1. Demographic and lab characteristic of the study group.

Variable	Cases (n=30)	Controls (n=30)	*P-value
Gender (male/female)	14/16	15/15	NS
Age (years)	62.9 ± 7.3	64.3 ± 7.9	NS
CRP (mg/l)	2.2 ± 1.4	1.9 ± 1.3	NS
WBC count (x1, 000/mm^3)	5.5 ± 1.2	5.8 ± 1.6	NS
Neutrophil respiratory burst percent	93.1 ± 4.1	96.6 ± 2.2	<0.001

Data are ratio or mean ± SD. CRP: C-reactive protein WBC: white blood cells. *Unpaired t test.
$P \geq 0.05$ is not significant (NS)

Clinical and radiological data of cases are demonstrated in Table 2. Cases had significantly lower neutrophil respiratory burst percent on day 3 of admission compared to baseline (P<0.001). On the contrary, CRP, WBC count and relative neutrophil count percent were significantly higher in cases on day 3 of admission in comparison to cases at baseline (P<0.001), (Table 3).

Table 2. Clinical and radiological characteristics of cases

Variable	Prevalence, no (%)
Relevant risk factors	
DM	21 (70.0%)
Hypertension	21 (70.0%)
IHD	11 (36.7%)
AF	7 (23.3%)
NIHSS	13 (9 -15), range 7 - 32
Magnetic resonance imaging (MRI) findings	
Lacunar infarction	9 (30.0%)
Branch occlusion	15 (50.0%)
Occluded main stem of MCA	6 (20.0%)

Data are number (%) or median (interquartile range). DM. diabetes mellitus; AF, atrial fibrillation; IHD, ischemic heart disease; MCA, middle cerebral artery, NIHSS NIH Stroke Scale/Score.

Table 3. Comparison of relevant hematological measures at baseline and on day 3 in cases.

Variable	Baseline	Day 3	P value
	Mean±SD	Mean±SD	
CRP (mg/l)	2.2±1.4	27.1±25.5	<0.001
WBC count (x1, 000/mm^3)	5.5±1.2	12.3±5.7	<0.001
Relative neutrophil count percent	52.0±7.6	70.3±17.0	<0.001
Neutrophil respiratory burst percent	93.1±4.1	68.2±24.7	<0.001

CRP: C-reactive protein WBC: white blood cells. *$P \leq 0.05$ is significant.

Prescence of infection among cases is represented in Figure 1. Among cases of acute ischemic stroke, 19 (63.3%) patients developed infection with a statistically significant increased length of hospital stay (range 7-35 days for cases with infection versus 6-7 days in cases with no evidence of infection) (Table 4). Of the 30 study cases, 5 (16.7%) cases had uncomplicated UTI, while 1 case (3.3%) had complicated UTI. Two patients (6.7%) had catheter related blood stream infections (CRSBI), (Table 4).

A significant inverse relation was found between baseline neutrophil respiratory burst in cases (*P*=0.007) and the presence of infection on day 3 (*P*<0.001), (Table 5).

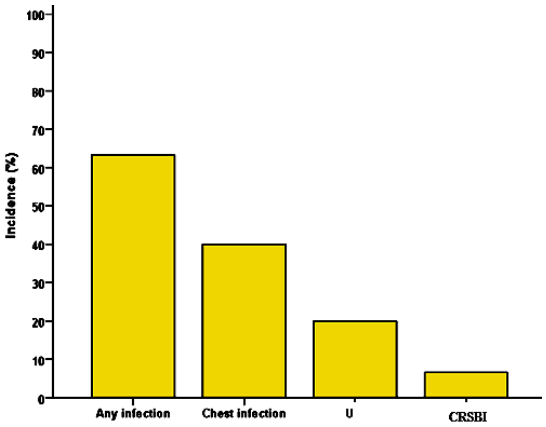

Figure 1. Prescence of infection among cases. UTI: urinary tract infection, CRSBI: Catheter related blood stream infection.

Table 4. Incidence of infection and length of hospital stay in study cases.

