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Role of Some Immunological Biomarkers in Differentiation of Bacterial and Viral Infection Among Children with Acute Febrile Illnesses

A Thesis

Submitted to the Council of the College of Medicine/University of Karbala,for partial the Fulfillments of the Requirements for the Master Degree of Science in Medical Microbiology.

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

﴿وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ ۚ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا﴾

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Supervisors Certification

We certify that this M.Sc. thesis titled:

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Was prepared under our supervision in the College of Medicine/ University of Kerbala, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology.

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Committee certification

We, the examiners committee, certify that we´ve read the M.Sc. thesis entitled:

"Role of Some Immunological Biomarkers in Differentiation of Bacterial and Viral Infection Among Children with Acute Febrile Illnesses"

We have examined the student **(Ali Abbas Meran Al-Kaaby)** in its contents. In our opinion it is meets the standards of thesis for the degree of Masters in Medical Microbiology and Immunology.

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Dedication

To the inspiration of humanity, Great Prophet Mohammad and Ahlu albeit…

To whom I carry his name proudly, the greatest man ....My Father...

To my angel in life, whose prayer was the secret of my success... The most precious woman in my life, My Mother...

To the one who taught me the meaning of loyalty and devotion, and bore with me the burdens of life, with its happiness and misery... my wife is the secret to my peace of mind….

To my brothers, friends and everyone who helped me..

To my lofty country (Iraq).

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Summary

Febrile illnesses in pediatric patients present significant diagnostic challenges due to their diverse etiologies, including bacterial, viral, and inflammatory conditions. Differentiating between these causes is crucial for guiding appropriate treatment strategies, minimizing unnecessary antibiotic use, and improving patient outcomes. Biomarkers such as Tumor Necrosis Factor-related apoptosis inducing ligand (TNFL), Neutrophil Gelatinase-Associated Lipocalin (NGAL), and Matrix Metalloproteinase-8 (MMP-8) are often studied for their roles in the body's response to infection and inflammation. This study aims to investigate the diagnostic utility of three specific immune markers TRAIL, MMP-8, and NGAL in children presenting with febrile illnesses.

This study is a Case-control study and the samples were divided into two groups: 60 febrile cases (30 patients with bacterial infection and 30 with viral cases) and 30 healthy controls. Enrolled in Karbala Teaching Hospital for Children, Karbala, Iraq. During the period November(2023) to February (2024).The samples of blood and serum were collected and detected the level of CRP, WBCs, Neutrophils, and lymphocytes were by Swelab alfa automated haematologyanalyzers, and TNFL, MMP-8, and NGAL by ELISA technique.

The study found elevated CRP, WBCs, and Neutrophil levels in patients with bacterial infection, and lymphocytes significantly increased in patients with viral infection at ($P \leq 0.05$). As well as, the study demonstrated that TNF-related apoptosis-inducing ligand (TNFL), Matrix Metalloproteinase 8 (MMP-8), and Neutrophil Gelatinase-Associated Lipocalin (NGAL) exhibit significant($P \leq 0.05$)potential as biomarkers to differentiate bacterial from viral infections in febrile children. TNFL, in particular, showed high specificity (0.8)

for bacterial infections, while elevated levels of MMP-8 (0.683) and NGAL (0.8) were more indicative of viral infections. The results showed an increase in the concentration of NGAL in females (0.038) compared to males in the group of patients with viral infection. The results of statistical analysis showed that TNFL significantly (0.002) increase in patients with bacterial infection as compared with patients with viral infection and control subjects. NGAL and MMP-8 were significantly (0.001) and (0.000) increased in patients with bacterial and viral infections as compared with control subjects respectively.

The results of this study have shown that TNF-related apoptosis-inducing ligand (TNFL), Matrix Metalloproteinase 8 (MMP-8), and Neutrophil Gelatinase-Associated Lipocalin (NGAL) exhibit significant potential as biomarkers to differentiate bacterial from viral infections in febrile children.

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List of abbreviations

ART	Antiretroviral Therapy
BBS	Bardet-Biedl Syndrome
CBC	Complete Blood Count
CD	Crohn's Disease
CNS	Central Nervous System
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
FDA	Food And Drug Administration
HIV	Human Immune Deficiency Virus
ICAM	Intercellular Adhesion Molecule 1
IDSA	Infectious Disease Society Of America
IL	Interleukin
iNTS	Invasive Non-Typhoidal Salmonella
LMICs	Low- And Middle-Income Countries
LPS	Lipopolysaccharide
MMPs	The Matrix Metalloproteinases
MPO	Myeloperoxidase
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NSID	Non Steroid Antiinflammation Drug
NST	Tractus Solitariut
PAMPs	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
SBIs	Serious Bacterial Infections
SPSS	The Statistical Package For The Social Sciences Software
T2DM	Type 2 Diabetes Mellitus

TB	Tuberculosis
TIMPs	Tissue Inhibitors Of Metalloproteinases
TLR	Toll-Like Receptor
TLR-4	Toll Like Receptors 4
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
UTI	Urinary Tract Infection
VCAM-1	Vascular Cell Adhesion Molecule-1
WHO	World Health Orgnazation

Chapter one

Introduction & literature Review

1.1 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).

Febrile illnesses are particularly prevalent in children under five years of age, reflecting the immaturity of their immune systems and increased exposure to infectious agents (Herlihy *et al.*, 2016). According to global health statistics, febrile illnesses account for a significant proportion of pediatric hospital visits and admissions. For instance, in low- and middle-income countries, febrile illnesses are a leading cause of morbidity and mortality among children (Woldkiros, 2021), often associated with diseases such as malaria, dengue, typhoid fever, and respiratory infections (Endy, 2020).

Misdiagnosis of febrile illness can lead to inappropriate antibiotic use, contributing to antibiotic resistance and adverse drug effects (Heffernan & 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).

A biomarker Tumor Necrosis Factor-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 & Ivanovska, 2016). tumor necrosis factor 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). In addition, Matrix

metalloproteinase-8 (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).

Furthermore, Neutrophil Gelatinase Associated Lipocalin (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 & Witkin, 2015). Neutrophil Gelatinase Associated Lipocalin level rise in response to bacterial infections, reflecting the body's inflammatory response and neutrophil activation (Venge & Xu, 2019). The accurate measurement and interpretation of these immune markers can significantly enhance the diagnostic process in pediatric febrile illnesses. However, their clinical utility requires robust validation through systematic research (Zandstra *et al.*, 2021).

However, 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).

Aim of the study:

The aim of this study was planned to estimate the association between serum level in a patient with polycystic ovarian syndrome in a group TNF-related apoptosis inducing ligand, Matrix metalloproteinase 8 and Neutrophil Gelatinase Associated Lipocalin as a biomarkers To distinguish Bacterial From Viral Infections in Febrile Children; the following objectives achieved this:

1- Analyzing laboratory data (CRP, WBCs, neutrophils, and lymphocytes) levels to serve as reliable markers for differentiating between bacterial, viral cases, and control group.
2- Determination the TNFL, MMP-8, and NGAL serum levels of patients and control groups by ELISA test.
3- Comparing the levels of (TNFL, MMP-8 and NGAL) biomarkers in febrile children with confirmed bacterial or viral infections, to establish their effectiveness in improving diagnostic accuracy and guiding appropriate treatment decisions.
4- Comparing the levels of immunological markers with laboratory variables in patients and control groups.
5- Utilizeing ROC curve analysis to assess the diagnostic accuracy of TNFL, NGAL, and MMP-8 as biomarkers for pediatric febrile illnesses. By analyzing the sensitivity, specificity, and AUC for each marker.

Literature Review

1-2 Defintion Febrile Body Temperatures

In healthy middle-aged individuals, fever can be characterized by an oral temperature exceeding 37.2 °C (99 °F) in the morning or surpassing 37.7 °C (100 °F) at any point throughout the day (Prasad Sharples *et al*., 2015; Nkunzimana, 2022). According to the fever management guidelines set by organizations such as the World Health Organization (WHO), the Society of Critical Care Medicine, and the Infectious Disease Society of America (IDSA) (Organization 2013b), a rectal temperature of 38 °C (100.4 °F) or higher, or an axillary temperature of at least 37.5 °C (99.5 °F), is considered indicative of fever in both adults and children. Infants and young children, however, tend to experience higher, more prolonged fevers with faster temperature increases and greater fluctuations compared to older children and adults (Barbi *et al.,* 2017).

Of the three major sites (i.e. rectal, oral and axillary) used for temperature assessment, rectal temperatures more closely estimate core temperatures than oral temperatures or axillary temperatures (Niedermann *et al.,* 2014). Although axillary temperatures are convenient to undertake, they are the least accurate method of temperature measurement, especially in adults. Axillary thermometers take longer time to reach equilibrium and they are altered by various factors such as by ambient temperature, sweat, humidity and the density of hair in the axilla (Marui *et al.,* 2017).

1-3 Epidemiology of Febrile Illness

In developed nations, the widespread implementation of vaccination programs against *Haemophilus influenzae* type b and *Streptococcus pneumoniae* has significantly reduced the occurrence of invasive diseases (Fitzwater *et al*., 2019). Consequently, systemic bacterial infections are relatively rare in these regions. Instead, localized bacterial infections, including urinary tract infections and pneumonia, are the main bacterial causes of fever among children (El-Radhi, 2018). Nevertheless, viral infections account for as much as 76% of fevers that lack an identifiable source. In low- and middle-income countries (LMICs), where there is ongoing but not yet widespread introduction of *H influenzae* type b and pneumococcal vaccines in most regions and where typhoidal and nontyphoidal *Salmonella* remain important causes of invasive bacterial disease but the currently available typhoid vaccine has suboptimal efficacy, there is scant literature on the burden of disease attributable to febrile illness among children. Researchers tend to investigate particular syndromes such as pneumonia (Iroh Tam *et al.*, 2016).

Among children, studies investigating acute febrile illness using various microbiological methods detected malaria in 1.3% to 64.4% of cases, Leptospira spp. in 0.4% to 7.7%, Rickettsia spp. in 0.4% to 7.4%, Coxiella spp. in 0.1% to 2.6%, and Brucella spp. in up to 2.0%. In an outpatient pediatric study conducted in Tanzania, respiratory viruses were identified as the cause in more than 50% of fever cases, with adenovirus accounting for 30% of these cases (Prasad, 2014; Iroh Tam *et al*., 2016; Kigozi *et al*., 2023; Oo, 2023).

Additional pathogens linked to acute febrile illness in patients with bloodstream infections include *Mycobacterium avium complex*, responsible for 1% of cases, *Salmonella* spp. in 0.1% to 19% of cases, *Streptococcus*

pneumoniae in 0.1% to 6.5%, Escherichia coli in 0.1% to 7.2%, and *Staphylococcus aureus* in 0.2% to 22.4%. In adults, other fungal causes of bloodstream infections, such as *Cryptococcus neoformans* (1.8% to 9%) and *Histoplasma capsulatum* (1.0%), have also been reported (Pond, 2005; Thriemer *et al*., 2013; Iroh Tam *et al*., 2016; De Winter, 2022).

In Bangladesh, typhoid is a significant cause of acute febrile illness, with preschool-aged children having a relative risk ranging from 8.9 to 12 compared to older individuals. Children under 2 years have an incidence rate of 443 per 100,000 child-years, which is higher than the rate of 405 per 100,000 child-years for those under 5 years (Iroh Tam et al., 2016). In South Asia, dengue, typhoid, and paratyphoid are the most frequently identified pathogens among febrile patients, detected in 25% and 23.2% of cases, respectively (Kuehn et al., 2022). Other pathogens, found less frequently, included Rickettsia spp. (positive in 17% of all tested isolates), West Nile virus (5%), and Hantavirus (2%). Research in Southeast Asia has revealed that the primary pathogens causing febrile illnesses are scrub typhus (1%–19.3% of cases), typhoid (1.8%–23%), dengue (5.4%–43.1%), Japanese encephalitis virus (3.4%–5.8%), chikungunya (1.2%–28.4%), Burkholderia pseudomallei (0.2%–1.5%), and influenza (1.0%–38.8%) (Chheng *et al*., 2013; Mayxay *et al*., 2013; Iroh Tam *et al*., 2016).

The epidemiology of febrile illness reflects both the region's environmental conditions and public health challenges. Iraq's tropical and subtropical climate, coupled with conflict-related disruptions in healthcare infrastructure, has exacerbated the prevalence of various infectious diseases responsible for febrile syndromes. In recent years, Iraq has witnessed a shift in the pattern of febrile illnesses, with a decline in certain vector-borne diseases,

such as malaria, due to concerted public health interventions, but an increase in others, such as dengue fever, due to changing climatic conditions (Barathan, 2024).

1-4 Etiology of Febrile Illness

The diverse causes of acute febrile illnesses, coupled with limited diagnostic resources, pose significant challenges to effective diagnosis, treatment, and public health strategies for both endemic and epidemic diseases. This complexity is further increased by the fact that most patients exhibit vague symptoms, such as low-grade fever, malaise, headache, and muscle aches, without a specific infection site. Healthcare providers often struggle to pinpoint the exact cause due to a lack of appropriate diagnostic tools, frequently leading to presumptive diagnoses based on clinical signs and knowledge of prevalent pathogens (Kasper *et al*., 2012).

1-4-1 Bacterial bloodstream infection

Bloodstream infections are a leading cause of fever among hospitalized patients in various regions. Numerous studies have reported the presence of bacteremia in children suffering from severe febrile illnesses (Moyo *et al*., 2020). Immunosuppressive conditions, such as HIV and severe malnutrition, continue to be critical risk factors for the development of bacteremia (Archary *et al*., 2017).

Among the most common pathogens causing bloodstream infections are non-typhoidal *Salmonella enterica* serovars and *Salmonella enterica* serovar Typhi (Albert *et al*., 2019). Factors that heighten the risk for invasive non-typhoidal Salmonella (iNTS) infections include HIV infection, malnutrition,

sickle cell disease, prior malaria infection, and severe anemia (Gilchrist & MacLennan, 2019)

Salmonella enterica serovar Typhi bacteraemia was greater than 100 cases per 100,000 population per year at multiple rural and urban. Incidence was often highest among pre-school children (Ng'ang'a, 2018).

Streptococcus pneumoniae remains another common cause of bacteraemia, particularly among children. Despite some replacement by non-vaccine strains among patients with invasive pneumococcal disease (Balsells, 2021), Other commonly identified pathogens include *Staphylococcus aureus* , which isolates was the most prevalent bloodstream pathogen among children (Lisowska-Łysiak *et al.*, 2021).

Mycobacterium tuberculosis is a major cause of bloodstream infection among adults living with HIV, but less so in children. *Mycobacterium tuberculosis* was the cause of bacteraemia among 0.4% of children and 13.5% of adults with HIV and persists as a major cause of bloodstream infection that cause hospitalization among febrile cases (Barr *et al.*, 2020).

1-4-2 Viral infection

Viral infections frequently lead to fevers with no identifiable source, particularly in young children, and most febrile illnesses are determined to be viral and self-limiting. It is generally believed that the more severe infections in febrile children tend to be bacterial, which are less likely to resolve on their own compared to viral infections. Some researchers propose that rapid viral testing could help differentiate between viral and bacterial infections. Additionally, only a small number of young children with identifiable viral infections experience bacteremia (Kool *et al*., 2015).

Even in the absence of bacterial infections, many children with fever are still treated with antibiotics. Viruses and bacteria engage with distinct pattern recognition receptors on circulating blood leukocytes, which activate specific transcriptional pathways in the host's immune response (Effah *et al*., 2024). As a result, it is possible to identify unique transcriptional signatures that can differentiate between viral and bacterial causes of fever that present without a clear source. In studies involving gene expression microarray analysis on blood samples from febrile children infected with adenovirus, human herpesvirus, or enterovirus, as well as from those with acute bacterial infections and afebrile controls, the transcriptional profiles of blood leukocytes were able to clearly distinguish virus-positive febrile children from both virus-negative afebrile controls and those without fever but carrying the same viruses. These analyses defined virus-specific gene expression profiles (Hu *et al*., 2013).

