Functional and prognostic relevance of –173 G/C gene polymorphism of macrophage migration inhibitory factor in sepsis patients in Egyptian intensive care units

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الأهمية العملية والتنبؤية لتعدد أشكال الموقع 173 G/C – في الجين المسؤول عن العامل المثبط لهجرة الخلايا الضامة لدى مرضى خمج الدم في وحدات العناية المركزة المصرية

تقوى معوض، مروى منصور، شيماء منصور، محمد لطفي محمد، إسلام إبراهيم، علي محمد علي

الخلاصة: لقد هدفت هذه الدراسة إلى تقييم الارتباط بين مستوى العامل المثبط لهجرة الخلايا الضامة في البلازما وتعدد أشكال النوكليوتيد المفرد 173 G/C– في الجين المسؤول عن العامل المثبط لهجرة الخلايا الضامة وبين حدوث خمج الدم وشدتها ومعدل الوفيات لدى المرضى المصابين بها. فأجريت شواهد في وحدات العناية الجراحية المركزة للكبار في مستشفيات جامعة الزقازيق بمصر على 25 مريضاً لديهم خمج الدم، و 27 مريضاً لديهم خمج دم شديد، و 28 شاهداً. فكانت العصيات سلبية الجرام العزلات الأكثر شيوعاً لدى كل من مجموعتي مرضى خمج الدم الشديد (63.0 ٪) ومرضى خمج الدم (56.0 ٪). ووجد فرق ذو دلالة إحصائية عالية في مستويات العامل المثبط لهجرة الخلايا الضامة بين حالات خمج الدم وبين الشواهد، وفرق ذو دلالة إحصائية فيما يتعلق بمستوى العامل المثبط لهجرة الخلايا الضامة في الأنماط الجينية المختلفة للمجموعات المدروسة. وكان مستوى العامل المثبط لهجرة الخلايا الضامة مرتبطاً – بشكل كبير – مع معدل الوفيات بين حالات خمج الدم. إن ارتفاع مستويات العامل المثبط لهجرة الخلايا الضامة وتعدد أشكال الموقع 173 G/C– في الجين المسؤول عن العامل المثبط لهجرة الخلايا الضامة يعتبران مُنْبِئَيْن قويَّيْن عن شدة خمج الدم ونتيجته.

ABSTRACT This study aimed to evaluate the association of plasma MIF level and –173 G/C single nucleotide polymorphism of the MIF gene with the occurrence, severity and mortality of sepsis patients. A study was conducted in adult surgical intensive care units of Zagazig University Hospitals, Egypt on 25 patients with sepsis, 27 with severe sepsis and 28 controls. Gram-negative bacilli were the most common isolates in both severe sepsis (63.0%) and sepsis (56.0%) patients. A highly statistically significant difference was found in MIF levels between sepsis cases and controls and a statistically significant difference as regards MIF level in different genotypes of the studied groups. MIF level was significantly associated with mortality in sepsis cases. High MIF levels and MIF –173G/C gene polymorphism are powerful predictors of the severity of sepsis and its outcome.

Pertinence fonctionnelle et pronostique du polymorphisme du gène –173 G/C du facteur d'inhibition de la migration des macrophages chez des patients atteints de septicémie admis dans des unités de soins intensifs en Égypte

RÉSUMÉ La présente étude visait à évaluer l'association entre le taux facteurs d'initiative de lamigration des macrophages (MIF) plasmatique et le polymorphisme du nucléotide simple – 173 G/C du gène MIF et l'occurrence, la sévérité ainsi que le taux de mortalité chez les patientsprésentant une septicémie. Une étude a été menée dans des unités de soins intensifs en chirurgie pour adultes de l'hôpital universitaire de Zagazig, (Égypte) auprès de 25 patients atteints de septicémie, de 27 patients atteints d'une septicémie sévère et de 28 témoins. Des bacilles à Gram négatif étaient les isolats les plus fréquents dans les cas de septicémie sévère (63,0 %) et de septicémie (56,0 %). Une différence statistiquement très importante a été observée entre les taux du facteur d'inhibition de la migration des macrophages des cas de septicémie et des témoins tandis qu'une différence statistiquement significative a été notée entre le taux MIF des différents génotypes des groupes étudiés. Le taux du facteur d'inhibition de la migration des macrophages était nettement associé à la mortalité dans les cas de septicémie. De forts taux MIF et le polymorphisme du gène – 173G/C du MIF sont de puissants facteurs prédictifs de la sévérité de la septicémie et de son issue.

