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Evaluation of Some Immunological Markers in Children with Febrile Illness

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Abstract

Background: Acute febrile illnesses (AFIs) represent a significant world burden disease however, diagnosing of (AFIs) is challenging due to the lack of accurate, quick point-of-care diagnostic, several biomarkers have been investigated to help distinguish between bacterial and viral infections. Among these, TNF-related apoptosis-inducing ligand (TRAIL) or (TNFL), Matrix metalloproteinase 8 (MMP-8), and Neutrophil Gelatinase-associated Lipocalin (NGAL) have shown promise.

Methods: Ninety participants were taken then divided into (60) with febrile patients with >=38ċ, while (30) were free from disease as a healthy control. The TNFL, MMP-8 and NGAL were determined by ELISA technique.

Result: The statistical analysis showed that TNFL significantly ($P \leq 0.05$) elevated in patients with bacterial infection other than in patients with viral infection and control. NGAL and MMP-8 significantly ($P \leq 0.05$) elevated in patients with bacterial and viral infections compared to control subjects. The results showed statistical analysis that there was a significant ($P \leq 0.05$) increase in the concentration of NGAL in females compared to males in the group of patients with viral infection.

Conclusion: TNFL level were found to be significantly different in children with bacterial infections compared to those with viral infections and control subjects. Elevated TNFL levels were more commonly associated with bacterial infections. NGAL and MMP-8 were significantly different in children and notable increase in children with bacterial and viral infections as compared with the control.

تحليل منحنى خصائص التشغيل للمستقبل لعلامات المناعة TNFL وNGAL وMMP-8 بين مرضى الحمى عند الأطفال

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الخلاصة

المقدمة: تشكل الأمراض الحموية لدى المرضى الأطفال تحديات تشخيصية كبيرة بسبب مسبباتها المتنوعة، بما في ذلك الحالات البكتيرية والفيروسية والالتهابية. تهدف هذه الدراسة إلى التحقيق في الفائدة التشخيصية لثلاثة علامات مناعية محددة - (TNFL)، (NGAL)، (MMP-8) لدى الأطفال الذين يعانون من أمراض محمومة.

المرضى وطرق العمل: تم جمع تسعين عينة دم من مرضى الأطفال الذين تم تشخيصهم سريريًا بأمراض محمومة. تم تقسيم العينات إلى مجموعتين: 60 حالة محمومة و30 حالة صحية. تم تحديد تركيز TNFL و NGALو MMP-8في عينات المصل باستخدام مجموعات .ELISA تضمن التحليل الإحصائي إحصاءات وصفية، وارتباط بيرسون، وتحليل منحنى ROC لتقييم الحساسية والنوعية والمساحة تحت المنحنى (AUC) لكل علامة.

النتائج: وجدت الدراسة فروقًا كبيرة في مستويات TNFL و NGALو MMP-8بين المرضى المصابين بالحمى وضوابط الأصحاء. كشف تحليل منحنى ROC أن كل من TNFL و MMP-8 يمكن أن يعملوا كعوامل تنبؤية للعدوى البكتيرية، مع أهمية عند قيمة p أقل من 0.01. أظهر NGAL و MMP-8أيضًا إمكانات كعلامات للعدوى الفيروسية، مع قيمة p كبيرة أقل من 0.01.

الاستنتاج: أظهرت العلامات المناعية TNFL و NGALو MMP-8درجة الحساسية والخصوصية التي تم تحقيقها للتمييز بين العدوى البكتيرية والفيروسية في المرضى الأطفال المصابين بأمراض الحمى. هناك حاجة إلى مزيد من التحقق من خلال البحوث المنهجية لتأكيد فائدتها السريرية.