Viral infections contribute to the development of fever in several ways: (1) by causing fever itself; (2) by inducing a level of fever that surpasses the individual's threshold for convulsive temperature; and (3) through the production of elevated cytokines or an abnormal immune response to the infection (El-Radhi, 2018). Certain viruses, like human herpesvirus (HHV) and influenza A, which are neurotropic and can sometimes invade the central nervous system, are implicated as causative agents, although the link to encephalitis or encephalopathy remains uncertain. Complex febrile seizures (lasting more than 15 minutes, focal, or occurring multiple times within 24 hours) might involve different mechanisms compared to simple febrile seizures (lasting less than 15 minutes, generalized, and occurring once), which are generally less severe. Distinguishing these seizures from encephalopathy can be challenging, as both are related to fever and infection, but in the case of complex

seizures, they may involve neurotropic or encephalopathic processes. Viral infections are found at similar rates in febrile patients, regardless of whether they experience seizures, suggesting that factors other than the infection itself might influence a child's susceptibility to convulsions. Fever acts as the primary trigger, though multiple contributing factors are likely involved (Masuyama *et al*., 2002; Millichap & Millichap, 2006; Desforges *et al*., 2019).

1-4-3 Protozoal infections

Visceral leishmaniasis, which can induce fever, significantly contributes to morbidity, especially in Middle Eastern countries, particularly among individuals living with HIV (World Health Organization, 2022). Human trypanosomiasis is endemic in certain regions of Central and West Africa. While public health initiatives focused on eradicating human African trypanosomiasis are decreasing its incidence, the prevalence of these infections among unselected febrile patients remains unclear and likely varies greatly by region (WHO, 2022)

1-4-4 Fungal infections

Fungal infections are often overlooked as a cause of febrile illness in hospitalized patients who do not have any underlying conditions. Cryptococcal infections, which can lead to widespread disease and meningitis, continue to be a major cause of mortality in developing countries, even with the increasing availability of Antiretroviral Therapy (ART) (Maze *et al*., 2018).

The epidemiology of cryptococcal disease is evolving, although it is still most frequently diagnosed during initial HIV presentations. This disease is becoming more frequently linked with treatment failures, discontinuation of therapy, and immune reconstitution during the early stages of ART. While data

are scarce, it is probable that histoplasmosis is prevalent in many areas worldwide and is often misdiagnosed as tuberculosis (Strid et al., 2007; Maze et al., 2018)

1-5 Symptoms of Febrile Illness

The thermal balance point is elevated by both humoral and neural signals, which in turn trigger a series of clinical and behavioral responses that define the febrile reaction. To achieve this new balance, the body minimizes heat loss through skin vasoconstriction (resulting in chills and piloerection), along with behavioral adaptations like curling up into a fetal position to decrease surface area or wearing warmer clothing and seeking heated environments. Subsequently, various mechanisms to increase body temperature are activated, including muscle contractions that result in rigors. When the fever signal dissipates in the central nervous system (CNS), the balance point returns to normal, activating heat loss processes such as sweating. As a result, fever is frequently marked by chills, rigors, elevated body temperature, and eventually sweating and a decrease in body temperature (Bouyou-Akotet et al., 2009; Iroh Tam et al., 2016)

Systemic symptoms such as headache, malaise, anorexia and other sickness behaviours may also accompany fever. These symptoms are due to the systemic effects of microbial products and pyrogenic cytokines that lead to various acute phase responses mediated through the neuroendocrine system (Kelley & Kent, 2020).

1-6 Classification and Patterns of Febrile Illness

Febrile illnesses can be categorized based on duration into three types: acute, sub-acute, and chronic. Acute fevers, lasting less than seven days, are commonly associated with infectious diseases like malaria and viral upper

respiratory infections. Sub-acute fevers, typically persisting for no longer than two weeks, may occur in conditions such as typhoid fever and intra-abdominal abscesses, among others (El-Radhi, 2018). Chronic or persistent fevers, which last longer than two weeks, are usually indicative of ongoing bacterial infections like tuberculosis, viral infections such as HIV, cancers, and connective tissue disorders. However, any acute fever can transition into a persistent or chronic state if not properly managed. Based on the height of body temperature, fever can also be classified into low grade, moderate grade, high grade and hyperpyrexia (Table 1). The height of body temperature may have some diagnostic and prognostic implications. Some studies have attributed high grade fevers in infants to serious bacterial infections. although others have also shown that children with high fevers are at equally high risk for serious bacterial infections and for viral illness (El-Radhi, 2018),

Table 1.1 **:**Normal and febrile body temperature ranges (rectal temperatures)

Body temperature	°C	°F
Normal	37–38	98.6–100.4
Mild/low grade fever	38.1–39	100.5–102.2
Moderate grade fever	39.1–40	102.2–104.0
High grade fever	40.1–41.1	104.1–106.0
Hyperpyrexia	>41.1	>106.0

The severity of a fever can sometimes indicate the seriousness of an illness, as observed in cases of shigellosis, dengue virus infection, and acute falciparum malaria, where hyperpyrexia often signals a more severe disease with a worse prognosis. Nevertheless, the patient's overall clinical condition is generally a more reliable indicator of serious illness than the fever's intensity (Ogoina, 2011).

Fever is typically categorized into three types: sustained (continuous) fever, intermittent fever, and remittent fever. Sustained or continuous fever is characterized by a consistent temperature that does not vary by more than about 1 °C (1.5 °F) over 24 hours and never returns to normal. This type of fever is commonly associated with conditions such as lobar pneumonia, gram-negative bacterial pneumonia, typhoid fever, acute bacterial meningitis, and urinary tract infections. Typhoid fever often presents with a gradual increase in temperature that reaches a high plateau, although this specific fever pattern is observed in only about 12% of cases in practice, likely due to the frequent use of self-medication with antibiotics and antipyretics before seeking medical attention. A fever accompanied by relative bradycardia (a slower-than-expected heart rate in relation to fever, known as Faget's sign) is a hallmark of untreated typhoid, leishmaniasis, brucellosis, Legionnaire's disease, psittacosis, and yellow fever, among other conditions (Ogoina, 2011; Ye *et al*., 2018).

Intermittent fever is characterized by a temperature spike that occurs for only a few hours each day, commonly seen in diseases such as malaria, pyogenic infections, tuberculosis (TB), schistosomiasis, lymphomas, leptospirosis, borreliosis, kala-azar, and septicemia (Torreggiani *et al*., 2016).

Remittent fever involves daily temperature fluctuations that exceed 2 °C but do not return to a normal range. This pattern is often linked to infectious conditions

like infective endocarditis, rickettsial infections, and brucellosis. Relapsing fever refers to fever episodes that recur, separated by periods of low-grade fever or no fever (David Knechtel & Opacic, 2019).

Relapsing or periodic fevers are observed in cases of malaria, lymphoma, borreliosis, cyclic neutropenia, and rat-bite fever. Fever that occurs along with night sweats is frequently associated with infections such as TB, Nocardia, brucellosis, liver or lung abscesses, and sub-acute infective endocarditis, as well as with non-infectious conditions like polyarteritis nodosa and cancers, including lymphomas (Rao, 2021)."

1-7 Pathogenesis

Fever can be an indicator of various diseases, each with a broad spectrum of severity. In healthy individuals, benign infections include bacterial conditions like otitis media, pharyngitis, impetigo, bacterial meningitis, relapsing fever, typhoid fever, and typhus fever, as well as viral illnesses such as pharyngitis, rhinitis, and pneumonia. If left untreated, severe bacterial infections can lead to significant morbidity and mortality, including sepsis, pyogenic meningitis, bacterial pneumonia, osteoarticular infections, and pyelonephritis. Life-threatening febrile diseases include malaria, typhoid fever, typhus fever, relapsing fever, and shigellosis (bacillary dysentery). Many febrile episodes are self-limiting infections that in healthy individuals present with minimal toxic signs and necessitate a thorough medical history and physical examination with limited laboratory tests (Adler & Mara, 2016).

However, certain high-risk groups, identified by factors such as age, coexisting conditions, and immunodeficiency, require comprehensive

evaluations and, in some cases, immediate antibiotic therapy before a pathogen (Tadesse & Tadesse, 2013).

1-8 Diagnostic

"Clinical history and physical examination play a crucial role in diagnosing febrile illnesses, particularly in resource-limited settings where these are often the primary tools available. Clinical algorithms have been devised to aid in identifying febrile diseases, aligning with World Health Organization (WHO) guidelines (Mfuh, 2017). The sensitivity of diagnostic tests can vary significantly depending on factors like the type and volume of fluid collected, the patient's age, underlying conditions, previous use of antibacterial agents, and the stage of the illness. For instance, blood cultures for detecting invasive Salmonella species have a sensitivity ranging from 40% to 80%, with the highest accuracy observed during the first week of illness when bacterial loads are at their peak (Iroh Tam *et al*., 2016). Bone marrow cultures, with a sensitivity of about 90%, remain effective even when antibiotics have been administered, whereas the sensitivity of stool cultures and rectal swabs is more limited (Antillon *et al*., 2018).

Some bacteria, such as Rickettsia and Leptospira species, require specialized cell-based systems or enriched culture media for growth, posing risks to laboratory personnel. These safety concerns limit their use in routine diagnostic labs. Respiratory tract infections represent a significant portion of febrile illnesses, but in cases of community-acquired pneumonia, blood cultures yield positive results in only a small number of patients (Baron *et al*., 2020).

Serological methods can detect a wide array of pathogens, but their effectiveness is hindered by the need for both acute and convalescent serum

samples to confirm seroconversion. This limitation was demonstrated in a study where acute serum antibody testing identified only 52.8% of dengue cases and 40.8% of leptospirosis cases that were later confirmed (Iroh Tam *et al*., 2016). For detecting Leptospira, the microscopic agglutination test has a sensitivity of 41% in the first week of illness, which improves to 96% after four weeks (Sykes *et al*., 2022). However, the need for multiple blood samples spaced four weeks apart reduces its practicality. Single-sample tests are sometimes used for presumptive diagnoses, but variations in cutoff values across different locations limit their accuracy, making it difficult to determine if a positive result truly indicates an acute infection. The low sensitivity and specificity of single agglutination tests are also observed in other serological assays, like the Widal test, particularly in the early stages of infection and in regions where these diseases are endemic (Dutta *et al*., 2006).

Using immunoglobulin M (IgM) antibodies for diagnosis can also be unreliable, especially if samples are taken too early in the illness. Furthermore, serological tests may exhibit either insufficient or excessive cross-reactivity with other related species. For example, the Widal test struggles to accurately differentiate between Salmonella enterica serovar Typhi and S. enterica serovar Enteritidis, despite the similarity of their O antigens. Serological tests for flaviviruses also face challenges due to significant cross-reactivity among the species (Fox *et al*., 2022). Although enzyme-linked immunosorbent assays (ELISA) have been utilized to study normal antibody responses during infection, their accuracy remains limited for certain diseases because of a lack of specificity. Research on febrile illnesses in Laos, which primarily used serological methods, found that 7% of patients appeared to have multiple infections (Mayxay *et al*., 2015; Iroh Tam *et al*., 2016).

Molecular diagnostic techniques have significantly improved pathogen detection, but their success varies depending on the pathogen and the sample type. For instance, the molecular diagnosis of Rickettsia species using polymerase chain reaction (PCR) has a sensitivity of 90% when using skin biopsy samples, which drops to 50% when blood samples are used. Similarly, PCR tests have shown limited sensitivity for detecting invasive Salmonella species due to the low concentration of the organisms in body fluids (Stewart & Stewart, 2021). However, recent advancements in molecular diagnostics have expanded the range of bacteria and viruses that can be detected, with notable progress in identifying respiratory viruses (Wylie *et al*., 2015).

1-9 Management

Managing febrile children is a fundamental component of pediatric care, as they represent around 15% of all emergency department visits. These cases can range from serious bacterial infections to self-limiting viral illnesses. Over the past three decades, there have been significant changes in the approach to treating febrile infants under 90 days old, influenced by the rise in Escherichia coli urinary tract infections, growing antibiotic resistance to ampicillin, and improvements in viral diagnostic techniques. (Cioffredi & Jhaveri, 2016).

Numerous studies have shown that a significant number of parents, guardians, or caregivers administer antipyretics to children even when the fever is mild or absent, often using incorrect dosages or not allowing adequate time between doses (Lubrano et al., 2016). Fever is a natural physiological response that helps the body fight infections and is not linked to long-term neurological damage. The main goal of treating fever in children should be to alleviate discomfort rather than to reduce the body temperature. Improper fever

management can lead to delays in diagnosing the underlying condition and increases the risk of antipyretic overdose. Factors like the simultaneous or alternate use of different antipyretics, using rectal formulations, and administering these medications despite contraindications due to underlying health issues can further elevate drug toxicity. Overuse of antipyretics can also have a notable economic impact in both low-middle-income and high-income countries (Lubrano *et al*., 2016).

Any child with a fever who appears significantly ill should be evaluated for suspected sepsis, regardless of the severity of the fever. It's important not to rely solely on symptoms like otitis media or upper respiratory signs as the definitive source of infection in young infants or sick children, as they still need to be assessed for potential serious bacterial infections (SBIs). If the child's condition is stable, it is recommended to conduct thorough investigations to identify the infection before starting antibiotics (Herlihy *et al*., 2016).

For children in high-risk groups, a lower threshold for initiating investigations is advisable. Urinary tract infection (UTI) is the most prevalent serious bacterial infection in these cases. If there is no clear source of fever, urine samples should be collected and tested. When blood cultures are necessary, ensure that a sufficient volume is collected for accurate results. Antimicrobial treatment recommendations should be based on local antimicrobial resistance patterns (Boon *et al*., 2021; Portal, 2022).

1-10 Mechanisms underlying febrile patterns

The exact mechanisms that cause specific fever patterns in different diseases are not yet fully understood. One theory suggests that repeated exposure to pyrogens, like lipopolysaccharides (LPS), may cause a decrease in cytokine production, resulting in intermittent or resolving fevers. Recurrent fevers could also arise from inadequate treatment of deep-seated infections, such as abscesses, or repeated encounters with new antigens, like allergens found in hypersensitivity pneumonitis (Ogoina, 2011). In some cases, these allergens may trigger an increase in eosinophils, especially in individuals with drug-induced fever. In cyclic neutropenic fevers, febrile episodes correspond to periods of neutropenia and are due to repeated bacterial infections (Zergham *et al.*, 2020).

In cases of cancer and pulmonary embolism, the recurrence of fever is partly linked to tissue necrosis, as the phagocytosis of necrotic tissue triggers the intermittent release of pyrogenic cytokines (Neethu, 2017). Recurrent fever may also be related to the disease's pathogenesis, as seen in relapsing fevers caused by spirochetes, where episodic spirochetemia leads to fever episodes interspersed with periods of afebrility that correspond to the disappearance of spirochetes from the bloodstream (Barbour & Kaplan, 2018).

While night sweats are common among healthy adults, they become clinically significant when associated with fever and drenching. This phenomenon may be linked to the pyrogenic properties of certain diseases, resulting in early morning fever spikes followed by remission, which manifests as night sweats (Larnard *et al*., 2023).

1-11 The fever pathways

Fever is regulated through two primary pathways: the humoral and neural pathways, activated by signals from both exogenous and endogenous pyrogens. In the humoral pathway, fever-inducing signals are transmitted by pathogen-associated molecular patterns (PAMPs) or pyrogenic cytokines, which are derived from microbial components

1-11-1 The humoral pathway

Microbial components known as pathogen-associated molecular patterns (PAMPs), such as gram-negative lipopolysaccharides (LPS), interact with Toll-like receptor 4 (TLR-4) present on various cells. When these PAMPs activate TLR-4 on the fenestrated capillaries of the circumventricular organ within the blood-brain barrier, they stimulate the release of prostaglandin E2 (PGE2) from the arachidonic acid pathway in the cell membranes(Zhang & Mosser, 2008; Santacroce *et al.*, 2023).