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Introduction

Sepsis is defined as a host's response to infection resulting from an imbalance between systemic pro-inflammatory reactions and an excessive anti-inflammatory response (*1*). Up to now there have been no published data about the incidence of sepsis in developing countries. Sepsis scores have been graded based on the international sepsis definitions as: local infection, bacteraemia, systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock (*2*). Mortality rates related to sepsis and its complications are high: 20% for sepsis, 40% for severe sepsis and more than 60% for septic shock. Even those who recover may have some permanent organ damage (*1*).

Bacterial infections are by far the most common causes of sepsis. Bacterial products such as lipopolysaccharides, lipoteichoic acid or cytokine receptors, including tumour necrosis factor-a and interleukin-1 via Toll-like receptors (TLR), enhance nuclear activation of nuclear factors and transcription of genes encoding expression of cytokines, chemokines, adhesion molecules, apoptotic factors and other mediators of inflammation and coagulation (*2*). Among the proinflammatory molecules is macrophage migration inhibitory factor (MIF), which has emerged as an important effector molecule of the innate immune system in response to infection (*3*). It enables macrophages, the forefront of the host antimicrobial defences, to sense invading Gram-negative bacteria and mount an innate immune response. Given that it is a pivotal regulator of innate immune responses by regulating the expression of the TLR4-LPS (Toll-like receptors–lipopolysaccharide) complex to bacterial infections, MIF appears to be a perfect target for novel therapeutic interventions in patients with severe sepsis (*4*).

High blood levels of MIF in children and adults with Gram-negative sepsis are associated with disease severity parameters and early mortality (*5*). Consequently, the *MIF* gene has been a candidate gene for investigation in inflammatory disease, and studies focusing on elucidation of *MIF* gene expression have been undertaken. A single nucleotide polymorphism (SNP) was identified in the untranslated 5′ region of the *MIF* gene at position –173 consisting of a G to C transition (*6*).

The aim of this study was to detect the incidence of sepsis in adult surgical intensive care units (ICUs) of Zagazig University Hospitals, Egypt. In addition, we aimed to evaluate the association of plasma level of MIF and –173 G/C single-nucleotide polymorphism of the *MIF* gene with occurrence, severity and suspected mortality of sepsis.

Methods

Study design and sample

This study was conducted in adult surgical ICUs of Zagazig University Hospitals, in Zagazig, Egypt (tertiary care hospital 2100 beds) from April 2009 to November 2011. The ICUs had annual numbers of admissions ranging from 940–1000.

The cases were sepsis patients in whom the severity of sepsis was assessed according to Acute Physiology and Chronic Health Evaluation (APACHE) II (*2*). The controls were those with fever of non-infectious origin who were selected to match the sepsis patients for age and sex. Patients were excluded in case of death within 6 h of inclusion, age under 18 years or using anti-inflammatory agents, corticosteroid therapy or other sepsis-modifying agents.

Data collection

Blood samples of 9 mL were collected from patients within 24 h of onset of illness and from controls into lithium heparin tubes; 8 mL blood was used for blood culture, 0.5 mL for DNA extraction and 0.5 mL for plasma separation.

Blood culture

Blood samples of 8 mL were inoculated into blood culture bottles (Egyptian Diagnostic Media) then incubated at 37 °C for 7–14 days. Subcultures were done every 48 hours on blood, chocolate and MacConkey agar (Oxoid) plates both aerobically and anaerobically using BD GasPack EZ anaerobic container (Becton and Dickinson). Bacterial isolates were identified on the basis of colonial morphology, microscopic examination of Gram-stained films of the different colonies and conventional biochemical reactions for either Gram-positive or -negative bacteria (*7*).

Determination of plasma MIF

To avoid the possible influence of MIF released from lysis of erythrocytes during blood clotting, circulating MIF levels were determined in plasma rather than serum samples (*8*). In addition, visibly haemolysed samples were excluded from measurements. Samples were stored at –80 °C until the concentrations were determined by enzyme-linked immunosorbent assay (human MIF ELISA, RayBiotech) according to the manufacturer's recommendations. The limit of detection was 6 pg/mL.