1. Introduction

Acute febrile illness is the most common symptom of people living worldwide, and physician have been challenged by the similar clinical features of a wide spectrum of aetiologies (Maze et al., 2018). Fever in children is a common clinical problem that often prompts medical evaluation to identify its underlying cause. Differentiating between bacterial and viral infections in febrile children is crucial, as it significantly influences treatment decisions, especially the use of antibiotics (Rhedin et al., 2021). Misdiagnosis can lead to inappropriate antibiotic use, contributing to antibiotic resistance and adverse drug effects (Heffernan and Denny, 2021). Biomarkers are biological molecules that indicate the presence or severity of a disease. They are critical tools in diagnosing infections, monitoring disease progression, and guiding treatment decisions (Bodaghi et al., 2023). Biomarkers such as TRAIL, MMP-8, and NGAL are often studied for their roles in the body's response to infection and inflammation.The TNF-related apoptosis-inducing ligand is a cytokine that belongs to the tumor necrosis factor (TNF) superfamily. It is involved in the regulation of immune responses and has been studied for its role in apoptosis and inflammation (Gyurkovska and Ivanovska, 2016). TNFL levels can vary significantly in bacterial versus viral infections, making it a potential biomarker for distinguishing between these types of infections in febrile children(Zandstra et al., 2021). MMP-8, also known as neutrophil collagenase, is an enzyme produced primarily by neutrophils. It plays a role in the degradation of extracellular matrix components and is involved in tissue remodeling and inflammation (Gajendrareddy et al., 2013). Elevated levels of MMP-8 have been associated with bacterial infections, making it a candidate biomarker for differentiating bacterial from viral causes of fever (Sathyamoorthy, 2014). In addition, NGAL is a protein expressed by neutrophils and various epithelial cells. It is involved in the innate immune response and has been identified as a marker for bacterial infections (Nasioudis and Witkin, 2015). NGAL levels rise in response to bacterial infections, reflecting the body's inflammatory response and neutrophil activation (Venge and Xu, 2019). This study aims to evaluate the diagnostic utility of TNFL, MMP-8, and NGAL as biomarkers to distinguish between bacterial and viral infections in children, by comparing the levels of these biomarkers in febrile children with confirmed bacterial or viral infections, to establish their effectiveness in improving diagnostic accuracy and guiding appropriate treatment decisions.

2. Patients and Methods

2.1.Study Subjects

Ninety blood samples had been taken then diagnosed participants by CBC and c-reactive protein that divided into (60) with febrile cases group [(30) infected with the virus and (30) infected with bacteria], while (30) as healthy control group. The samples were obtained (43) males and (47) females, with ages ranging between (<1-14) years old attending to Karbala Teaching Hospital for Children, Karbala /Iraq during the period extended from November (2023) to February (2024).

2.2.Specimen Collection

Each patient and control group had five milliliters of venous blood drawn into gel tubes; to avoid hemolysis, the blood sample was slowly drawn out using a syringe needle. After the sample was placed into a sterile, disposable gel tube, the serum separated at room temperature after 20 minutes. Following a five-minute centrifugation at 3500 rpm, the samples were divided into three separate Eppendorf tubes and kept at -20°C until analysis.

2.2.1. Inclusion Criteria: Clinical suspicion of an acute infectious disease, Children <1–14 years of age peak fever ≥38°C since onset of symptoms.

2.2.2. Exclusion Criteria: indications of an acute infection within the two weeks before to enrollment; congenital immunodeficiency; immunosuppressive or immunomodulatory medication; active cancer; and infection with the hepatitis B/C virus or HIV-1.

2.3.Immunological Measurement of TNFL, MMP-8, And NGAL By ELISA Kits

The study was used ELISA technique for determine the concentration of TNFL, MMP-8, and NGAL from patient and control with febrile illness according to manufacture company (Bioassay technology laboratory).

2.4.Statistical Analysis

Statistical analysis of data in the present study was performed by SPSS. version 25.0 on the basis of one way analysis of variance (ANOVA) using significant levels (P<0.05).

3. Results

3.1.Evaluation of Immunological Markers in Study Groups

Table 1 displays the immunological markers in study populations. The statistical analysis showed that TNFL significantly (*P≤0.05*) increase in patients with bacterial infection as compared with patients with viral infection and control subjects. NGAL and MMP-8 were significantly (*P≤0.05*) increase in patients with bacterial and viral infections as compared with control subjects.