Prostaglandin E2 is small molecule that easily diffuses across the blood brain barrier, binds to specific PGE2 receptors (EP3 receptor) in the preoptic area and then activates thermal neurons in the anterior hypothalamus to a higher thermal balance point. It is unclear whether microbial products also lead to elevation of the thermal balance point by gaining direct access to the brain through disruption of the Bardet-Biedl Syndrome (BBS) (Ogoina, 2011).

The febrile response is characterized by an early rapid phase and a delayed late phase. Based on studies undertaken in animal models with polyphasic LPS-induced fever, it is believed that the first phase of this febrile response is dependent on PGE2 synthesized in the liver and lungs before migration to the brain, while the latter phases are due to centrally synthesized

PGE2 (Pákai, 2020). Consequently, while peripheral synthesized PGE2 may act to initiate the febrile response, centrally synthesized PGE2 may be largely involved in its maintenance (Wright & Auwaerter, 2020).

The second humoral pathway is directed by circulating pyrogenic cytokines. They transmit fever signals to the thermoregulatory circuitry by both indirect and direct pathways. In the indirect pathway, pyrogenic cytokines act outside the brain by binding and activating cytokine receptors located on the fenestrated capillaries of the circumventricular organ leading to release of PGE2 (Mirrasekhian, 2020).

In the direct pathway, circulating cytokines disrupt the blood-brain barrier, allowing direct access to cytokine receptors on vascular, glial, and neuronal structures in the brain. The activation of these central receptors further stimulates the synthesis of PGE2 or promotes the de novo production of additional cytokines by the brain (Archie *et al*., 2021).

While PGE2 is essential for the febrile response, some cytokines and various inflammatory mediators can activate this response independently of PGE2 (Blomqvist & Engblom, 2018). Cytokines may directly activate thermal neurons without involving PGE2, which could contribute to hyperpyrexia observed in CNS infections and hemorrhages—often referred to as central fever. In these scenarios, the anti-pyretic functions of the CNS are disrupted, leading to an uncontrolled increase in body temperature. Other inflammatory mediators, such as bradykinin, corticotropin-releasing hormone, nitric oxide, MIP-1, IL-6, IL-8, and preformed pyrogenic factors (PPF), may also reset the thermal balance point independently of PGE2 (Ogoina, 2011).

1-11-2 The neural pathway

Fever signals from the peripheral body can reach the central nervous system (CNS) via pathways involving peripheral nerves, including cutaneous sensory nerves and the vagus nerve. This activation of the neural pathway is considered a key mechanism for the rapid onset of fever (Blomqvist & Engblom, 2018).

It has been proposed that localized production of PGE2 at inflammation sites contributes to fever generation by activating cold-sensitive cutaneous nerves, which then relay fever signals to the brain regions responsible for generating fever (Blomqvist & Engblom, 2018). The transmission of these fever signals through the vagus nerve involves a more complex pathway. Circulating pyrogens, such as LPS, activate complement proteins, which subsequently stimulate Kupffer cells in the liver to produce endogenous mediators, including pyrogenic cytokines. These cytokines activate the hepatic branch of the vagus nerve, transmitting fever signals to the central projections within the nucleus of the tractus solitarius (NST). From the NST, the signals travel to the preoptic and hypothalamic areas via the ventral noradrenergic bundle, leading to the release of norepinephrine in the preoptic region (Santacroce et al., 2023).

Norepinephrine plays a role in mediating the vagal pathway by inducing distinct increases in core temperature. The first increase is rapid and mediated by alpha (1)-adrenoceptors (AR), and is independent of PGE2, while the second increase is delayed, mediated by alpha (2)-AR, and is dependent on PGE2. The role of vagal afferents in fever generation has been supported by studies in rats, which demonstrated that surgical vagotomy can attenuate or completely eliminate febrile responses to pyrogenic signals (Ogoina, 2011). However, further research has questioned this perspective, suggesting that the absence of febrile response may be due to vagotomy-related side effects, such as

malnutrition (Blomqvist & Engblom, 2018). When these side effects are minimized, studies in rats indicate that complete or partial vagotomy does not abolish the febrile response to pyrogenic signals like intravenous PGE2 (Ogoina, 2011; Roth & Blatteis, 2014).

1.12 Role of some immunological markers in febrile illness

1-12-1 Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has traditionally been used as a biomarker for cardiovascular and autoimmune diseases, but recent studies have highlighted its potential as a reliable viral biomarker (Rhedin *et al*., 2021). TRAIL is part of an FDA-approved diagnostic test that combines it with CRP and interferon gamma-induced protein 10 to improve the accuracy of distinguishing between bacterial and viral infections. Research indicates that this test outperforms procalcitonin (PCT) in accuracy, based on external validation studies conducted in febrile children (Ashkenazi-Hoffnung *et al*., 2018)

Fever is a common response to infection. In mice, a high septic-like dose of bacterial lipopolysaccharide (LPS) induces systemic inflammation that results in an acute phase response (APR), accompanied by symptoms such as hypothermia, fever, cachexia and anorexia The fever induced by LPS is mediated by IL-6 and TNF-α, with IL-6 acting as an endogenous pyrogen and TNF-α functioning as an endogenous antipyretic or cryogen (Leon et al., 1998). For instance, studies by Chai et al. (1996) and Nilsberth et al. (2009) found that mice lacking IL-6 did not develop fever following LPS injection. Conversely, mice deficient in TNF receptors (p55 and p75), the two known signaling receptors for TNF, exhibited exaggerated fevers in response to high doses of LPS.

Additionally, the TRAIL/TRAIL receptor (TRAIL-R) system influences the development of both viral and bacterial infections. TRAIL may play a dual role in the immune system, capable of killing infected cells while also contributing to the pathogenesis of various infections. Many pathogens have developed strategies to manipulate TRAIL signaling, thereby enhancing their replication (Gyurkovska & Ivanovska, 2016).

Viral infections typically trigger an inflammatory response characterized by the release of cytokines and chemokines, impacting numerous cellular processes such as signaling, apoptosis, transcription, and DNA repair. Various viral proteins can independently induce apoptosis or autophagy without the need for other viral proteins. During viral infections, type I interferons (IFNs) are produced in high amounts and play crucial roles in both innate and adaptive immunity against viruses (Trinchieri, 2010). Toll-like receptors (TLRs) are essential for transducing type I IFN signaling upon recognizing specific viral components. For example, TLR3 and TLR4 identify viral double-stranded RNA (dsRNA), activating NFκB. TLRs also detect dsDNA and capsid components of human papillomavirus (HPV), contributing to HPV regression. Research indicates that non-infected cells resist TRAIL-induced apoptosis, but they become sensitive to it following viral infections (Davidson et al., 2014; Gyurkovska & Ivanovska, 2016).

1-12-2 Neutrophil Gelatinase Associated Lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) is an iron-binding protein with antimicrobial and anti-inflammatory properties, produced by cells such as neutrophil granulocytes and renal tubule cells in response to infections or inflammatory conditions (Walvik *et al*., 2020). Oral infections, for instance,

can trigger the migration and activation of neutrophil granulocytes(Rijkschroeff *et al.*, 2016). Whereas epithelial cells primarily produce a monomeric form of the lipocalin, activated neutrophil granulocytes are the main producers of the dimeric form of NGAL (Mårtensson & Bellomo, 2014)

Neutrophil gelatinase-associated lipocalin (NGAL) forms complexes with bacterial metalloproteases, limiting the pathogens' ability to uptake iron, thus acting in a bacteriostatic manner that supports the innate immune system (Walvik *et al*., 2020). As an acute phase protein, NGAL plays a role in antibacterial immune processes. Inflammatory cytokines induce NGAL expression in neutrophils, epithelial cells, and hepatocytes. Damage to epithelial cells in the intestine, stomach, liver, or lungs during infections leads to increased plasma NGAL concentrations (Romejko *et al*., 2023).

Additionally, NGAL modulates iron transport as part of the antibacterial immune response. During inflammation, bacteria produce siderophores, which have a high affinity for iron, causing it to dissociate from lactoferrin and transferrin and transfer into the pathogens (Parrow et al., 2013). Macrophage stimulation through Toll-like receptors (TLRs) enhances NGAL synthesis by upregulating its gene. NGAL sequesters siderophores, thereby preventing bacteria from accessing iron and inhibiting their growth, as bacterial proliferation often relies on iron availability (Romejko et al., 2023). In experiments with mice, NGAL was shown to manage bacterial infections by altering iron transfer (Scindia *et al*., 2019).

Moreover, NGAL has been found to inhibit siderophore production by *Escherichia coli*, which can be implicated in pneumonia. NGAL expression increases in bronchial epithelium and alveolar type II pneumocytes during respiratory infections with *E. coli* (Wu *et al*., 2010). NGAL also provides

protection against other infections, such as those caused by *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Mycobacterium tuberculosis* (Romejko et al., 2023; Robinson *et al*., 2014).

In the context of gastritis caused by *Helicobacter pylori*, inflammation of the mucosa raises local NGAL expression (Zijlstra, 2016). Additionally, NGAL enhances bacterial clearance from the urinary system. While its primary bacteriostatic function is to sequester bacterial siderophores, NGAL also plays a role in the activation and differentiation of T-cells toward the Th1 phenotype (Romejko *et al*., 2023). A deficit in NGAL impairs neutrophil function, potentially disrupting chemotaxis, adhesion, and the migration of inflammatory cells (Araos *et al.*, 2020). Research indicates that patients with reduced NGAL levels are more susceptible to various infections (Forster *et al*., 2017).

NGAL concentrations increase in sepsis and correlate with inflammatory markers such as interleukin-6 (IL-6), interleukin-10 (IL-10), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and leukocyte counts (Macdonald *et al*., 2017). Plasma NGAL levels are notably higher in patients with septic shock and sepsis-related organ failure compared to those with milder forms of sepsis (Chang *et al*., 2018). NGAL is also involved in fungal and viral infections. Although its exact role in fungal infections remains unclear, increased NGAL gene expression has been observed in candidiasis (Colceriu *et al.*, 2023).

The induction of NGAL expression has been documented in epithelial cells from patients with infections caused by human papillomavirus and rotavirus. In children suffering dehydration from rotavirus infections, NGAL levels may serve as an early indicator of renal impairment (Arcolaci *et al*., 2023).

Conversely, in patients with human immunodeficiency virus (HIV), serum NGAL levels are low due to reduced neutrophil counts and impaired neutrophil function (Nada *et al*., 2020)

As one of the useful, noninvasive, and convenient biomarkers, neutrophil gelatinase-associated lipocalin (NGAL) is an emerging indicator for diagnosing UTI, thus, the NGAL has great potential for early diagnosis of UTI. There are 2 forms of the NGAL: plasma NGAL (pNGAL) and urine NGAL (uNGAL). pNGAL was secreted by neutrophils as a result of systemic inflammations, and uNGAL is specific for damage to the genitourinary epithelium (Moon *et al.*, 2021).

1-12-3 The matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that play a significant role in remodeling the extracellular matrix (ECM) in both physiological and pathological processes. The activity of MMPs is carefully controlled through various mechanisms, including regulation at the transcriptional and translational levels, secretion in an inactive state, and inactivation through binding to specific endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (He *et al.*, 2023).

The MMPs were first described as early as 1949 with the discovery of depolymerizing enzymes involved in connective tissue growth (Laronha & Caldeira, 2020). However, the research into what is currently known as the family of matrix metalloproteinases began in 1962 when Woesnner, followed by Gross and Lapiere, discovered and characterized an enzyme with collagenolytic activity in the amphibian tissues (Maskos, 2005; Laronha & Caldeira, 2020). It was only in 1980 that Harris *et al*. proposed using the name

MMPs for this group of collagenases/gelatinases. The past 60 years have seen remarkable progress in studying the biological functions of MMPs and their involvement in numerous biological processes such as tissue repair and remodeling, cellular differentiation, embryogenesis, morphogenesis, cell proliferation and apoptosis (Tallant *et al.,* 2010). Not surprisingly, the deregulation of MMPs activities leads to several pathological processes and diseases such as periodontal diseases, arthritis, cancer, neurodegenerative disorders, cirrhosis, and cardiovascular abnormalities (Laronha & Caldeira, 2020).

Matrix metalloproteinases (MMPs) are major enzymes involved in extracellular matrix remodeling; they can also act intracellularly, are capable of activating growth factors in their proximity, cell surface receptors, and adhesion molecules (Luchian *et al.,* 2022).

MMP-8, also known as collagenase 2, is recognized as one of the most promising biomarkers for periodontitis in oral fluids (Luchian *et al.,* 2022). Rautava et al. (2020) discuss Crohn's disease (CD), a complex inflammatory condition of the gastrointestinal tract, highlighting the increased risk for these patients to develop periodontitis, dental caries, and oral mucosal lesions. The study suggests that the dysregulation of the immune system in CD may influence MMP-8 levels in the oral cavity. In this context, MMP-8 appears to be a key inflammatory mediator; elevated levels have been found in both the intestines and oral cavities of CD patients.

Current evidence links matrix metalloproteinases (MMPs) and their endogenous inhibitors to various pathways involved in the development and

progression of diabetic microvascular complications. In diabetic nephropathy, altered MMP expression contributes to extracellular matrix deposition and glomerular hypertrophy, ultimately leading to proteinuria and renal insufficiency. In diabetic cardiomyopathy, MMPs are involved in collagen and elastin degradation, myocardial remodeling, and the susceptibility of coronary plaques. The progression of diabetic peripheral arterial disease is mediated by impaired angiogenesis related to MMP activity. Experimental data suggest that MMPs play a crucial role in cerebral circulation and stroke volume in diabetes. An excess of MMPs may hinder wound healing in diabetic patients. Future research should further elucidate the role of MMPs in the pathophysiology of diabetes and explore potential therapeutic options (Tsioufis *et al*., 2012).

Matrix metalloproteinases (MMPs) may play a pathophysiological role in the development of diabetic nephropathy (DN). that urinary MMP activity in patients with type 2 diabetes mellitus (T2DM) is related to a decline in renal function. determined MMP-2, -8 and -9 activity in 24-h urine collections in relation to risk factors for DN in T2DM patients with and without albuminuria (van der Zijl *et al.*, 2010).

MMP-8 is the most abundant MMPs in periodontal tissues, and their level reflects the severity of the disease and its progression and response to treatment. They are secreted due to the infiltration of polymorphonuclear leukocytes and also macrophages, plasma, and residual cells such as fibroblasts, endothelial cells, keratinocytes, and bone cells (Luchian *et al.*, 2022).

An important biological function of MMP-8 in the periodontium is to facilitate the migration of leukocytes, especially neutrophil granulocytes, from the circulation to the periodontal sulcus by the cleavage of collagen and other components of the extracellular matrix (Luo *et al.,* 2017). In addition, after the onset of inflammation, other cell types (e.g., fibroblasts) may also express MMP-8 . Increased expression, release, and activation of uncontrolled MMP-8, along with other MMPs and proteinases, are thought to induce the inflammation associated with tissue destruction in periodontal disease and also other inflammatory diseases (Verstappen & Von den Hoff, 2006).

MMP8 also affects endothelial cells through another additional mechanism through its involvement in glycocalyx degradation pathways and causing degradation of endothelial glycocalyx, which leads to loss of vascular reactivity, which is one of the most important causes of multi- organ damage and failure in septic shock (Pietrasanta *et al.,* 2019).

In addition, MMP8 plays an additional role in exacerbating the inflammatory state at the level of macrophages, as studies have shown that it is complicit in the rapid activation of NF-κB following the septic state, which increases the severity of the inflammatory state and is associated with an increased probability of death. Indeed, an increase can be observed in MMP8 concentrations are high in sepsis and even more severe in septic shock and additional organ failure (Solan *et al.,* 2012).

The activation of MMP-8 is facilitated by other MMPs and host proteases and increased oxidative stress caused mainly by neutrophil-released myeloperoxidase (MPO). Bacterial-derived proteases, such as Porphyromonas gingivalis (gingipain) and Treponema denticola, can also activate MMPs (Luchian *et al.,* 2022).