Genomic DNA extraction

DNA was extracted using QIAamp DNA blood mini kit (Qiagen GmbH) according to the manufacturer's recommendations.

Detection of MIF –173G/C gene polymorphism

Detection of *MIF* –173G/C gene polymorphism was performed using the tetra-primer amplification refractory mutation system (ARMS) method (*9*). The primers (Sigma-Aldrich Chemie) designed in this study were as follows: forward inner primer for the G allele, 5′-AGCCGCCAAGTGGAGAACTGG-3′; reverse inner primer for the C allele, 5′-AGCCCGGCGCACCGCTCCTAG-3′; forward outer primer, 5′-CAGTGCGTGCAGTGGAATGAAC-3′; reverse outer primer,

5′-TGGGGAAGTCACCGCCTGC-CT-3′. A polymerase chain reaction (PCR) assay was done using PCR-gold master-mix beads (Bioron): 50 µL reaction contained 2.5 U taq DNA polymerase, 250 mM each dNTP, 10 mM tris-HCL (pH 9.0), 30 M KCl, 1.5 mM $MgCl_2$, to which 70 ng template DNA, 0.20 pmol concentration of outer primers, 1.0 pmol concentration of inner primers, then distilled water was added to a total volume of 50 µL. The reaction was performed in a thermal cycler (Biometra) using the amplification programme: initial denaturation at 94 °C for 5 min, followed by 33 amplification cycles, each consisting of denaturation at 94 °C for 50 s, annealing at 60 °C for 50 s and extension at 72 °C for 50 s and final extension at 72 °C for 5 min. Each run of PCR amplification included negative controls and included no template DNA to avoid false positive results caused by possible contamination. The amplified products were separated by electrophoresis on 2.0% agarose gel stained with ethidium bromide and visualized under an ultraviolet transilluminator (Biometra) with a 100-bp DNA molecular weight marker (Sigma-Aldrich Chemie) (Figure 1).

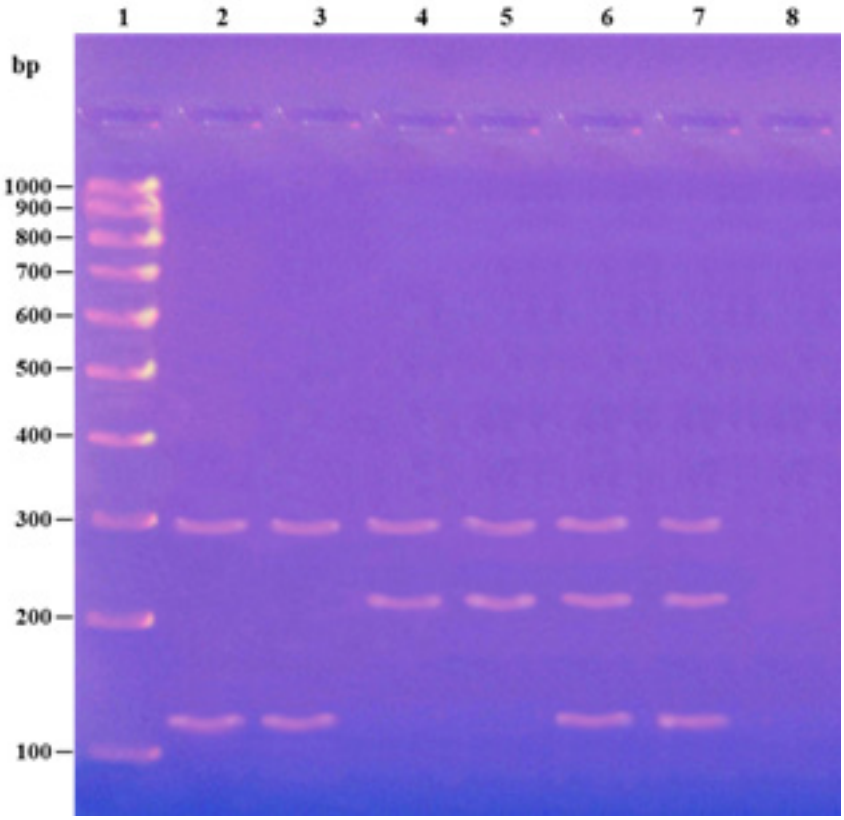

Figure 1 Gel electrophoresis showing *MIF* –173G/C gene polymorphism by tetra-primer amplification refractory mutation system (ARMS) method: lane 1: 100-bp DNA ladder marker; lanes 2 and 3: G/G genotype (2 bands; 1 at 126 bp and 1 at 298 bp); lanes 4 and 5: CC genotype (2 bands at 213 bp and at 298 bp); lane 6 and 7: GC genotype (3 bands, 1 at 126 bp, 1 at 213 bp and 1 at 298 bp). Lane 8: negative control (no bands)