Table 1: Evaluation of Immunological Markers in Study Population

Variables	Study population	N	Mean	Std. Deviation	*P value*
TNFL	**Bacterial infection**	30	544.685 [a]	249.017	0.002 *
	Viral infection	30	441.228 [b]	209.794	
	Control	30	362.820 [b]	88.046	
	Total	90	449.578	206.597	
NGAL	**Bacterial infection**	30	267.269 [a]	140.066	0.001 *
	Viral infection	30	284.055 [a]	143.303	
	Control	30	168.540 [b]	63.321	
	Total	90	239.955	130.447	
MMP-8	**Bacterial infection**	30	6.3157 [a]	2.304	0.000 *
	Viral infection	30	6.135 [a]	1.918	
	Control	30	4.049 [b]	0.986	
	Total	90	5.499	2.077	

*Significant difference at the 0.05 level by One way – ANOVA. Different small letters refer to significant differences

3.2.The Association of Gender with Immunological Markers

The association between gender and immunological markers explained in Table 2. The results of the statistical analysis showed that there was a significant (*P≤0.05*) increase in the concentration of NGAL in females compared to males in the group of patients with viral infection. On the other hand, the remaining markers showed no significant distribution according to gender in all groups.

Table 2: Association of Gender with Immunological Markers in Patients and Control Group

Population	Variables	Gender	N	Mean	Std. Deviation	*P value*
Bacterial infection	**TNFL**	**Male**	13	600.569	286.691	0.290 NS
		Female	17	501.949	215.105	
		Total	30	544.685	249.017	
	MMP-8	**Male**	13	7.215	2.082	0.060 NS
		Female	17	5.627	2.283	
		Total	30	6.315	2.304	
	NGAL	**Male**	13	307.489	154.167	0.173 NS
		Female	17	236.513	124.152	
		Total	30	267.269	140.067	
Viral infection	**TNFL**	**Male**	21	413.884	159.745	0.283 NS
		Female	9	505.033	298.681	
		Total	30	441.228	209.793	
	MMP-8	**Male**	21	5.945	1.9129	0.419 NS
		Female	9	6.576	1.9684	
		Total	30	6.134	1.9181	
	NGAL	**Male**	21	249.009	134.787	0.038*
		Female	9	365.829*	135.191	
		Total	30	284.055	143.303	
Control	**TNFL**	**Male**	13	383.809	101.177	0.261 NS
		Female	17	346.769	75.773	
		Total	30	362.820	88.046	
	MMP8	**Male**	13	3.957	0.814	0.667 NS
		Female	17	4.118	1.120	
		Total	30	4.048	0.98636	
	NGAL	**Male**	13	166.861	62.235	0.902 NS
		Female	17	169.823	66.018	
		Total	30	168.540	63.321	

*Significant difference at the 0.05 level by T-test. NS: Non-significant difference

3.3.Correlation of Immunological Markers in Both Patients Groups

Fig.1, Fig.2 and Fig.3 summarize the correlation between of immunological markers in both patients' groups. The results of statistical analysis showed significant *(P≤0.05*) positive correlations between levels of TNFL and NGAL in patients with bacterial infection, also a significant (*P≤0.05*) positive correlation was found between the levels of TNFL and MMP-8 in patients with viral infection. On the other hand, the correlation analysis showed non-significant (P>0.05) positive correlation the remaining markers.