Variable	Value
Incidence of infection (pneumonia, UTI, Catheter related blood stream infection (CRSBI)	19 (63.3%)
Pneumonia	
No pneumonia	19 (63.3%)
Pneumonia requiring IV antibiotics	7 (23.3%)
Severe Pneumonia with respiratory failure	4 (13.3%)
UTI	
No UTI	24 (80.0%)
Non-Complicated UTI	5 (16.7%)
Complicated UTI with Pyelonephritis	1 (3.3%)
Catheter related blood stream infection (CRSBI)	
No Catheter related blood stream infection	28 (93.3%)
IV Catheter related blood stream infection	2 (6.7%)
Hospital LOS (days)	15 (7 -19), range 6 -35

Data are number (%) or median (interquartile range). UTI: Urinary tract infection, LOS: length of hospital stay.

Table 5. Relation between neutrophil respiratory burst and the presence of infection.

Presence of infection	Neutrophil respiratory burst (%)	
	Baseline	Day 3 (%)
Infection (n=19) Mean± SD	91.6±4.0	56.1±23.3
No infection (n=11) Mean± SD	95.6±2.9	89.1±5.7
**P*-value¶	0.007	<0.001

*Unpaired *t* test; *t*, t statistic; *df*, degree of freedom. **P* ≤ 0.05 is significant.

Table 6 shoes the correlation between WBC count and CRP level (on day 3) and neutrophil respiratory burst. There was a statistically significant (*P*<0.0001) inverse correlation between WBC count and CRP level (on day 3) and neutrophil respiratory burst. Additionally,

stroke severity (NIHSS) and length of hospital stay showed statistically significant (*P*<0.0001) inverse correlation with neutrophil respiratory burst on day 3 (Figures 2 and 3).

Table 6. Correlation between neutrophil respiratory burst and other relevant numerical variables.

		Neutrophil respiratory burst	
		Baseline	Day 3
Baseline neutrophil respiratory burst	Correlation coefficient	-	0.535
	P-value	-	0.002
Neutrophil respiratory burst on day 3	Correlation coefficient	0.535	-
	P-value	0.002	-
Age	Correlation coefficient	-0.209	-0.087
	P-value	NS	NS
NIHSS	Correlation coefficient	-0.484	-0.852
	P-value	0.007	<0.0001
CRP on day 3	Correlation coefficient	-0.575	-0.931
	P-value	0.001	<0.0001
WBC count on day 3	Correlation coefficient	-0.588	-0.853
	P-value	0.001	<0.0001
Baseline relative neutrophil count	Correlation coefficient	-0.003	-0.255
	P-value	NS	NS
Relative neutrophil count on day 3	Correlation coefficient	-0.444	-0.710
	P-value	0.014	<0.0001
Hospital LOS	Correlation coefficient	-0.422	-0.848
	P-value	0.020	<0.0001

NIHSS NIH Stroke Scale/Score, CRP C-Reactive Protein, WBC white blood cells, LOS lengths of stay.
P ≥ 0.05 is not significant (NS).

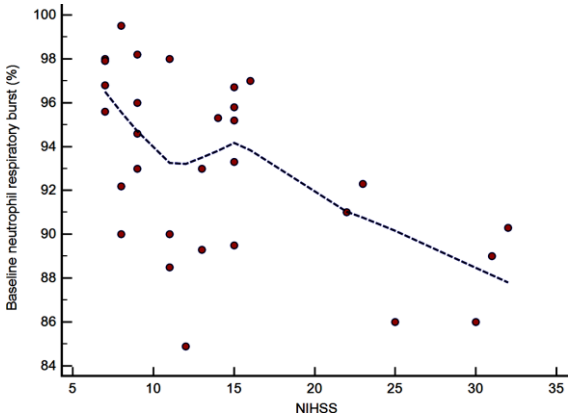

Figure 2. Scatter plot showing the correlation between the National Institute of Health Stroke Scale (NIHSS) Score and baseline neutrophil respiratory burst in the studied cases. Fitted line represents local regression smoothing (LOESS) trend line. NB: LOESS is a technique for smoothing data characterized by a lot of scatters.