Ethical considerations

The study was reviewed and approved by the review board of the research ethics committee, Faculty of Medicine, University of Zagazig. Informed consent was obtained from all participants after explanation of the procedure and the purpose of the study.

Statistical analysis

All patients' data were tabulated and processed using *SPSS*, version 12.0. Quantitative variables were expressed by mean and standard deviation (SD) and then compared using the Mann–Whitney U-test for comparing 2 independent variables and the Kruskal–Wallis analysis for more than 2 independent variables. Qualitative variables were expressed by frequency and percentages and compared using the chi-squared test or Fischer exact test when appropriate. Correlations between variables were assessed using Spearman analysis. Differences were considered significant at P-value < 0.05. To assess and compare plasma MIF level among the studied groups, boxplots were used exploring distribution characteristics (median, interquartile range, range and outliers for each group). Interval plots were used to assess and compare means and confidence intervals of plasma MIF levels among the different genotypes within each studied group.

Results

Out of 459 patients admitted 52 (11.3%) were shown to have septicaemia; 25 were classified as sepsis and 27 as severe sepsis.

Table 1 showed the characteristics of the study groups. There were no significant differences in the age and sex of patients and controls. Patients with severe sepsis had significantly higher mean APACHE scores (19.5) than those with sepsis (16.5) (P = 0.01). The overall mortality rate for cases was 19/52 (36.5%) and the greater severity of illness in the severe sepsis group was reflected in a statistically significantly higher mortality rate during the time of stay in the ICU (55.6%) compared with the sepsis group (20.0%) (P = 0.003). Concerning risk factors, older age (> 60 years), hospitalization more than 48 h, use of instrumentation, presence of polytrauma and history of diabetes mellitus were all statistically significantly

Table 1 Demographic and clinical characteristics and risk factors for sepsis in the study groups of patients

Variable	Severe sepsis (*n* = 27)	Sepsis (*n* = 25)	Controls (*n* = 28)	χ^2-value	*P*-value
Age (years) [mean (range)]	62.7 (43.9–71.5)	53.1 (44.1–69.0)	50.2 (40.0–60.5)		0.1
Sex ratio (no. of males/females)	16/11	15/10	19/9	0.53	0.76
APACHE II score [mean (range)]	19.5 (16.0–27.0)	16.5 (12.0–21.5)	n/a	2.42	0.01
Deaths (no.)	15	4	n/a	8.7	0.003
Risk factors (no. of patients)					
Older age (> 60 years)	18[a]	9	4	16.0	< 0.001
Hospitalization > 48 h	27	25	5[a]	15.5	< 0.001
Corticosteroid therapy	5[b]	3	0	5.4	0.06
Instrumentation					
Mechanical ventilation	27[a]	14	10	25.5	< 0.001
Central venous catheter	20[a]	12[b]	6	15.3	< 0.001
Urinary catheter	27[a]	20[b]	13	21.5	< 0.001
Post-surgery	4	4	6	0.47	0.78
Cardiovascular disease	2	3	5	1.38	0.50
Hepatic disease	7	5	5	0.57	0.75
Renal disease	3	6	6	1.62	0.44
CNS disease	2	4	6	2.15	0.34
Polytrauma	10[a]	3	0	14.3	< 0.001
Diabetes mellitus	9[a]	2	0	13.9	< 0.001

[a]Significant difference versus other groups; [b]Significant difference versus controls.
APACHE = Acute Physiology and Chronic Health Evaluation; CNS = central nervous system; n/a = not applicable.

different between the patient and control groups.