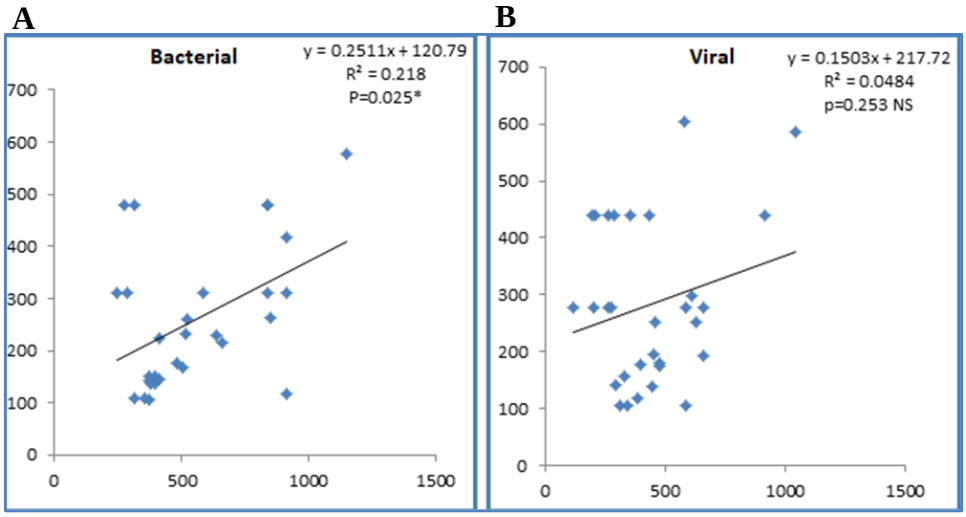

Figure 1: A and **B** Show the Correlation Between TNFL And NGAL In Both Patients Groups

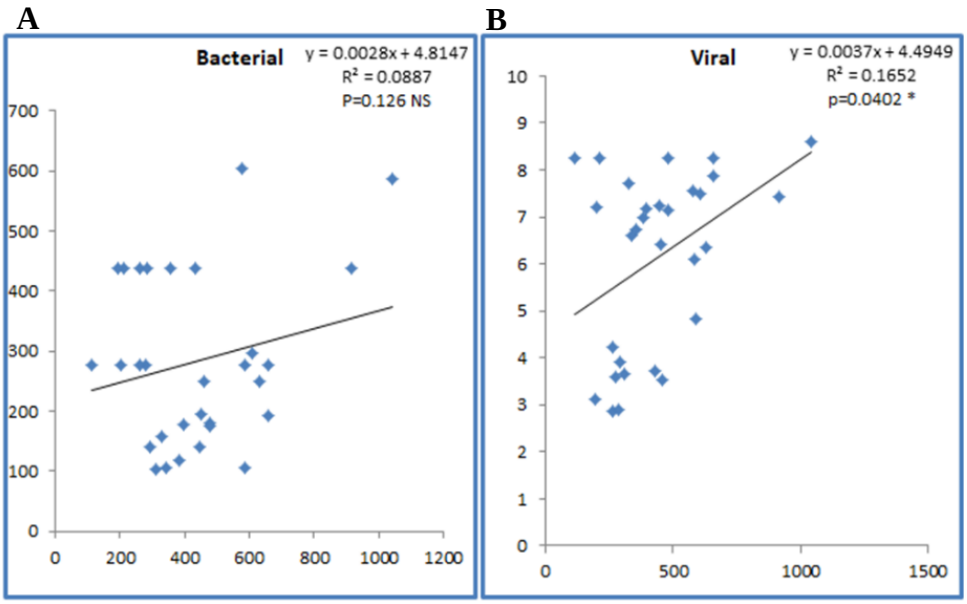

Figure 2: A and **B** Represent Correlation Between TNFL and MMP-8 In Both Patients Groups