Chapter Two

Materials and Method

2.1 Materials and Methods

2.1.1 Subjects

A total of ninety blood and serum samples were collected from clinically diagnosed participants using CBC and C-reactive tests. These samples were categorized into two groups: 60 febrile cases (≥38°C) were further divided into 30 with viral infections and 30 with bacterial infections, while the remaining 30 samples served as the control group (healthy individuals). The study involved both male and female participants (47 males and 43 females) aged between >1 years -14 years, who were admitted to Karbala Teaching Hospital for Children in Karbala, Iraq, from November 2023 to February 2024. Case information sheets involving age, gender, and other information were carried out for each patient (Appendix 1).

Inclusion criteria included: Clinical suspicion of an acute infectious disease, Children <1–14 years of age peak fever >37.5°C since onset of symptoms, and duration of symptoms ≤12 days.

Exclusion criteria included: evidence of acute infection in the two weeks preceding enrollment; congenital immune deficiency; treatment with immunosuppressive or immunomodulatory therapy; active malignancy; and human immunodeficiency virus (HIV)-1, or hepatitis B/C virus infection

2.1.2 Approval of the Ethical Committee

Approval for the study protocol was obtained from the Karbala Medical College Ethical Committee, ensuring that informed consent was provided by all subjects or their family members prior to participation.(No:24-45)(Date 29/9/2024)

2.1.3. Study design

Case-control study

2.1.4. Study Design scheme

The design of study was illustrated in figure (2-1).

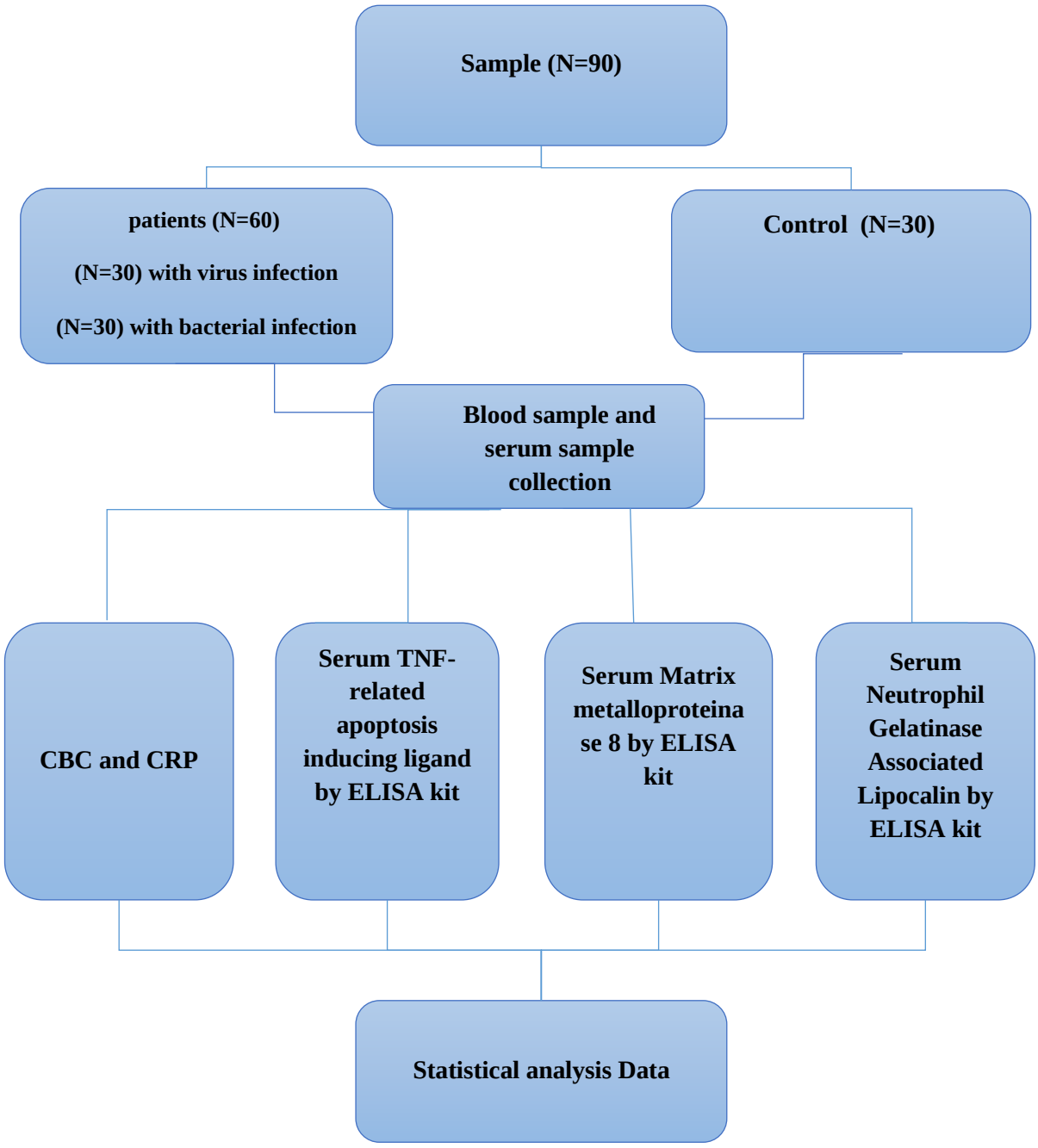

Figure (2-1): scheme of the Study Design.

2.1.5 Laboratory Apparatuses and Instrument

The primary instruments and disposable materials utilized in this study are detailed in Table 2-1.

Table (2-1): Instrument and Apparatus current study

Type of Equipment	Company/Orgin
Centrifuge	Hitachi (Germany)
Chemistry Auto analyzer system	Roche INTEGRA Germany
Deep freezer	GFL (Germany)
ELISA Reder system	Human (Germany)
ELISA Thermo-shaker	Biosan (European)
ELISA Washer	Human (Germany)
Eppendorf tube (2ml)	Bio-Basic (Canada)
GEL tube (7ml)	*AFCO (*Jordan)
Gloves	TGR (Malaysia)
Hematology system	Bio-zik china 5D
Micropipette tips	BIOBASIC (Canada)
Micropipettes	BIOBASIC (Canada)
Multichannel pipette	BIOBASIC (Canada)
Syringes	ENTEPLIN (Egypt)
Vortex mixer	Gemmy (Taiwan)

2.1.6. Commercial kits

Table 2-2 outlines the main commercial kits employed in this study

Table (2-2): Commercial kits used in the present study

Type of Kits	Company/Origin
Human Matrix metalloproteinase 8 (HMMP8)	Bioassay Technology (China)
Human neutrophil gelatinase-associated lipocalin (HNG)	Bioassay Technology (China)
Human Tumor necrosis factor ligand (HTN)	Bioassay Technology (China)
C-reactive	Roche Germany

2.2. Methods

2.2.1. Specimen collection

Five milliliters of venous blood were drawn from both patients and control groups using gel tubes, ensuring a slow withdrawal of the sample to prevent hemolysis. The blood was then placed into clean, disposable gel tubes, and the serum was allowed to separate at room temperature for 20 minutes. The samples were then centrifuged at 3500 rpm for 5 minute and then stored in to separated three eppendorf tubes at freeze condition (-20C) until analyzed.

2.2.2. Complete Blood Count

The blood specimen in EDTA tube was shaken up then was examined as soon as possible in Swelab alfa automated hematology analyzers to count white blood cells, lymphocyte, hemoglobin, and platelet.

2.2.3. Immunological markers assay by ELISA

The serum concentrations of TNF-related apoptosis-inducing ligand, Matrix metalloproteinase 8, and Neutrophil Gelatinase Associated Lipocalin were measured using the sandwich-ELISA technique with specific ELISA kits.

2.2.4. Principle of Sandwich ELISA technique

Sandwich ELISA is often regarded as one of the most effective Immunosorbent assays for antigen detection, as it typically demonstrates sensitivity levels that are two to five times higher than assays where the antigen is directly attached to a solid surface. To detect antigens, the wells of microtiter plates are coated with specific (capture) antibodies followed by incubation with test solutions containing antigens. An unbound antigen is washed out and a different antigen-specific antibody conjugated to the enzyme (developing reagent) is added, followed by another incubation. Unbound conjugate is washed out and substrate is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution (Premjeet *et al.*, 2011).

2.2.5. Human immunological marker ELISA Kits description

Enzyme immunoassay for the quantitative determination of the TNF-related apoptosis-inducing ligand, Matrix metalloproteinase 8 and Neutrophil Gelatinase Associated Lipocalin (Bioassay Technology, China, Zhejiang, Number E3154Hu, E0903Hu and E1719Hu respectively).

2.2.6. Tumor necrosis factor (TNF) related apoptosis inducing ligand

2.2.6.1. Test Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human TNFSF10 antibody. TNFSF10 present in the sample is added and binds to antibodies coated on the wells. And then

biotinylated Human TNFSF10 Antibody is added and binds to TNFSF10 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated TNFSF10 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human TNFSF10. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.2.6.2 Kit components

The contents of Tumor necrosis factor (TNF) related apoptosis-inducing ligands that contain components and quantity.

Table (2-5): Tumor necrosis factor (TNF) related apoptosis-inducing ligands containing components and quantity.

Components	Quantity
Standard Solution (1600ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human TNFSF10 Antibody	1ml x1
User Instruction	1
Plate Sealer	2 PICS
Zipper bag	1 PIC

2.2.6.3. Reagent Preparations

1- All reagents should be brought to room temperature before use.

2- Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

3- Standard Reconstitute the 120µl of the standard (1600ng/L) with 120µl of standard diluent to generate a 800ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (800ng/L) 1:2 with standard diluent to produce 400ng/L, 200ng/L, 100ng/L and 50ng/L solutions. Standard diluent serves as the zero standard(0 ng/L). Any remaining solution should be frozen at -20°C and used within one month.

2.2.6.4. Assay Procedure

1. Had prepared all reagents, standard solutions, and samples as instructed. Had brought all reagents to room temperature before had used them. The assay had been performed at room temperature.
2. Had determined the number of strips required for the assay. Had inserted the strips in the frames for use. The unused strips had been stored at 2-8°C.
3. Had added 50µl standard to the standard well. Note: Had not added biotinylated antibody to the standard well because the standard solution had contained biotinylated antibody.
4. Had added 40µl sample to sample wells and then had added 10µl anti-TNFSF10 antibody to sample wells. Had added 50µl streptavidin-HRP

to sample wells and standard wells (not blank control well). Had mixed well. Had covered the plate with a sealer. Had incubated for 60 minutes at 37°C.

5. Had removed the sealer and had washed the plate 5 times with wash buffer. Had soaked wells with 300µl wash buffer for 30 seconds to 1 minute for each wash. For automated washing, had aspirated or had decanted each well and had washed 5 times with wash buffer. Had blotted the plate onto paper towels or other absorbent material.
6. Had added 50µl substrate solution A to each well and then had added 50µl substrate solution B to each well. Had incubated the plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Had added 50µl Stop Solution to each well, and the blue color had changed to yellow immediately.
8. Had determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.2.6.5. Calculation of results

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined.

2.2.7. Human Matrix metalloproteinase 8 (HMMP8)

2.2.7.1 Test Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human MMP-8 antibody. MMP-8 present in the sample is added and binds to antibodies coated on the wells. And then

biotinylated Human MMP-8 Antibody is added and binds to MMP-8 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated MMP-8 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human MMP-8. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.2.7.2 Kit components

The contents of the Human Matrix metalloproteinase 8 that contain components and quantity were listed in table (2-3)

Table (2-3): The contents of Human Matrix metalloproteinase 8 (HMMP8) that contain components and quantity.

Components	Quantity
Standard Solution (12.8ng/ml)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human MMP-8 Antibody	1ml x1
User Instruction	1
Plate Sealer	2 PICS
Zipper bag	1 PIC

2.2.7.3. Reagent Preparations

All preparations have been mixed thoroughly and warmed up at room temperature prior to use.

1- Washed Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals had formed in the concentrate, mix gently until the crystals had completely dissolved

2- Color development solution: An equal amount of chromogene A and B were mixed.

3- Standard Reconstitute the 120µl of the standard (12.8ng/ml) with 120µl of standard diluent to generate a 6.4ng/ml standard stock solution. Allowed the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepared duplicate standard points by serially diluting the standard stock solution (6.4ng/ml) 1:2 with standard diluent to produce3.2ng/ml, 1.6ng/ml, 0.8ng/ml and0.4ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml).

2.2.7.4. Assay Procedure

1. Had prepared all reagents, standard solutions, and samples as instructed. Had brought all reagents to room temperature before they had been used. The assay had been performed at room temperature.
2. Had determined the number of strips required for the assay. Had inserted the strips in the frames for use. The unused strips had been stored at 2-8°C.
3. Had added 50µl standard to the standard well. Note: Had not added biotinylated antibody to the standard well because the standard solution had contained biotinylated antibody.
4. Had added 40µl sample to sample wells and then had added 10µl anti-MMP-8 antibody to sample wells. Then had added 50µl streptavidin-

HRP to sample wells and standard wells (not blank control well). Had mixed well. Had covered the plate with a sealer. Had incubated for 60 minutes at 37°C.

5. Had removed the sealer and had washed the plate 5 times with wash buffer. Had soaked wells with 300µl wash buffer for 30 seconds to 1 minute for each wash. For automated washing, had aspirated or had decanted each well and had washed 5 times with wash buffer. Had blotted the plate onto paper towels or other absorbent material.
6. Had added 50µl substrate solution A to each well and then had added 50µl substrate solution B to each well. Had incubated the plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Had added 50µl Stop Solution to each well, and the blue color had changed to yellow immediately.
8. Had determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.2.7.5. Calculation of results

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best-fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

2.2.8. Human neutrophil gelatinase-associated lipocalin (NGAL)

2.2.8.1 Test Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human NGAL antibody. NGAL present in the sample is added and binds to antibodies coated on the wells. And then biotinylated

Human NGAL Antibody is added and binds to NGAL in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated NGAL antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human NGAL. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.2.8.2 Kit components

The contents of the Human neutrophil gelatinase-associated lipocalin that contain components and quantity were found in table (2-4)

Table (2-4): The contents of Human neutrophil gelatinase-associated lipocalin (NGAL) that contain components and quantity.

Components	Quantity
Standard Solution (640ng/ml)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human NGAL Antibody	1ml x1
User Instruction	1
Plate Sealer	2 PICS
Zipper bag	1 PIC

2.2.8.3. Reagent Preparations

1- All reagents should be brought to room temperature before use.

2- Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved

3- Standard Reconstitute the 120µl of the standard (640ng/ml) with 120µl of standard diluent to generate a 320ng/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (320ng/ml) 1:2 with standard diluent to produce 160ng/ml, 80ng/ml, 40ng/ml and 20ng/ml solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard

2.2.8.4. Assay Procedure

1. Had prepared all reagents, standard solutions, and samples as instructed. Had brought all reagents to room temperature before they had been used. The assay had been performed at room temperature.
2. Had determined the number of strips required for the assay. Had inserted the strips in the frames for use. The unused strips had been stored at 2-8°C.
3. Had added 50µl standard to the standard well. Note: Had not added biotinylated antibody to the standard well because the standard solution had contained biotinylated antibody.
4. Had added 40µl sample to sample wells and then had added 10µl anti-NGAL antibody to sample wells. Then had added 50µl streptavidin-HRP to sample wells and standard wells (not blank control well). Had mixed

well. Had covered the plate with a sealer. Had incubated for 60 minutes at 37°C.