Table 2 shows that there was no statistically significant difference regarding the rate of microbial isolation between sepsis patient groups. Gram-negative bacteria were isolated from 63.0% and 56.0% of severe sepsis and sepsis patients respectively. However, the rates of leukocytosis ($P < 0.05$), thrombocytopenia ($P < 0.001$) and positive C-reactive protein (CRP) ($P < 0.001$)

Table 2 Microbiological and haematological findings and C-reactive protein levels of the study groups of patients

Variable	Severe sepsis (*n* = 27)		Sepsis (*n* = 25)		Controls (*n* = 28)		χ^2-value	*P*-value
	No.	%	No.	%	No.	%		
Microbiological findings								
Gram-negative	17	63.0	14	56.0	n/a	n/a	0.26	0.61
Gram-positive	5	18.5	6	24.0	n/a	n/a	0.23	0.63
Fungi	2	2.7	1	4.0	n/a	n/a	–	1.0[c]
Mixed infection	3	11.1	4	16.0	n/a	n/a	–	0.69[c]
Leukocyte count								
Normal	4	14.8	3	12.0	17[a]	60.7	19.4	< 0.001
Leukocytosis	22	81.4	20	80.0	8[a]	28.6	21.7	< 0.001
Leukopenia	1	3.7	2[b]	8.0	3	10.7	0.99	0.61
Platelet count								
Normal	1	3.7	5	20.0	25	89.0	47.8	< 0.001
Thrombocytopenia	26	92.3	20	80.0	3[a]	11.0		
C-reactive protein								
Positive	27	100.0	24	96.0	10[a]	35.7	39.2	< 0.001
Negative	0	0.0	1	4.0	18	62.3		

[a]Significant difference versus other groups; [b]Significant difference versus controls; [c]Fisher exact test.
n/a = not applicable.

were significantly higher in both sepsis groups than the controls.

Figure 2 shows that mean plasma MIF levels were significantly higher in sepsis patients than in controls, with the highest levels in the severe sepsis group [severe sepsis 12 855 (SD 2823) pg/mL, sepsis 9036 (SD 1623) pg/mL and controls 2207 (SD 823) pg/mL] ($P < 0.001$ for severe sepsis versus controls; $P = 0.01$ for sepsis versus controls).

Table 3 shows a significantly lower mean MIF level among the 33 survivors of sepsis [8953 (SD 1870) pg/mL] compared with the non-survivors [14 852 (SD 2780) pg/mL] ($P = 0.007$).

Table 4 compares the frequencies of –173 G/C SNP genotypes and alleles between patients and controls. There was a statistically significant difference in the frequency of the C/C genotype and C allele, with a 3-fold increased risk in patients versus controls (OR 3.26 for C allele; 95% CI: 1.36–8.03, $P = 0.003$).

Figure 3 is the interval plot of plasma MIF level among the different genotypes in the severe sepsis patients ($n = 27$), sepsis patients ($n = 25$) and controls ($n = 28$). There was a statistically significant difference in plasma MIF level for different genotypes of the study groups. Mean plasma MIF levels were higher in individuals carrying the CC and GC genotypes than the GG genotypes [CC genotypes 16 251 (SD 2979) pg/mL, GC genotypes 12 144 (SD 2728) pg/mL, GG genotypes 10 378 (SD 3272) pg/mL] ($P = 0.001$).