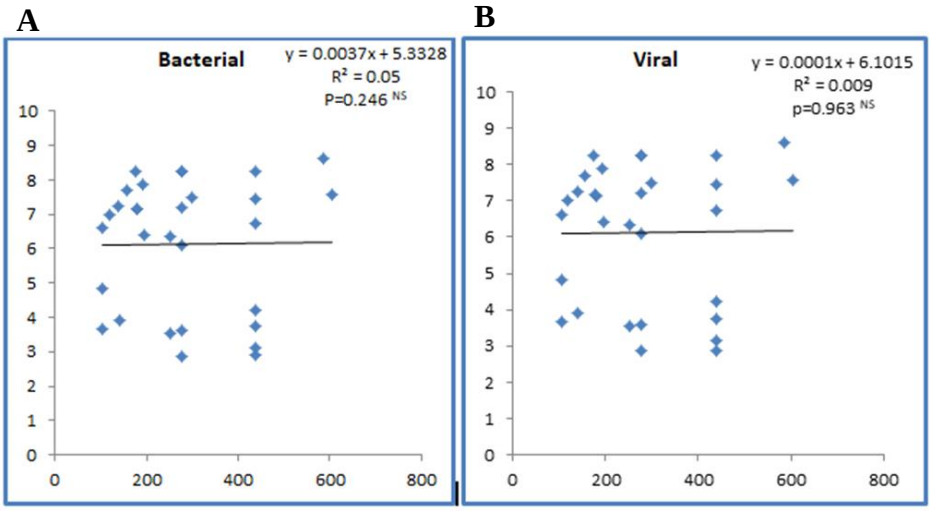

Figure 3: A and **B** Show the Correlation Between MMP-8 and NGAL in Both Patients Groups

3.4.Correlation of Immunological Markers with Laboratory Variables in Bacterial Infection Cases

Table 3 summarizes the correlation between levels immunological markers and laboratory variables in patients with bacterial infection. Through the results of the statistical correlation analysis, it is clear that there CRP had a significant (*P≤0.05)* positive correlation with each of TNFL and NAGL, while other markers showed non-significant correlation.

Table 3: Correlation of Immunological Markers with Laboratory Variables in Bacterial Infection Cases

Variables		TNFL	NGAL	MMP-8
CRP	**Pearson Correlation**	.395*	.372*	.034
	Sig. (2-tailed)	.031	.043	.858
	N	30	30	30
WBCs	**Pearson Correlation**	.249	.195	-.107
	Sig. (2-tailed)	.185	.303	.573
	N	30	30	30
Neutrophil	**Pearson Correlation**	.195	.175	-.156
	Sig. (2-tailed)	.301	.355	.411
	N	30	30	30
Lymphocyte	**Pearson Correlation**	.040	.229	.222
	Sig. (2-tailed)	.835	.223	.238
	N	30	30	30
*. Correlation is significant at the 0.05 level (2-tailed). N: Number, CRP: C-reactive protein, WBC: White blood cell				

Table 4 summarizes the correlation between levels immunological markers and laboratory variables in patients with viral infection. Correlation analysis in this group showed a significant (*P≤0.05*) positive correlation between

Neutrophil and TNFL; also, a trend toward significant (*p= 0.062*) correlation was found between Lymphocyte and NAGL, while other markers showed non-significant (*p>0.05*) correlation.

Table 4: Correlation Among Laboratory Variables and Immunological Markers in Viral Infection Cases

Variables		TNFL	NGAL	MMP-8
CRP	**Pearson Correlation**	-.263	.028	.286
	Sig. (2-tailed)	.161	.885	.125
	N	30	30	30
WBCs	**Pearson Correlation**	.026	.224	-.003
	Sig. (2-tailed)	.893	.233	.988
	N	30	30	30
Neutrophil	**Pearson Correlation**	.402*	.099	.189
	Sig. (2-tailed)	.028	.601	.318
	N	30	30	30
Lymphocyte	**Pearson Correlation**	-.076	.345	-.135
	Sig. (2-tailed)	.689	.062	.478
	N	30	30	30
* Correlation is significant at the 0.05 level (2-tailed).				

Table 5 illustrates the correlation between levels immunological markers and laboratory variables in healthy control individuals. Correlation analysis showed a significant (*P≤0.05*) positive correlation between CRP and NAGL; a significant (*P≤0.05*) inverse correlation was reported between Neutrophil and MMP-8; also, a trend toward significant (*p= 0.066*) inverse correlation was detected between WBCs and MMP-8, while other markers showed no significant (*p>0.05*) correlation.