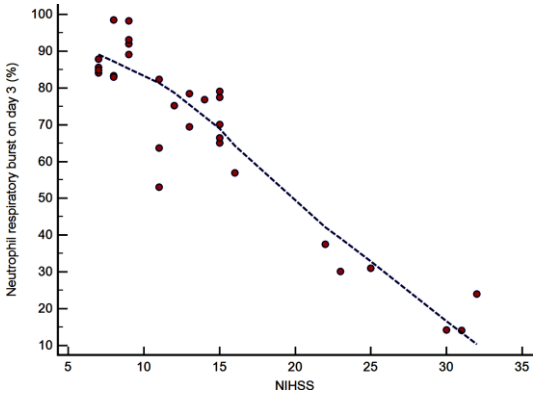

Figure 3. Scatter plot showing the correlation between the National Institute of Health Stroke Scale (NIHSS) score and neutrophil respiratory burst on day 3 in the studies cases. Fitted line represents local regression smoothing (LOESS) trend line. NB: LOESS is a technique for smoothing data characterized by a lot of scatters.

There was a significant relationship between baseline neutrophil respiratory burst percent in cases and size of occluded cerebral vessels; lacunar branch and main stem occlusion on MRI; the larger the occluded vessel, the lower the neutrophil respiratory burst percent (P=0.001) and (P<0.001) at baseline and on day 3, respectively (Figure 4).

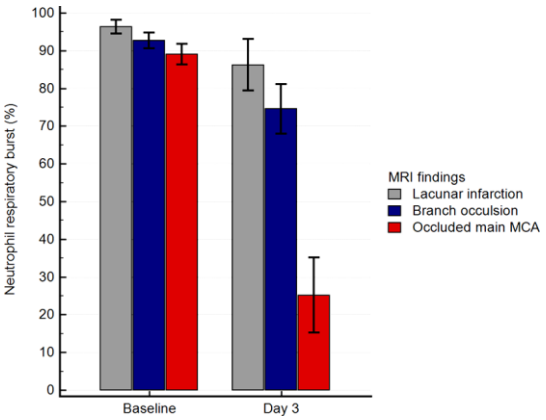

Figure 4. Neutrophil respiratory burst at baseline and on day 3 in cases with lacunar infarction, branch occlusion, or main middle cerebral artery (MCA) occlusion. Error bars represent 95% confidence limits (95% CI).

The ROC curve for discrimination between cases and controls using the baseline neutrophil respiratory burst showed a significant difference in neutrophil respiratory burst percentage between cases and control with sensitivity 53% and specificity 90% (Figure 5).

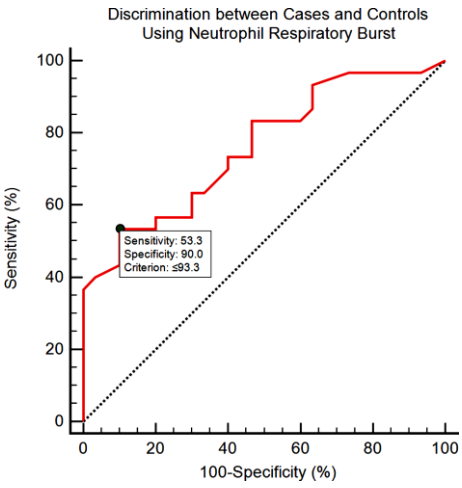

Figure 5. Receiver-operating characteristic (ROC) curve for discrimination between cases and controls using the baseline neutrophil respiratory burst.

Discussion

The exact role of neutrophils in the dynamics of immune cell infiltration in stroke is under ongoing research.[14, 15] We aimed to measure the percentage of neutrophils showing spontaneous oxidative burst in patients with acute ischemic stroke (samples collected at baseline and on day 3 of stroke) in comparison to age and gender matched controls, and to determine the incidence and severity of infection, as well as the length of hospital stay in patients with stroke.