5. Had removed the sealer and had washed the plate 5 times with wash buffer. Had soaked wells with 300µl wash buffer for 30 seconds to 1 minute for each wash. For automated washing, had aspirated or had decanted each well and had washed 5 times with wash buffer. Had blotted the plate onto paper towels or other absorbent material.
6. Had added 50µl substrate solution A to each well and then had added 50µl substrate solution B to each well. Had incubated the plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Had added 50µl Stop Solution to each well, and the blue color had changed to yellow immediately.
8. Had determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.2.8.5. Calculation of results

Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis

2.2.9. Statistical analyses

Data analysis for this study was performed using the Statistical Package for the Social Sciences (SPSS) software version 26 (IBM, Chicago, Illinois, USA) and the Real Statistics Resource Pack for Mac (Release 7.2) integrated with Excel 2016. Descriptive statistical analysis was carried out for each participant group, calculating means and standard deviations for continuous variables, while frequencies were used to summarize categorical data. The correlation between the analyzed parameters was estimated using Pearson regression and 95% Confidence Interval range which calculated by a non-conditional logistic regression. The mean of the investigated biomarkers were compared between the studied groups using t-Test; Chi-Square analysis was employed to significant compare between percentages; Differences among groups were analyzed using one-way ANOVA analysis of variance. Duncan's test was used to determine critical values for comparisons between means. The Results of all hypothesis tests with p-values <0.05 (two-side) were considered to be statistically significant. A receiver operating characteristic (ROC) curve was analyzed to assess the research indicator for predicting bacterial and viral infection. (Duncan *et al.,* 1983; Basher, 2003). In addition, SPSS program was used to draw the Boxplots, while Microsoft Excel 2010 program was employed to draw other chart figures.

Chapter Three

Result

3. Results

3.1 Demographic Characteristics of Study Population:

Demographic characteristics of study population (patients with Bacteria and viral infection as well as healthy control subjects) were displayed in Table (3-1). Study populations were divided according to their age into four age groups: ≤ 1 y, 2-5 y, 6-9 y, and ≥ 10 y; statistical analysis revealed a significant increase in percent of patients with bacterial infection 30% and 33.3% in 2-5 y, 6-9 y age groups respectively, while percent of patients with viral infection 43.33% and 33.33% in ≤ 1 y, 2-5 y age groups respectively, the percent of healthy control showed a significant increase (76.7%) in 2-5 y age group as compared with other groups. Also results of statistical analysis showed a significant ($P \leq 0.05$) difference among groups comparison in all age groups. As Table (3-1) shows there is a significant ($P \leq 0.05$) increase in age range of patients with Bacterial infection compared with viral and control subjects.

Regarding temperature degrees, there are a significant ($P \leq 0.05$) decrease in control subjects compared with patients groups. As for gender distribution, within group comparison revealed no significant ($P > 0.05$) difference in Bacterial and control groups, while in viral group the majority of patients were male (70%) with a significant ($P \leq 0.05$) difference; also among group comparison revealed a significant ($P \leq 0.05$) differences, where the majority of patients with viral infection were male, while the majority of patients with Bacterial infection and control subjects were female. In context of time of symptoms, the results of the statistical analysis showed that there were significant differences in both the within-group comparison and the between-groups comparison. The majority of patients with bacterial infections (70%)

showed symptoms within one day, and only 30% showed symptoms within two days, while all patients with viral infections showed symptoms within one day. Regarding the hospital duration, patients groups were divided into three groups: 1-2 day, 3-4 day, and >4 day; the results of the statistical analysis showed that there were significant ($P \leq 0.05$) differences in both the within-group comparison and the between-groups comparison, the majority of the both groups of patients were in the first group (who stayed in the hospital for 1-2 days).

Table (3-1): Demographic data for study population

Variables	Age group (year)					
	≤ 1 y	**2-5 y**	**6-9 y**	**≥ 10 y**	**Total**	***P value* (P ≤ 0.05) within group**
Bacterial	7 (23.3 %)	9 (30 %)	10 (33.3%)	4 (13.3 %)	30 (100 %)	0.0226*
Viral	13 (43.33%)	10 (33.33%)	6 (20%)	1 (3.34%)	30 (100 %)	0.0001*
Control	1 (3.3%)	23 (76.7%)	4 (13.3%)	2 (6.7%)	30 (100 %)	0.0001*
***P value* (P ≤ 0.05) among groups**	0.0001*	0.0001*	0.0093*	0.0276*	/	/
Age (Year)						
Variables	**Mean**	**Std. Deviation**	**Lower Bound**	**Upper Bound**	***P value* (P ≤ 0.05)**	
Bacterial	5.2167 [a]	2.2	3.8103	6.6230	0.032*	
Viral	3.1633 [b]	1.4	2.0750	4.2517		
Control	4.5000 [b]	2.16	3.6929	5.3071		
Total	4.2933	2.34	3.6429	4.9438		
Temperature						
Variables	**Mean**	**Std. Deviation**	**Lower Bound**	**Upper Bound**	***P value* (P ≤ 0.05)**	
Bacterial	38.5900 [a]	0.65672	38.3448	38.8352	0.000*	
Viral	38.1167 [b]	0.29837	38.0053	38.2281		
Control	36.8333 [c]	0.23973	36.7438	36.9229		
Total	37.8467	0.86326	37.6659	38.0275		

Sex				
Variables	**Male**	**Female**	**Total**	***P value* (P ≤ 0.05) within group**
Bacterial	13 (43.3%)	17 (56.7%)	100.0%	0.1914 ^{NS}
Viral	21 (70.0%)	9 (30.0%)	100.0%	0.00 *
Control	13 (43.3%)	17 (56.7%)	100.0%	0.1914 ^{NS}
***P value* (P ≤ 0.05) among groups**	0.0093*	0.0086*	/	/
Time of symptoms (day)				
Variables	**1 day**	**2 day**	**Total**	***P value* (P ≤ 0.05) within group**
Bacterial	21 (70.0%)	9 (30.0%)	100.0%	0.0001*
Viral	30 (100.0%)	0	100.0%	0.0001*
***P value* (P ≤ 0.05) between groups**	0.0214*	0.0001*	/	/

Hospital duration (day)					
Variables	**1-2 day**	**3-4 day**	**> 4 day**	**Total**	***P value* (P ≤ 0.05) within group**
Bacterial	21 (70.0%)	7 (23.3%)	2 (6.7%)	100.0%	0.0001*
Viral	29 (96.7%)	1 (3.3%)	0	100.0%	0.0001*
***P value* (P ≤ 0.05) between groups**	0.0436*	0.0001*	0.0143	/	/

*Significant difference at the 0.05 level by chi-square test and One way – ANOVA.
Different small letters refer to significant differences
NS: Non-significant difference

3-2 Laboratory data for patients and control groups

Table (3-2) shows the laboratory data for all study populations. Laboratory data including: CRP, WBCs, Neutrophil, and lymphocyte. The result of statistical analysis revealed a significant (*P≤0.05)* increase in the levels of CRP, WBCs, and Neutrophil in patients with bacterial infection as compared with patients with viral infection and control subjects; while lymphocyte significantly increased in patients with viral infection as compared with patients with bacterial infection and control subjects.

Table (3-2): Laboratory data for study population

Variables	Study population	N	Mean	Std. Deviation	*P value* (P ≤ 0.05)
CRP Mg/dl	**Bacterial infection**	30	46.4667 [a]	55.08347	0.000 *
	Viral infection	30	11.3000 [b]	9.62808	
	Control	30	1.4600 [b]	.84592	
	Total	90	19.7422	37.37026	
WBCs Cell/mcl	**Bacterial infection**	30	21.2833 [a]	8.83602	0.000 *
	Viral infection	30	15.1000 [b]	3.78664	
	Control	30	7.4667 [c]	8.24091	
	Total	90	14.6167	9.19421	
Neutrophil Cell/mcl	**Bacterial infection**	30	15.5053 [a]	6.34221	0.000 *
	Viral infection	30	4.5000 [b]	1.35214	
	Control	30	3.4300 [b]	.75801	
	Total	90	7.8118	6.63403	
Lymphocyte Cell/mcl	**Bacterial infection**	30	2.2860 [b]	.81067	0.000 *
	Viral infection	30	8.2467 [a]	3.16432	
	Control	30	2.6333 [b]	2.94119	
	Total	90	4.3887	3.72042	

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

3-3 Evaluation of immunological markers in study groups

Table (3-3) 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. Boxplots in Figure (3-1 A, B, C) explain the ranges of mean for the immunological markers in patient with bacterial infection, patients with viral infection and control subjects, respectively.

Table (3-3): Evaluation of immunological markers in study population

Variables	Study population	N	Mean	Std. Deviation	*P value* (P ≤ 0.05)
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

3-4 The association of Sex with immunological markers

The association between Sex and immunological markers explained in Table (3-4) as well as in the Figures (3-2, 3-3, and 3-4). The results of the statistical analysis showed 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. On the other hand, the remaining markers showed no significant distribution according to gender in all groups.

Table (3-4*)*: association of gender with immunological markers in patients and control group

Population	Variables	Gender	N	Mean	Std. Deviation	*P value* (P ≤ 0.05)
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

3-5 Correlation analysis

3-5-1 Correlation of immunological markers in both patients groups

Figure (3-5, 3-6, and 3-7) 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 between the remaining markers.

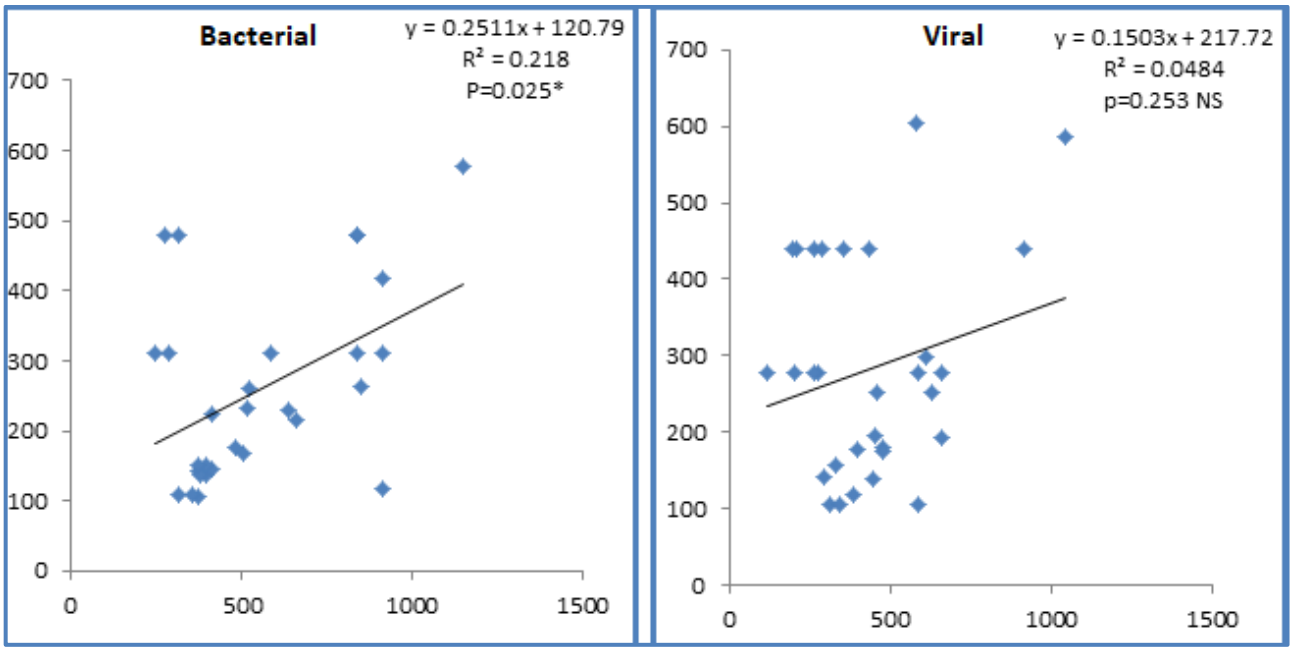

Figure (3-5): correlation between TNFL and NGAL in both patients groups

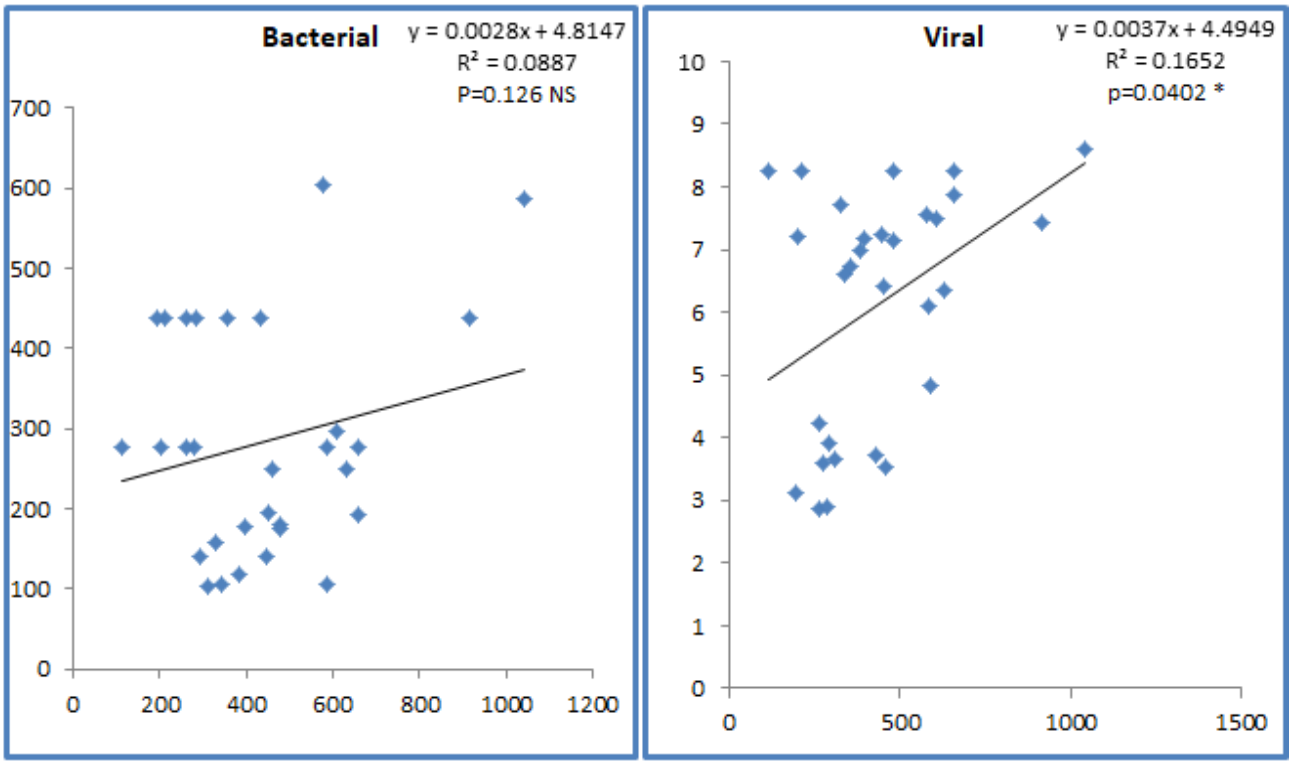

Figure (3-6): correlation between TNFL and MMP-8 in both patients groups

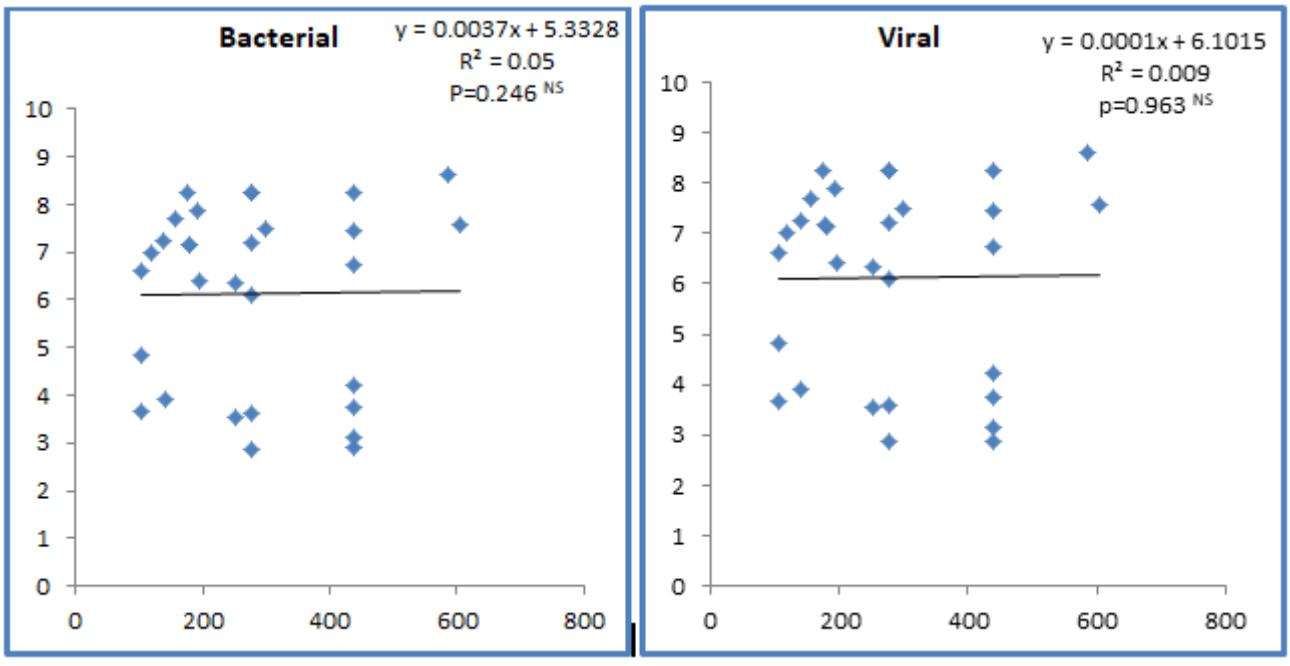

Figure (3-7): correlation between MMP-8 and NGAL in both patients groups

3-5-2 Correlation of Immunological Markers with Laboratory Variables in Bacterial Infection Cases

Table (3-5) summarizes the correlation between levels of 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 ($P \leq 0.05$) positive correlation with each of TNFL and NAGL, while other markers showed non significant correlation.