Table 5: Correlation Among Laboratory Variables and Immunological Markers in Control Individuals

Variables		TNFL	NGAL	MMP-8
CRP	**Pearson Correlation**	.086	.398*	-.173
	Sig. (2-tailed)	.651	.029	.361
	N	30	30	30
WBCs	**Pearson Correlation**	.065	.146	-.340
	Sig. (2-tailed)	.731	.440	.066
	N	30	30	30
Neutrophil	**Pearson Correlation**	.237	.317	-.456*
	Sig. (2-tailed)	.207	.088	.011
	N	30	30	30
Lymphocyte	**Pearson Correlation**	-.294	-.280	-.034
	Sig. (2-tailed)	.115	.134	.860
	N	30	30	30
*. Correlation is significant at the 0.05 level (2-tailed).				

4. Discussion

Regarding the immunological markers in this study, the statistical analysis showed that TNFL significantly increase in bacterial infection cases more than viral cases and control subjects. NGAL and MMP-8 were significantly increase in patients with bacterial and viral infections as compared with control subjects. In the comparisons with other studies, current study corresponding with (Nasioudis & Witkin, 2015) who's revealed to elevated levels of NGAL have been demonstrated in the blood of patients with bacterial urinary tract infection, pneumonia, sepsis, also in the cerebrospinal fluid and peritoneal fluid of patients with bacterial meningitis and peritonitis. But disagreed with findings by (Venge et al., 2015) who measured Human Neutrophil Lipocalin (HNL) concentrations in whole-blood samples and found that HNL elevated in bacterial as opposed to viral infections.

(Brand et al., 2012) reported that viral lower respiratory tract infections severity in children is related with increased levels of the MMP-8 genes expression. While results of (Gillette et al., 2021) found significantly different abundances between bacterial and viral infections. Bacterial pneumonia was strongly associated with MMP8. These results, which are not compatible with present study that demonstrated the MMP are slightly differences in bacterial and viral infection compared with control group. In the comparisons with other studies, this study inconsistent with (Oved et al., 2016) who showed that the serum TNFL levels were significantly decline in bacterial patients and elevated in viral cases compared with control (bacterial 45 ± 33 pg/mL; viral 145 ± 110 pg/mL and controls 77 ± 32 pg/mL). Many pathogens have evolved mechanisms to manipulate TNFL signaling thus increasing pathogen replication (Gyurkovska and Ivanovska, 2016), this may interpret slight increase of tumor necrosis factor ligands in bacterial infection.

Regarding the association between gender and immunological markers showed statistically significant increase in the concentration of NGAL in females compared to males of the viral infection group. On the other hand, the remaining markers showed insignificant distribution according to gender in all groups. Study of (Thrailkill et al., 2010) investigated gender associated with NGAL and MMP9 in patient with diabetes, found plasma NGAL concentrations were significantly higher in females compared to males, while findings of (Sathyamoorthy et al., 2015), showed differences in plasma matrix metalloproteinase-8 elevated in male. In addition, (Aomatsu et al., 2013) showed neutrophils from human males express higher levels of TLR4 and produce more TNF- ligands than female neutrophils, Also, (Rusman et al., 2018) demonstrated that females switched (27%) of TNF more than males (16%), but this difference was not significant. Some differences in this study with other studies most likely due to the low number of patients that enrolled. The results of this study showed positive correlations between levels of TNF and NGAL in patients with bacterial infection, also positive correlation was found between the levels of TNFL and MMP-8 in patients with viral infection. With comparison with the other studies, this result agreed with (Oikonomou et al., 2012) who found a significant correlation between NGAL and TNF, also with results of (Yu et al., 2016) found the monomeric Human Neutrophil Lipocalin (HNL) in viral infections were elevated and the dimeric type in bacterial infections were increased. As well as, with (Han et al., 2012) found there was a strong correlation between TNFα and NGAL mRNA in rat, (Malyszko et al., 2010) revealed that NGAL was induced by TNF activation. Furthermore, (Arena et al., 2010) showed that NGAL expression in polymorphonuclear granulocytes were regulated by TNF-α, this interprets correlation between TNF and NGAL. Several studies confirmed present study such as a study accomplished by (Sharma et al., 2021), (Nylund et al., 2015) and (Andronovici et al., 2022) showed there were a strong correlation