Of the studied cases, 70% had hypertension and diabetes mellitus, 36.7% had ischemic heart disease and 23.3% had atrial fibrillation (AF). Comparison of neutrophil respiratory burst in cases on day 1 and day 3 revealed a statistically significant (P<0.001) higher neutrophil respiratory burst at baseline. Similar findings were reported by Seki, et al., 2009 in patients with hemorrhagic stroke, and it was speculated that neutrophil respiratory burst might be strictly controlled in the blood stream after stroke to protect the endothelium from excessive oxidative damage.[16] Two theories were postulated to explain this immune suppression. One theory suggested that exhaustion of immunity occurred due to sustained inflammatory response, while the alternative theory suggested that the immune suppression is a protective action of brain to alter more cerebral.[17]

Neutrophils with a low oxidative burst have diminished bactericidal action which impairs the host's defense mechanism thus accounting for the frequent infectious complications encountered after hemorrhagic stroke.[16] Some studies attributed the reduction of peripheral lymphocytes to the atrophy of peripheral lymphatic organs caused after a stroke insult and the ability of T cells to make mitogen-induced cytokine production and proliferation declines after brain ischemia.[18,19] Overall, these mechanisms work together to dampen the inflammatory immune responses and minimize further immune pathology in the brain, although this comes at the cost of susceptibility to infections in the host.[20]

In our study, 63.3% of the study patients showed signs of infection on day 3. This is

consistent with previously published literature reporting that between 23% and 65% of patients acquire infection after stroke, with pneumonia and urinary tract infections (UTI) being the most common infections.[1,21] Also, 23.3% of study patients had respiratory tract infections (RTI) necessitating IV antibiotics, and 16.7% had RTI complicated with respiratory failure. Hilker, et al., reported that the incidence of stroke associated pneumonia was 21.4% [22]

Of the study cases, 16.7% had uncomplicated UTI, while 3.3% had complicated UTI. In addition, 6.7% had catheter related blood stream infections (CRSBI). Various studies reported that bacterial pneumonia in acute stroke patients ranged from 9-27%, while incidence of UTI was 11-43% and incidence of CRSBI 2-6%.[23,24,25,26,27,28] Sikora et al., studied the involvement of reactive oxygen species (ROS) in systemic inflammatory response syndrome (SIRS) pathogenesis during sepsis and severe thermal and mechanical injuries. They suggested that the evaluation of neutrophil oxygen metabolism in different clinical entities, as a supplement to the anamnestic data and imaging studies, creates the possibility of differentiating the SIRS etiology.[29]

The neutrophil respiratory burst in patients with infection was lower than that in patients without infection at baseline, and it further declined at day 3 (P= 0.007 and <0.001, respectively), indicating that baseline neutrophil count could be used as a predictor of infection in patients with acute stroke. *Gens et al.,* investigated the usefulness of Neutrophil-to-Lymphocyte ratio (NLR) obtained within 24 h after acute ischemic stroke (AIS) for predicting post-stroke pneumonia and urinary tract infection.[30]

We observed a statistically significant negative correlation between neutrophil respiratory burst and NIHSS score, WBC count, CRP and length of hospital stay. Such finding indicates that impaired oxidative function of neutrophils in stroke patients may predict more severe stroke by NIHSS score and longer hospital stay.

Interestingly, MRI findings of the patients showed statistically significantly lower neutrophil respiratory burst in patients with occluded main stem of MCA than patients with branch occlusion or lacunar infarcts at baseline and on day 3 (P=0.001 and P<0.001 respectively). Precise interpretation of MRI could be of medical importance in determining preliminary outcome of patients at risk of infection from day one admission and may help in deciding the treatment and care protocol to avoid infectious complications and improve quality of life.

The ROC curve analysis suggested that neutrophil respiratory burst could be used to discriminate between patients with acute ischemic stroke from control individuals with 90% specificity.

In conclusion, it is of value to measure immune markers that predict the occurrence of infection and severity of ischemic damage in acute stroke patients to determine their prognosis. From our study, baseline neutrophil function and neurological outcome at 3 days after acute ischemic stroke were evaluated, we assumed that a reduced neutrophil function may be associated with an unfavorable outcome.

Author Contributions

AMO, MNF and RYS contributed to the design and implementation of the research, ASM and HME wrote the manuscript with input from all authors, DSS, AH and ASS sample collection and data recruitment, MRE contributed to sample preparation and analysis, All authors discussed the results and commented on the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Ain Shams University (approval no. FMASU 1941/2014).

Informed consent

An informed consent was taken from all study participants after explaining the aim of the study and ensuring the data confidentiality.

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