Table (3-5): 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).**				

Table (3-6) summarizes the correlation between levels immunological markers and laboratory variables in patients with viral infection. Correlation analysis in this group showed a significant ($P \leq 0.05$) 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 in-significant ($p > 0.05$) correlation.

Table (3-6): 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 (3-7) illustrates the correlation between levels immunological markers and laboratory variables in healthy control individuals. Correlation analysis showed a significant ($P \leq 0.05$) positive correlation between CRP and NAGL; a significant ($P \leq 0.05$) 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 in-significant ($p > 0.05$) correlation.

Table (3-7): 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).**				

3-6 Receiver Operative Characteristic Curve Analysis

3-6-1 Receiver Operative Characteristic Curve Analysis for bacterial infection

The Receiver Operative Characteristic Curve (ROC) analysis yielded a cut off value of TNFL, NGAL, and MMP-8 (480.153, 304.538, and 6.658, respectively) for prediction of bacterial infection activity by theses markers. The overall Area Under Curve (AUC), sensitivity, and specificity for TNFL, NGAL, and MMP-8 were as follows: (0.667, 0.599, and 0.645), (0.5, 0.4, and 0.6), and (0.8, 0.85, and 0.733), respectively, as displayed in Table (3-8). It is worth noting that both TNFL and MMP-8 can be considered as a prediction factors

for bacterial infections due to the presence of a significant *p values* (0.010 and 0.026, respectively).

Figure (3-8) illustrates the sensitivity and 1-specificity values for TNFL, NGAL, and MMP-8

Table (3-8): ROC based analysis for immunological markers in patients with Bacterial infection

Variables	AUC	Specificity	Sensitivity	Cut-off	P-value
TNFL	0.667	0.8	0.5	480.153	0.010*
NGAL	0.599	0.85	0.4	304.538	**0.125** NS
MMP-8	0.645	0.733	0.6	6.658	**0.026***

*Significant difference at the 0.05 level.

NS: Non-significant difference

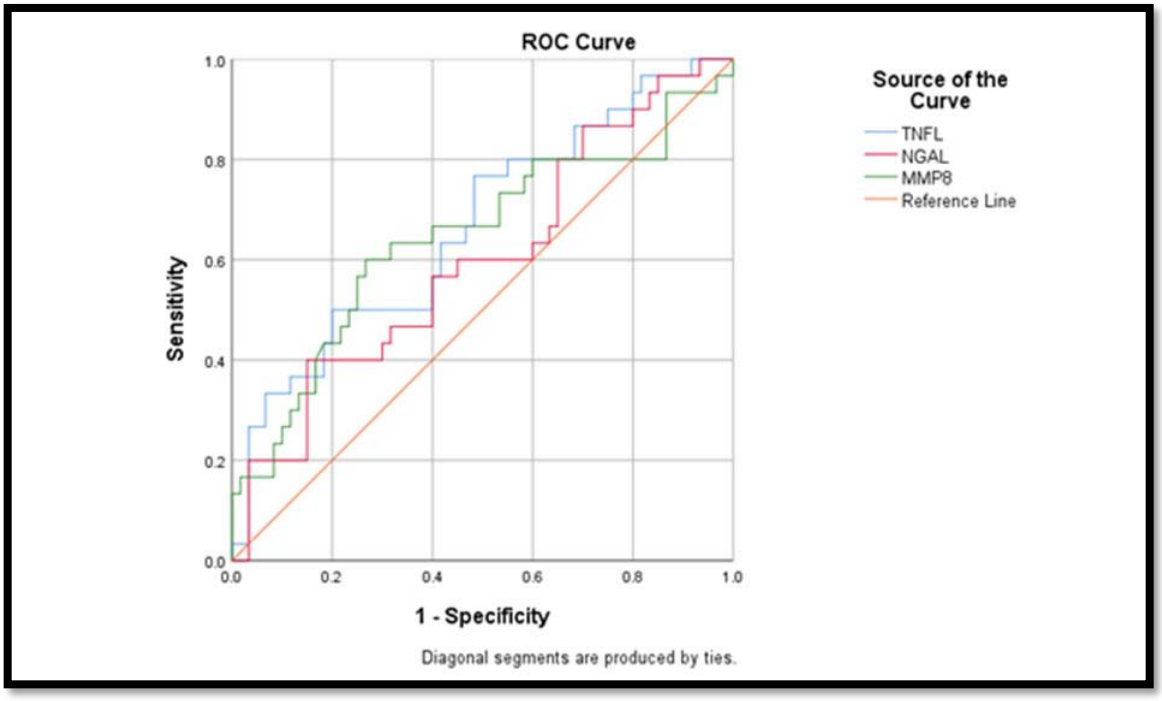

Figure (3-8): ROC curve for prediction of the disease activity (Bacterial infection) by TNFL, NGAL, and MMP-8

3-6-1 Receiver Operative Characteristic Curve Analysis for viral infection

The Receiver Operative Characteristic Curve (ROC) analysis yielded a cut off value of TNFL, NGAL, and MMP-8 (429.493, 273.088, and 6.0425, respectively) for prediction of viral infection activity by theses markers. The overall AUC, sensitivity, and specificity for TNFL, NGAL, and MMP-8 were as follows: (0.494, 0.642, and 0.630), (0.5, 0.533, and 0.667), and (0.65, 0.8, and 0.683), respectively, as displayed in Table (3-9). It is worth noting that both NGAL and MMP-8 can be considered as a prediction factors for viral infections due to the presence of a significant *p values* (0.028 and 0.044, respectively).

Figure (3-9) illustrates the sensitivity and 1-specificity values for TNFL, NGAL, and MMP-8

Table (3-9): ROC based analysis for immunological markers in patients with viral infection

Variables	AUC	Specificity	Sensitivity	Cut-off	P-value
TNFL	0.494	0.65	0.5	429.493	0.925 NS
NGAL	0.642	0.8	0.533	273.088	**0.028***
MMP-8	0.630	0.683	0.667	6.0425	**0.044***

*Significant difference at the 0.05 level.

NS: Non-significant difference

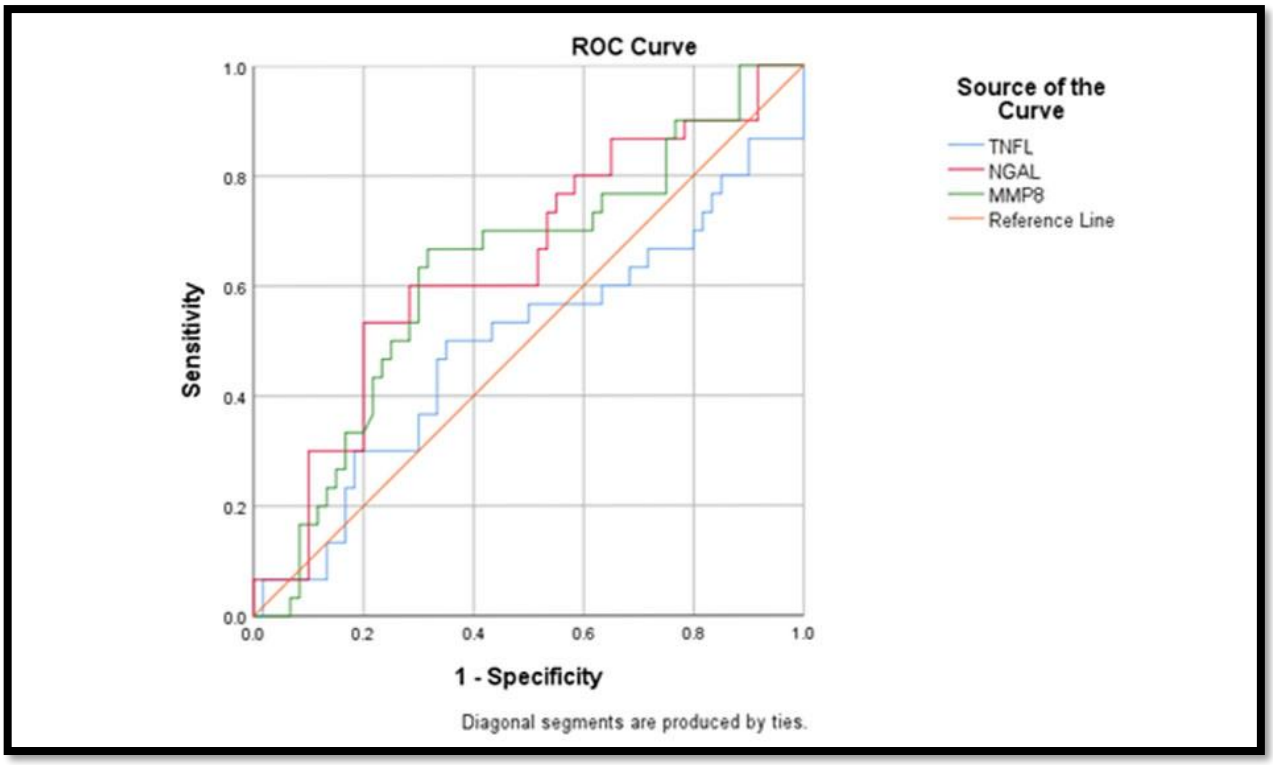

Figure (3- 9): ROC curve for prediction of the disease activity (viral infection) by TNFL, NGAL, and MMP-8

Chapter Four

Discussion

Discussion:

4.1 Demographic characteristics of the study population

The study of febrile illness is designed to investigate causes of febrile either bacterial or viral, where currently there is little evidence and minimal diagnostic capacity to guide fever causes. There is an unmet need for rapid noninvasive diagnostic for distinguishing between viral and bacterial infections. A test with this feature and purpose could potentially support decision-making on initiating treatment. However, the study findings indicated in Table (3-1) that common childhood viral diseases are more frequent at ages less than one year than bacterial causes, there is a significant difference between age group and infection with viral, bacterial and healthy controls, as well as significant differences among group.

Also, this study found the temperature of febrile was high when the cause was bacteria, as well there was a strong association between temperature and causes of febrile. It is worth noting that sex was found to be an association between males and viral infection.

Regarding time of infection, this study found all cases had one day of symptoms when infection with viral, regarding hospitalization, this study found the highest percentage of cases demined to inter-hospital for 1-2 days when the cause was viral.

The Current study confirmed the findings of previous studies, it agreed with the study of van Houten *et al.,* (2017) that found the majority of the cases diagnosed 71 as bacterial infections and 435 as viral infections, as well as agreed with a study conducted by Yaeger *et al.*,(2018) who found that of

232 febrile infants, the median age was 54 days, 58% were male; 31 infants (13.4%) had an infection, current result also agreed with Chipwaza *et al.*, (2015) who demonstrated the majority of patients (75.7%) had a mild fever, whereas this study disagreed with the study of Ssentongo *et al.*, (2023) who found the burden of febrile illness was higher in older children than the younger . Both Fetveit, (2008) and (Sharawat *et al*., 2016) highlighted that the peak incidence of Febrile was below 2 years with male predominance. The reason for this male predominance is not entirely clear, but it is often attributed to genetic and hormonal differences. Some research suggests that boys may be more susceptible to seizures due to sex-specific factors in brain development and immune responses, other studies, along with others, suggest a hereditary component to febrile seizures, with some families showing higher incidences. This genetic predisposition, when combined with early childhood fever episodes, contributes to the peak incidence under 2 years of age. Some evidence suggests a stronger hereditary component in males, though this area remains under investigation. (Sharawat et al., 2016)

This result consistency with several studies, Oved *et al*., (2015) found highest percentage of children (37%), (21%) and (12%) were had time of symptoms onset (2-3) (4-5) and (6-7) days respectively and majority of them not need to stay in hospital and 29% of them stay from 1 to 2 days, Ashkenazi-Hoffnung *et al*., (2018) demonstrated that 59% of patient with respiratory infection caused by bacteria and virus together forced to inter emergency department with median time from symptom onset to enrollment of 3 days. And the median of hospitalization duration was 2 days of 61% from those admission hospital while Borensztajn *et al.*, (2021) found 74% of children with febrile lasting in hospital more than 24 hours regardless of causes.

4-2 Laboratory Data for Patients and Control Groups

The result of laboratory data showed statistically significant increase in the levels of CRP, WBCs, and Neutrophil in patients with bacterial infection as compared with patients with viral infection and control subjects; in table (3-2) while lymphocyte significantly increased in patients with viral infection as compared with patients with bacterial infection and control subjects. Present study consistent with study of Fernandez-Carballo *et al.*, (2021) which designed to differentiate bacterial from non-bacterial infection, finding the CRP showed high levels in febrile illness, compared with healthy control subjects. Several studies reported statistically significant differences in CRP levels between the bacterial and viral group, with higher levels seen in bacterial infections. (Espana *et al.,* 2012; Katoh *et al.,* 2014; Oved *et al.,* 2015; Kapasi *et al.,* 2016). Also, a study of (Díez-Padrisa *et al.,* 2010) who found a higher significant result ($p<0.001$) of CRP levels in bacterial infections compared to patients with viral cases (185.4 vs. 18.3 mg/l)

Neutrophil and WBC counts were the most frequently studied markers. The majority of studies investigating WBC counts reported statistically significant differences in patients with bacterial versus non-bacterial infections. (Haran *et al.,* 2013; Tamune *et al.,* 2014)

Pratt & Attia, (2007) compared the WBC and CRP in relation to the onset of fever and found that CRP had a better sensitivity and specificity than WBC regardless of the duration of fever. Venge *et al.,* (2015) measured C-reactive protein (CRP) and blood neutrophil counts by established techniques, all tested biomarkers were elevated in bacterial as opposed to viral infections. CRP and neutrophils were elevated in viral infections compared to healthy control.

Blot *et al.*, (2014) using a leukocyte score with points for neutropenia, lymphopenia and monocytopenia, found a high score to be significantly associated with mortality in bacteremic pneumococcal pneumonia. Current study did not corresponding with Naess *et al.,* (2017) who found white blood cell counts, neutrophil counts, and C-reactive proteins did not differ significantly between other infection and bacterial infection.