between MMP-8 and TNF related apoptosis induced ligands and elevated together. MMP expression is influenced by chemokines and pro-inflammatory cytokines. Numerous chemokines and cytokines induce target cells to produce TNF. The TNF becomes active and strongly pro-inflammatory when the inactive (latent) form is broken down by proteases (Hardy and Fernandez-Patron, 2021). In these findings showed the correlation between immunological markers level and laboratory variables in patients with bacterial infection. It is clear that there CRP had a significant positive correlation with each of TNFL and NAGL. (Liu & Nilsen-Hamilton, 1995) reported that Serum NGAL levels were elevated in systemic illnesses without a clear bacterial infection, indicating an acute phase response and potential utility as an indicator of inflammation. Present result supported a number of studies, with (Yigit et al., 2015) showed that relationships between NGAL and inflammation markers (hs-CRP, IL-6 and TNF-α).(Kumar & Rizvi, 2010) examined the function of CRP and tumor necrosis factor-alpha in the diagnosed pediatric with sepsis. TNF-α was shown to be a far more sensitive marker than CRP and culture for the early detection of sepsis and the severity of the disease. A two-pronged approach was utilized to assess the relationship between TNF-α and CRP in bacterial infection. Signiant correlations were noticed between the serum NGAL levels and CRP, and PCT, as well as WBC (white blood count), assessed during subsequent days. Similarly, urinary NGAL levels correlated innocently with CRP and, PCT, and in some assessments, with WBC. Additionally, serum NGAL was inversely related to the platelet count. In addition, (Fernandez-Carballo et al., 2021) that were explicitly intended to distinguish bacterial from non-bacterial illnesses assessed the CRP, TRAIL signature, and HNL in the patient with febrile illness and found elevated these markers. A previous review of (Kapasi et al., 2016) highlighted strong association between the CRP and TRAIL signature, TRAIL was found to be differentially expressed between bacterial and viral infections. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) immune response-derived indicators have been demonstrated in several studies to be able to distinguish between bacterial and viral illnesses in the emergency department (ED) both as a single marker and in conjunction with procalcitonin (PCT) or CRP. Furthermore, no research has been done on the use of TRAIL in conjunction with PCT and CRP in an adult ED population. Moreover, the combined clinical use of these indicators remains incompletely understood (van der Does et al., 2016) (van der Does et al., 2016, 2018).

Current study showed a significant positive correlation between Neutrophil and TNFL while there was non-significant correlation between other markers in viral infection group. These results consistent with (Bradley et al., 2012) who demonstrate There is a close correlation between neutrophils and TNF when active neutrophils may release a variety of cytokines and chemokines, including TNF, which attracts and activates more neutrophils to the infection site. Also (Grudzinska & Sapey, 2018) clarify that Neutrophil production of proinflammatory cytokines such as tumor necrosis factor (TNF) is important in initial phases of viral disease. Moreover, Neutrophils are frequently implicated in controlling secondary bacterial infections linked to respiratory virus infections, according to research by Johansson & Kirsebom (2021), also, (Lee et al., 2011) showed increased levels of TNF-α in Patients with Influenza, this explains the reason for the positive relationship between neutrophil and TNF during viral infection.

This study illustrates a significant positive correlation between CRP and NAGL; a significant inverse correlation was reported between Neutrophil and MMP-8 in control group. These results strong supported by (Smertka et al., 2014) who found positive correlation between CRP and NAGL in both control group and septic group.

5. Conclusion

In conclusion, Biomarkers such as TNFL, MMP-8, and NGAL have the potential to enhance clinical decision-making by modifying the pre- and post-test of experiencing fever considerably. Early and accurate differentiation between these infections allows for appropriate and timely treatment, improving patient outcomes and reducing healthcare costs associated with misdiagnosis and overtreatment. Exploring the integration of these biomarkers into rapid diagnostic tests could facilitate their use in various healthcare settings, including resource-limited areas.

6. Ethical Approval

The relevant ethics committee of the health directorate will receive the study protocol. Prior to collecting the sample, each subject will also be asked for their verbal consent. Safety and health precautions will be followed when collecting the samples

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