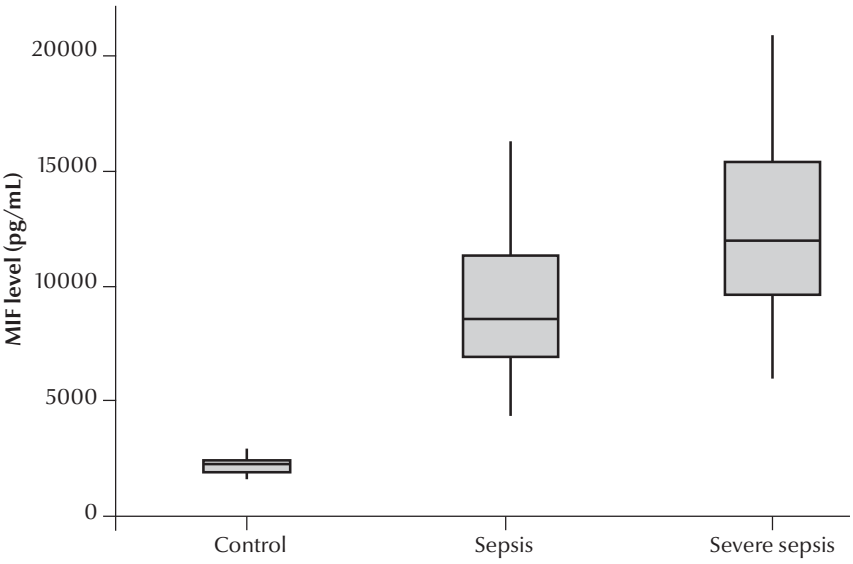

Figure 2 Box plot of mean and standard deviation plasma macrophage migration inhibitory factor (MIF) level in the study patients with severe sepsis ($n = 27$), sepsis ($n = 25$) and controls ($n = 28$)

Discussion

This research work analysed the incidence of sepsis in the adult surgical ICUs of a 2100-bed tertiary care Egyptian hospital. Egypt is a developing country where there is a high risk of mortality from infection. The incidence of sepsis in those ICUs was 11.3%, compared with only 4.4% and 2.1% in Spanish (*10*) and Japanese (*11*) studies respectively. There was no significant difference as regards age and sex between the case and control groups (as they were matched for age and sex) or between the sepsis and severe sepsis cases, which agrees with the study of Leaver et al. (*12*).

Many years ago the APACHE II scoring system was designed as a reliable and useful means of evaluating patient outcomes, measuring the severity of disease and describing morbidity by comparing the outcomes of sepsis patients with those of others admitted to intensive care (*13*). In our study, there

Table 3 Plasma macrophage migration inhibitory factor (MIF) levels among the study groups of patients and between survivors and non-survivors of sepsis

Study groups	No. of patients	MIF levels (pg/mL)		*P*-value
		Mean (SD)	Range	
All patients				
Severe sepsis	27	12855 (2823)	5780–20 750	< 0.001[a]
Sepsis	25	9036 (1623)	4152–16 098	0.01[b]
Controls	28	2207 (823)	1028–9815	
Sepsis cases				
Survivors	33	8953 (1870)	5274–17 251	0.007
Non-survivors	19	14 852 (2780)	6543–20 750	

[a]Significant difference versus other groups; [b]Significant difference versus controls.
SD = standard deviation.

Table 4 Frequency of –173 G/C single nucleotide polymorphism genotypes and alleles of the *MIF* gene in the study groups of patients

Variable	Severe sepsis (*n* = 27)	Sepsis (*n* = 25)	Controls (*n* = 28)	χ^2-value	*P*-value
Genotype frequency					
GG	11[b]	14	20	5.26	0.07
GC	9	5	7	1.23	0.54
CC	7	6	1[a]	5.82	0.05
Allele frequency					
G	31	33	47[a]	12.1	0.002
C	23	17	9		

[a]*Significant difference versus other groups;* [b]*Significant difference versus controls.*

was a significant difference in APACHE II scores between sepsis and severe sepsis groups. This was also shown by Juncal et al., who found significant associations between a diagnosis of sepsis and APACHE II score (*14*).

Mortality among severe sepsis cases was high (55.6%) and the average for all sepsis cases was high (36.5%) compared with other studies. In separate studies in the United States of America (USA), Angus et al. (*15*) and Braun et al. (*16*) reported mortality rates of 28.6% and 20.6% respectively, while Khan et al. reported a mortality rate of 24% in a Pakistani ICU (*17*) and Sanya et al. reported that sepsis accounted for 18.1% of overall deaths among old-age people in Nigeria (*18*).

Sepsis, with its serious sequelae, is a totally preventable condition in spite of the associated complications. The prevention of sepsis relies on the identification of risk factors and the availability of appropriate interventions. In this study, older age, hospitalization more than 48 h, instrumentation, polytrauma and diabetes mellitus were risk factors. However, the picture was different in Shiramizo et al.'s research, in which liver, renal and haematological conditions were risk comorbidities (*19*). Implementation of infection control standards for identified risk factors is a key factor in combating the cycle of sepsis.