Lymphocytes, especially specific T cells, have a critical role in viral clearance. Thus, lymphopenia may affect the host adaptive immune responses and impact the clinical course of acute viral infections, lymphopenia was often seen in patients and animals infected with viruses that could result in serious illness or even death and it was found that lymphopenia is associated with disease severity (Guo *et al.,* 2021). Many previous studies showed correlation between lymphocytes and virus, Tavakolpour *et al.*, (2020) found increased neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, and increased IL-2R and to lymphocyte count, were found to be correlated with disease severity of COVID-19, Chalupa *et al.*, (2011) found higher account of neutrophil and lymphocyte in viral infection compared to bacterial infection, Kapasi *et al.,* (2016) showed lower blood lymphocyte counts were associated (4/7, 57%) with bacterial infections and higher in non-bacterial infections.

4-3 Evaluation of Immunological Markers in Study Groups

Regarding the immunological markers in this study, the statistical analysis showed that TNFL significantly increased in patients with bacterial infection as compared with patients with viral infection 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, this study discorresponding with Oved *et al.,* (2016) showed that the TRAIL serum levels were significantly decreased in bacterial patients and increased in viral patients compared with controls (average ± standard deviation: bacterial 45 ± 33 pg/mL; viral 145 ± 110 pg/mL; controls 77 ± 32 pg/mL, $P < 10^{-15}$). Many pathogens have evolved mechanisms to manipulate TRAIL signaling thus increasing pathogen replication (Gyurkovska & Ivanovska, 2016), this may interpret slight increase of tumor necrosis factor ligands in bacterial infection.

Current study corresponding with Nasioudis & Witkin, (2015) who revealed that elevated levels of NGAL have been detected in the blood of patients with bacterial urinary tract infection, community-acquired pneumonia, sepsis, as well as 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 severity of viral lower respiratory tract infections in children is associated with increased expression levels of the MMP-8 genes. 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.

4-4 The Association of Sex with Immunological Markers

Regarding the association between sex and immunological markers showed statistically significant 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 insignificant distribution according to gender in all groups. showed differences in plasma matrix metalloproteinase-8 elevated in male. Aomatsu *et al.*, (2013) showed neutrophils from human males express higher levels of TLR4 and produce more TNF- ligands than female neutrophils, Rusman et al., (2018) demonstrated that females switched more (27%) TNF 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.

4-5-1 Correlation of Immunological Markers in Both Patients Groups

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) found a significant correlation between NGAL and TNF, also with results of Yu *et al.*, (2016) found the monomeric Human Neutrophil Lipocalin (HNL) being elevated in viral infections and the dimeric HNL being elevated in bacterial infections. 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) demonstrated that NGAL was strongly induced by stimulation with TNF. Furthermore, Arena *et al.,* (2010)showed TNF-α

regulates the NGAL expression in polymorphonuclear granulocytes, this interprets correlation between TNF and NGAL. Several studies confirmed our study, Sharma et al., (2021), Nylund et al., (2015) and Andronovici et al., (2022) showed there was a strong correlation between MMP-8 and TNF related apoptosis induced ligands and elevated together. This result Pro-inflammatory cytokines and chemokines influence the expression of MMPs. many active cytokines and chemokines stimulate the production of TNF in target cells. The proteolytic conversion of the inactive (latent) form of TNF results in active and highly pro-inflammatory TNF (Hardy & Fernandez-Patron, 2021)

4-5-2 Correlation of Immunological Markers with Laboratory Variables

Those 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 in systemic diseases with no obvious bacterial infection, serum NGAL levels was increased as acute phase response and can be used as an inflammation marker. This study agreed with several previous study, with Yigit *et al.*, (2015) showed that relationships between NGAL and inflammation markers (hs-CRP, IL-6 and TNF-α*)*. Kumar & Rizvi, (2010) investigated the role of CRP and tumor necrosis factor-alpha in diagnosing sepsis in pediatric patients, used a two-pronged approach to evaluate the association of TNF-α and CRP in bacterial infection as well as TNF-α proved to be a far more sensitive marker than CRP and culture for early diagnosis of sepsis and disease severity.

In addition, Fernandez-Carballo *et al.,* (2021) found the CRP, TRAIL signature and HNL were evaluated in the context of febrile illness and in studies specifically designed to differentiate bacterial from non-bacterial infections. 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. Several studies have shown that tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) immune response derived biomarkers, can accurately differentiate between viral and bacterial infections in the emergency department (ED), single marker and in combination with CRP or procalcitonin (PCT). These study populations consisted either of young children or highly selected patient populations. Moreover, the combination of both CRP and PCT, together with TRAIL, has not been investigated in an adult ED population. Furthermore, the clinical value of the combination of these biomarkers has not been fully elucidated (van der Does *et al.*, 2016; van der Does *et al.*, 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 when activated neutrophils can produce multiple cytokines and chemokines, including TNF that recruit and activate more neutrophils to the site of infection, as well there are strong association between neutrophil and TNF. 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, Johansson & Kirsebom, (2021) found neutrophils are often involved in containing secondary bacterial infections associated with respiratory viral infections, 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 have been strongly supported by (Smertka *et al.*, 2014) who found positive correlation between CRP and NAGL in both control group and septic group.

4-6 Receiver Operative Characteristic Curve Analysis

4-6-1 Receiver Operative Characteristic Curve Analysis for bacterial infection

The Present study displayed ROC analysis yielded a cut off value of TNFL-10, NGAL, and MMP-8 (480.153, 304.538, and 6.658, respectively) for prediction of bacterial infection. AUC, sensitivity and specificity for TNFL, NGAL, and MMP-8 were as (0.667, 0.599, and 0.645), (0.5, 0.4, and 0.6), and (0.8, 0.85, and 0.733), respectively, both TNFL and MMP-8 can be considered as a prediction factor for bacterial infections due to the presence of a significant *p values* less than 0.05.

This study is compatible with many studies, the study of Zhai *et al.*, (2021) demonstrated that TNF-α are very specific (0.98) but have a low sensitivity (0.33) and higher AUC with the difference statistically significant ($p < 0.05$), this better diagnostic than the routine laboratory. This agreed with the current study finding both TNF and MMP-8 had high specific and low sensitivity and better prediction for bacterial infection. In the same context, Kumar & Rizvi, (2010) found TNF-α levels were elevated in 67/79 (84.8%) cases infected with

different type of bacteria . the sensitivity of TNF-α was (84.8%) and specificity was 100%. Thus TNF-α had the highest sensitivity for diagnosis pediatric with 100% specificity.

Kocabaş *et al.*, (2007) conclude that TNF are the best markers in the diagnosis of neonatal sepsis, and these markers are also valuable in the following: the effectiveness of treatment and determining the prognosis of the disease because of the cut-off value of TNF-alpha > or = 7.5 pg/ml, sensitivity, specificity, positive predictive value, negative predictive value and diagnostic efficacy were found to be 100%, 96.6%, 96.2%, 96.5% and 98.3%, respectively.

While this study disagreed with Busch *et al.*, (2022) who found the sensitivity and specificity of synovial fluid TNF were 63 and 51% with a cut-off value of 3.9 pg/mL, the differences between this study and current study may be due to the diiferent in type of sample and type of infection.

Forsblom *et al.*, (2021) found the patients with severe sepsis or infection focus presented higher MMP-8 levels at day 3 and 5 with ($p<0.01$) and associated to mortality. Also, Bolyarova *et al.*, (2012) determined the cut-off value of the biomarker MMP-8 in saliva – 134 ng/ml OR = 11.7 (95% confidence interval) with ($p = 0.005$), the sensitivity is 57.9% and specificity is 89.5% and identify MMP-8 in saliva as a significant marker for the diagnosis of gingivitis.

In addition, the current study in the same line with a result of Savonius *et al.*, (2019) who demonstrated the CSF MMP-8 presented as an attractive prognostic marker in children with bacterial meningitis. MMP-8 emerged as the best predictor of disease outcomes: a CSF MMP-8 concentration above the median increased the odds of death 4.9-fold (95% confidence interval 1.8–12.9). While the present study disagreed with Paul *et al.*, (2023) who reported

that plasma NGAL is a novel and potential new biomarker for sepsis. The NGAL sepsis screening tool consists of plasma NGAL, and clinical parameters had a reasonably good performance as a diagnostic tool, found the AUC for the ROC curve for plasma NGAL and bacterial sepsis was 0.69 (95% CI: 0.59–0.79). The cut-off for plasma NGAL to predict sepsis was 570 ng/mL with sensitivity = 0.87 and specificity = 0.46. These differences in results with previous studies may be varied according to the course of the disease, for these reasons some authors have applied a variety of biomarkers to predict infection.

4-6-2 Receiver Operative Characteristic Curve Analysis for Viral Infection

The ROC analysis yielded a cut off value of TNFL, NGAL, and MMP-8 (429.493, 273.088, and 6.0425, respectively) for prediction of viral infection activity by theses markers. The overall AUC, sensitivity, and specificity for TNFL, NGAL, and MMP-8 were as follows: (0.494, 0.642, and 0.630), (0.5, 0.533, and 0.667), and (0.65, 0.8, and 0.683), respectively, NGAL and MMP-8 can be considered as a prediction factor for viral infections due to the presence of a significant *p values* (0.028 and 0.044, respectively).

The Current study is consistent with a study conducted by Can *et al.*, (2022) who demonstrated that NGAL was highly associated with COVID-19 virus severity and consider that NGAL might be a useful biomarker to diagnose the disease severity in patients with viral infection with cutoff value of 72 ng/ml, NGAL predicted severe COVID-19 with a sensitivity rate of 57% and a specificity rate of 84%. On the other hand, the current study disagreed with Oved *et al.*, (2015) who revealed that TNF-related apoptosis-inducing ligand

(TRAIL) with AUC of 0.89; 95% confidence interval (CI) 0.86 to 0.91, which was consistently up-regulated in viral infected patients.

The accurate differential diagnosis provided by this novel combination of viral and bacterial induced proteins has the potential to improve management of patients with acute infections and reduce antibiotic misuse.

Conclusions

&

Recommendations

Conclusions

1- The laboratory findings demonstrated elevated levels of CRP, WBCs, and Neutrophils in patients with bacterial infection, and lymphocytes significantly increased in patients with viral infection.

2- The TNF-related apoptosis-inducing ligand (TNFL), Matrix Metalloproteinase 8 (MMP-8), and Neutrophil Gelatinase-Associated Lipocalin (NGAL) exhibit significant potential as biomarkers to differentiate bacterial from viral infections in febrile children.

3- Increase in the concentration of NGAL in females compared to males in the group of patients with viral infection.

4- The results of statistical analysis showed positive correlations between levels of TNFL 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.

5- There was a positive correlation between the levels of CRP with each of TNFL and NAGL in patients with a bacterial infection.

6- There was a positive correlation between Neutrophil and TNFL in patients with viral infection.

Recommendations

1. It is recommended that the integration of TRAIL, MMP-8, and NGAL as part of routine diagnostic tests in febrile children should be considered, especially in settings where rapid differentiation between bacterial and viral infections is necessary. This could enhance the accuracy of diagnoses and improve clinical decision-making.
2. Additional large-scale studies are needed to validate these findings across diverse populations and age groups. Longitudinal studies examining the prognostic value of these biomarkers over the course of infection should also be conducted.
3. Training programs for healthcare providers on the interpretation of these biomarkers should be developed to enhance their effective use in clinical practice. This would ensure that the biomarkers are used optimally to inform therapeutic decisions, particularly in avoiding unnecessary antibiotic use.
4. Policymakers should encourage the adoption of biomarker-based diagnostic tools in pediatric healthcare settings to promote more judicious use of antibiotics and to combat the global challenge of antibiotic resistance.

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Appendix

Appendix 1

The Questionnaire Sheet of Study Cases:

Demographic Data:

- Case no. and Name: …… Age: …… Sex: ……..

- maximal temperature (C): ……. Time from symptoms onset (days): ……..

-Hospitalization duration (days): ……..

- symptoms : ….

Lab. Data:

- CBC:

1- White Blood Cells: .... 2-Lymphocytes : ….

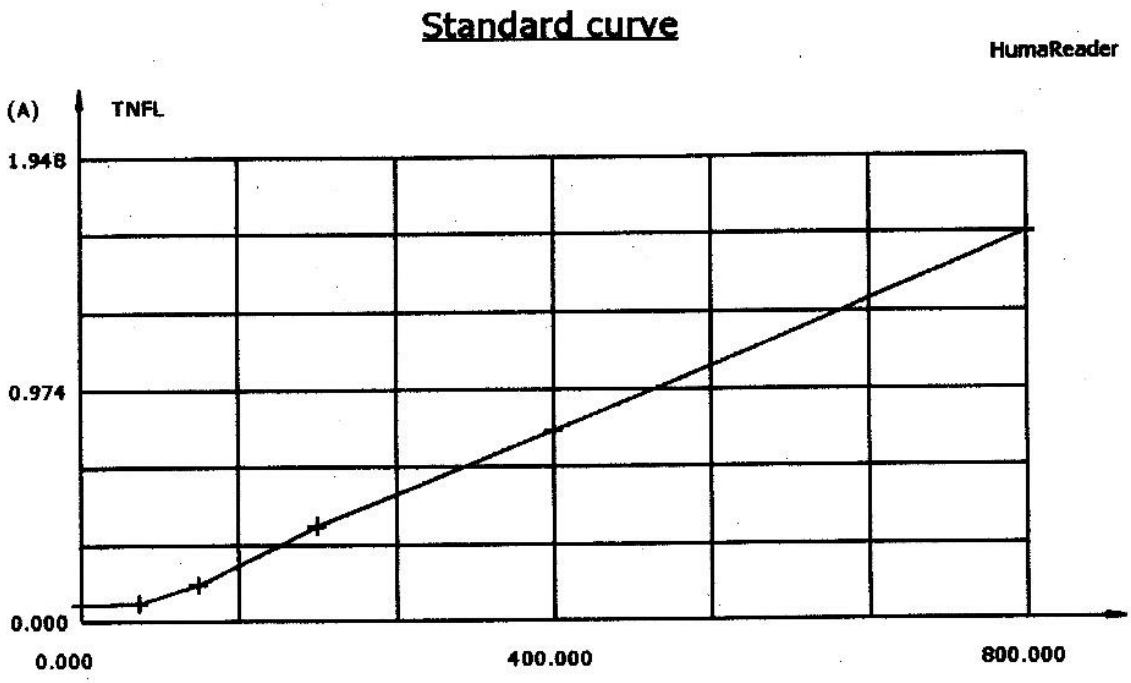
- C-reactive protein: ..…..