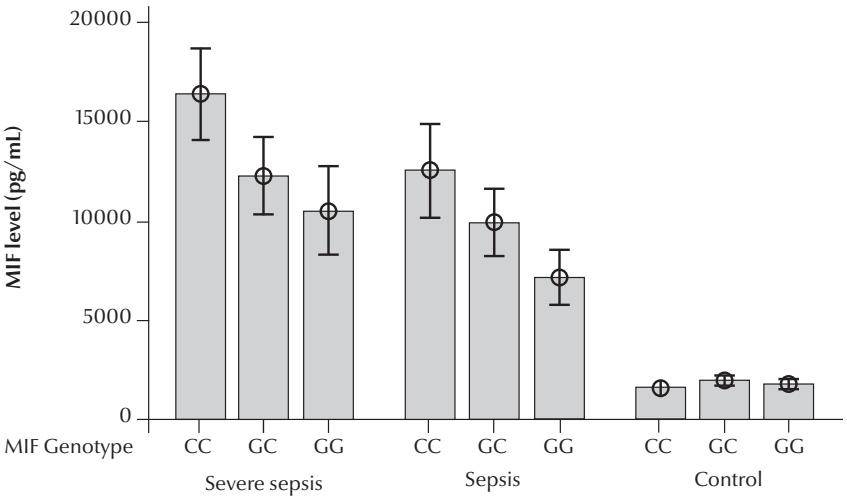

Figure 3 Interval plot of plasma macrophage migration inhibitory factor (MIF) level among the different genotypes in the study patients with severe sepsis (*n* = 27), sepsis (*n* = 25) and controls (*n* = 28)

Blood culture remains the gold standard for confirmation of the diagnosis of sepsis. A predominance of Gram-negative organisms (63.0% and 56.0% of severe sepsis and sepsis patients respectively) was observed in this study. This was supported by the findings of Emonts et al. in the Netherlands (80%) (*5*), Enweronu-Laryea and Newman in Ghana (54.2%) (*20*) and Berkley et al. in a rural hospital in Kenya (in which Escherichia coli accounted for more than 70% of cases) (*21*). However, Martin et al. in the USA found fewer cases with Gram-negative (37.6%) than Gram-positive bacteria (52.1%) (*22*).

The haematological findings in this work matched those reported by Yamamoto et al. (*23*), as there was a statistically significant difference between sepsis and non-sepsis patients in the rate of thrombocytopenia and leucocytosis. CRP in our study was a good marker of sepsis as 100% and 96% of severe sepsis and sepsis patients respectively had positive CRP and this was significantly higher than in the controls. This was in accordance with Pfäfflin and Schleicher, who stated that CRP is the preferred marker for determination of inflammation due to its high sensitivity (*24*).

Many investigators have endeavoured to find reliable markers for the diagnosis and management of sepsis. The ideal biomarker should not only reflect sepsis but also the severity of the condition (*25, 26*). The present work

showed an elevation of plasma MIF level in sepsis and severe sepsis patients in our ICUs versus controls. The studies of both Emonts et al. (5) and Brenner et al. (27) were in agreement with our results. They reported the MIF level was significantly elevated in a sepsis group compared with controls. De-Mendonca-Filho et al. also reported that elevated MIF level is an early marker of patients with postoperative sepsis (28). However, Chuang et al. found no significant correlation between serum MIF levels and clinical severity of sepsis (29). Regarding the relationship between MIF level and survival, Chuang et al. found that a high incremental increase in MIF levels in day 1 and 2 predicts 3–7-day mortality in severe sepsis patients and this is similar to our results despite the different study designs (29). The studies of Rahman et al. (30), Calandra et al. (4) and Bozza et al. (31) also agreed with these results. In contrast, Donn et al. found no significant difference between the surviving and non-surviving subgroups of sepsis patients regarding MIF level (6).

Regarding the frequencies of MIF –173 G/C SNP genotypes and alleles among the study groups, many authors have studied this association. Our results were in accordance with those of Gao et al. who reported an association of –173 SNP genotype C/C with the incidence of sepsis in African Americans (32). Our results were also in agreement with Donn et al. who reported that MIF plasma levels in –173 C allele carriers were higher compared with non-C carrying individuals (6).

Conclusions and Recommendations

Our data indicate that high plasma MIF level and –173 G/C polymorphism in the MIF gene are powerful predictors of the severity and outcome of sepsis. Further studies in this field are recommended to investigate the underlying causes of high mortality in sepsis.

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