Appendix 2

Standard Curve of Immunological Markers:

A)TNFL B) MMP 8 C) NGAL

A



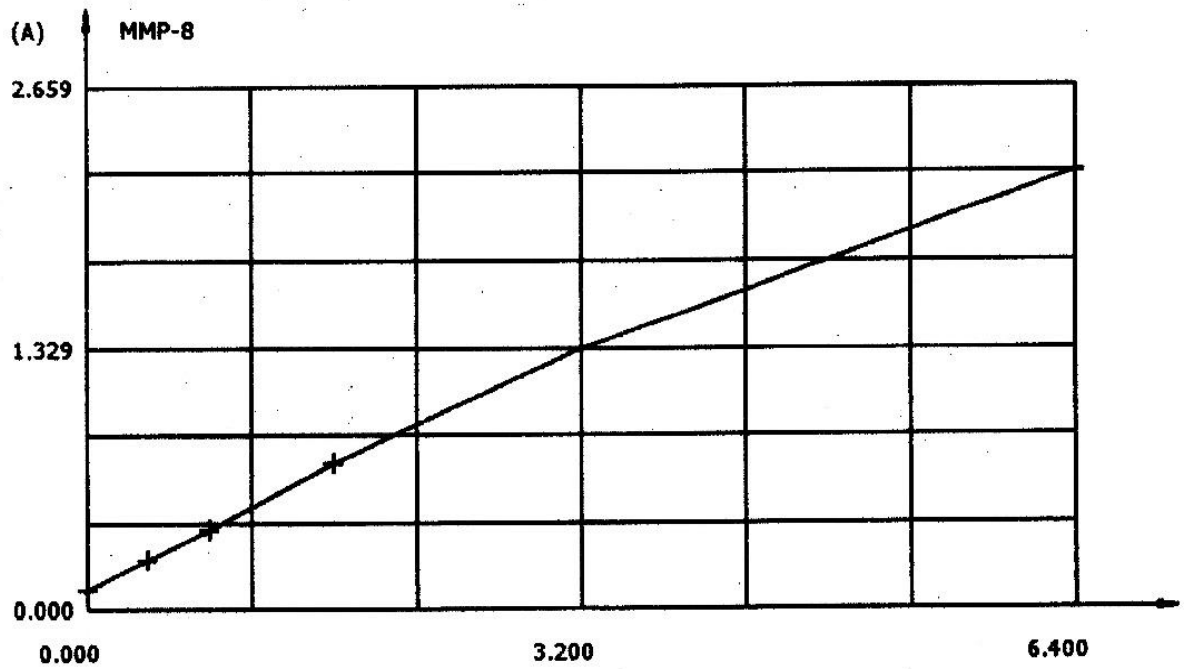
Program: TNFL TNFL				Caculation: Curve		Wavelength(nm): 450
Standard sample	#1	#2	#3	#4	#5	#6
Concentration(ng/l)	0.000	50.000	100.000	200.000	400.000	800.000
Abs(A)	0.065	0.070	0.152	0.400	0.795	1.623

Operator: Checker:
Print date:

B

Standard curve

HumaReader

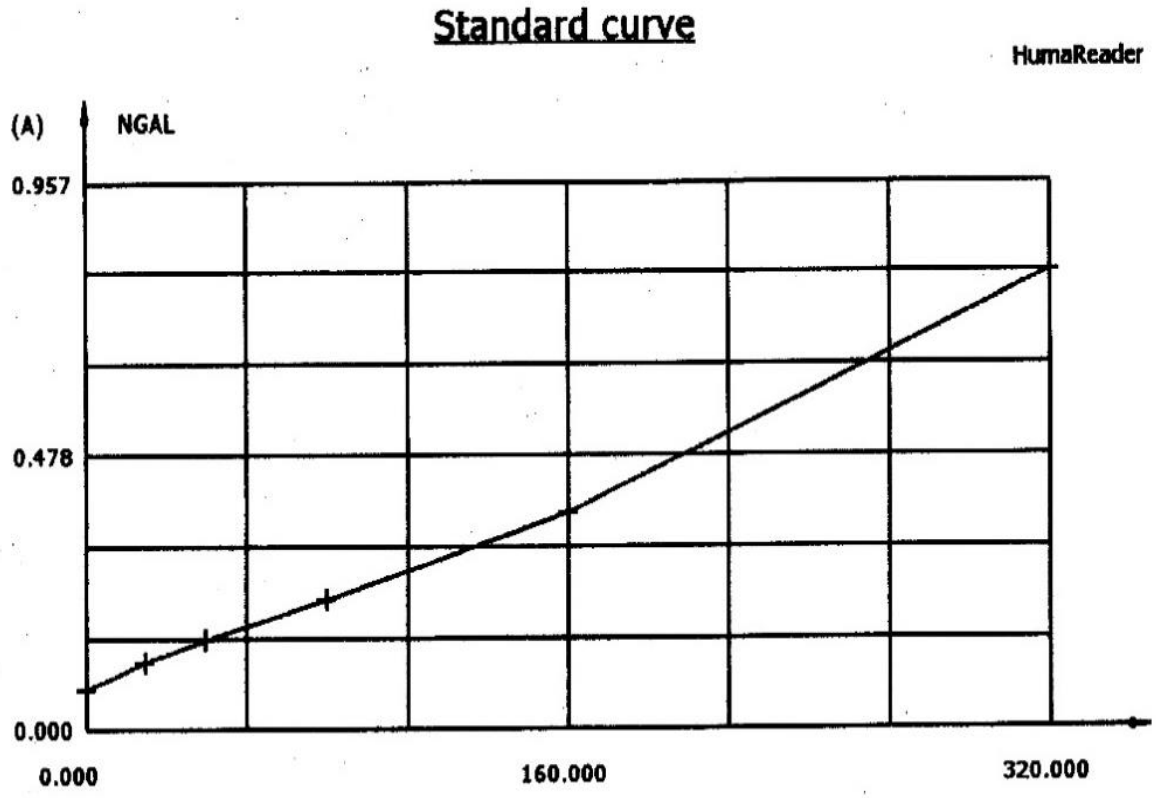


Program: MMP-8 MMP-8 Caculation: Curve Wavelength(nm): 450

Standard sample	#1	#2	#3	#4	#5	#6
Concentration(ng/ml)	0.000	0.400	0.800	1.600	3.200	6.400
Abs(A)	0.096	0.250	0.405	0.741	1.328	2.216

Operator: Checker:
Print date:

C



Program: NGAL NGAL Caculation: Curve Wavelength(nm): 450

Standard sample	#1	#2	#3	#4	#5	#6
Concentration(ng/ml)	0.000	20.000	40.000	80.000	160.000	320.000
Abs(A)	0.069	0.116	0.156	0.226	0.376	0.797

Operator: Checker:
Print date:

Appendix 3

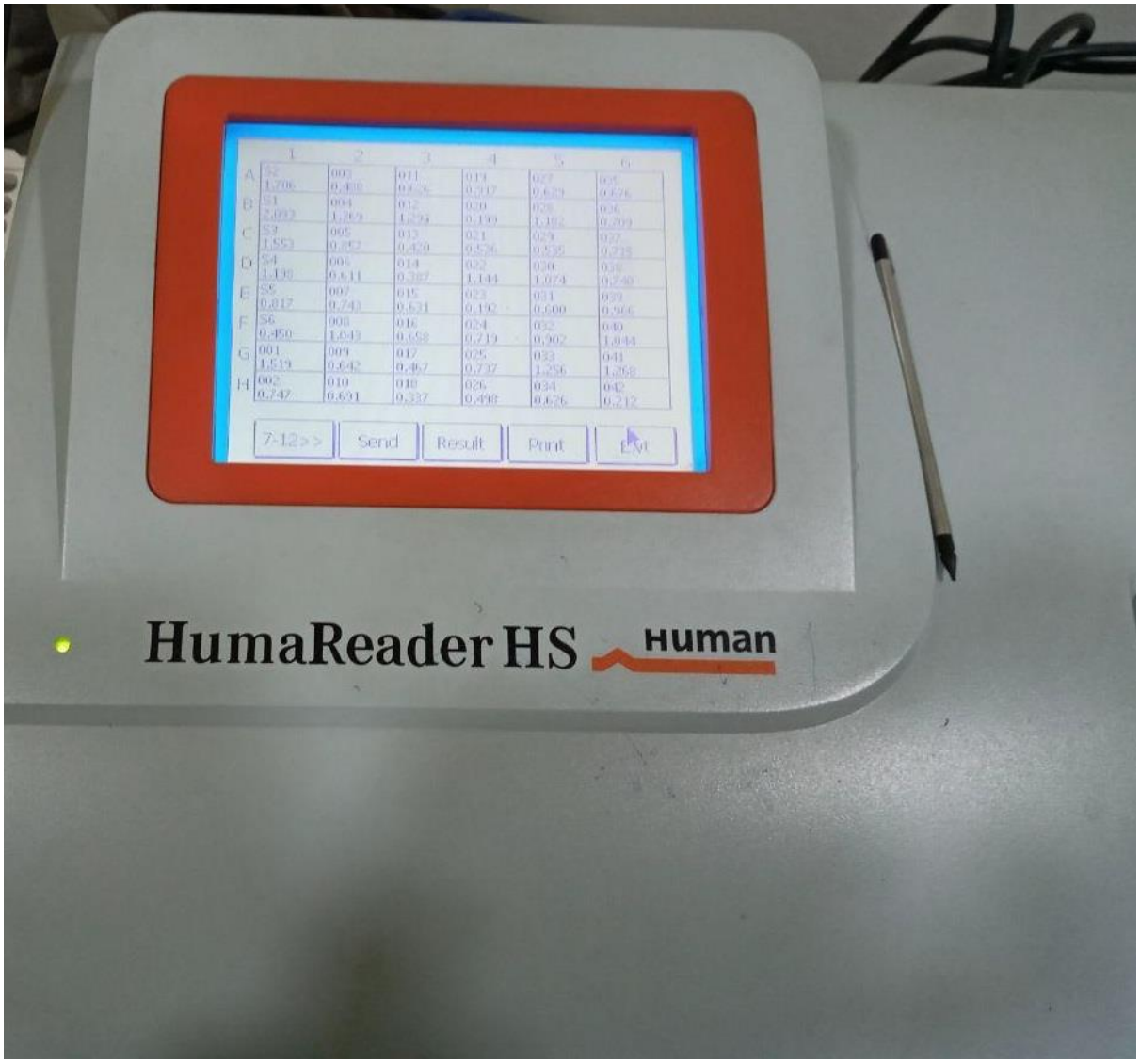
A-ELISA Reader

B- ELISA Washer

C- ELISA Shaker

D-Micro plates of immunological markers

E- ELISA Kit



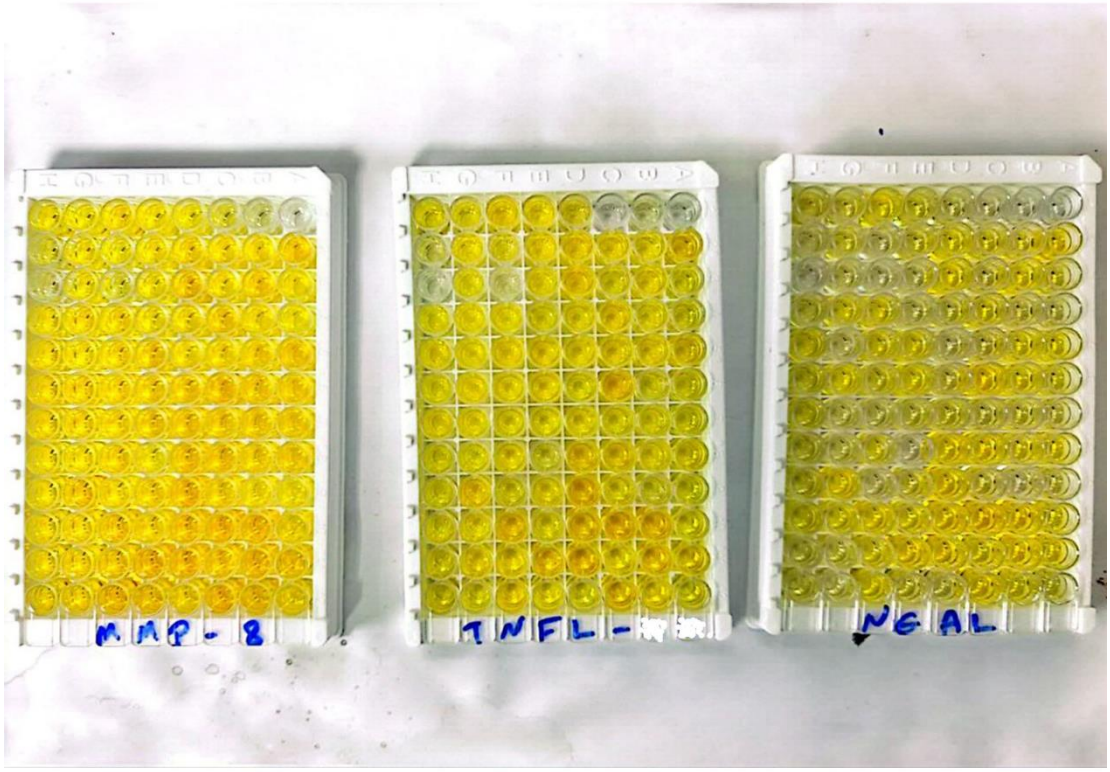
A


Max level
Human
CombiWash
Bottle 1# Priming Prime?
START
PRIME
RINSE
CANCEL
SELECT
ASPR
COMBIWASH Human

B


biosan
Set
Actual
01:00 000 37.0
00 05 000 36.4
hr : min
RPM
T (°C)
Thermo-Shaker
TIME
RPM
T (°C)
RUN/STOP
RPM
TIME
PST-60HL

C



D

E

الخلاصة:

تُشكل امراض الحمى لدى الأطفال تحديات تشخيصية كبيرة بسبب أسبابها المتنوعة، بما في ذلك الحالات البكتيرية والفيروسية والالتهابية. ويعد التمييز بين هذه الأسباب أمرًا بالغ الأهمية لتوجيه استراتيجيات العلاج المناسبة، وتقليل استخدام المضادات الحيوية غير الضرورية، وتحسين نتائج المرضى. غالبًا ما تتم دراسة المؤشرات الحيوية مثل ربيطة موت الخلايا المبرمج المرتبطة بعامل نخر الورم (TNFL)، وليبوكالين المرتبط بالجيلاتيناز المتعادل (NGAL)، وميتالوبروتيناز 8 (MMP-8) لدورها في استجابة الجسم للعدوى والالتهاب. تهدف هذه الدراسة إلى التحقيق في الفائدة التشخيصية لثلاثة علامات مناعية محددة TRAIL وMMP-8 وNGAL في الأطفال الذين يعانون من أمراض الحمى

و هي دراسة حالة ومجموعة السيطرة وتم تقسيم العينات إلى مجموعتين: 60 حالة حمى (30 مريضًا مصابًا بعدوى بكتيرية و30 مصابًا بحالات فيروسية) و30 من السيطرة الأصحاء. تم تسجيلهم في مستشفى كربلاء التعليمي للأطفال، كربلاء، العراق. خلال الفترة من نوفمبر (2023) إلى فبراير (2024). تم جمع عينات الدم والمصل وكشف مستوى البروتين التفاعلي سي، وكريات الدم البيضاء، والعدلات، والخلايا اللمفية بواسطة أجهزة تحليل الدم Swelab alfa، ومستويات TNFL وMMP-8 وNGAL بواسطة تقنية ELISA. وجدت الدراسة ارتفاع مستويات البروتين التفاعلي سي، وكريات الدم البيضاء، والعدلات لدى المرضى المصابين بعدوى بكتيرية، وزادت الخلايا الليمفاوية بشكل ملحوظ لدى المرضى المصابين بعدوى فيروسية عند ($P \leq 0.05$). كما أظهرت الدراسة أن ربيطة موت الخلايا المبرمج المرتبطة بعامل نخر الورم (TNFL)، وميتالوبروتيناز 8 (MMP-8)، وليبوكالين المرتبط بالجيلاتيناز المتعادل (NGAL) تظهر إمكانات كبيرة ($P \leq 0.05$) كعلامات حيوية للتمييز بين العدوى البكتيرية والفيروسية لدى الأطفال المصابين بالحمى. وأظهرت TNFL، على وجه الخصوص، خصوصية عالية (0.8) للعدوى البكتيرية، في حين كانت المستويات المرتفعة من MMP-8 (0.683) و NGAL (0.8) أكثر دلالة على العدوى الفيروسية. وأظهرت النتائج زيادة في تركيز NGAL في الإناث (0.038) مقارنة بالذكور في مجموعة المرضى المصابين بالعدوى الفيروسية. وأظهرت نتائج التحليل الإحصائي أن TNFL زاد بشكل ملحوظ (0.002) في المرضى المصابين بالعدوى البكتيرية مقارنة بالمرضى المصابين بالعدوى الفيروسية ومرضى مجموعة السيطرة. زاد NGAL و MMP-8

بشكل ملحوظ (0.001) و (0.000) في المرضى المصابين بالعدوى البكتيرية والفيروسية مقارنة بمرضى مجموعة السيطرة على التوالي.

كما نتج عن هذه الدراسة أن ربيطة موت الخلايا المبرمج المرتبطة بعامل نخر الورم (TNFL)، وميتالوبروتيناز 8 (MMP-8)، والليبوكالين المرتبط بالجيلاتيناز المتعادل (NGAL) ظهور إمكانات كبيرة كعلامات حيوية للتمييز بين العدوى البكتيرية والفيروسية لدى الأطفال المصابين بالحمى.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء / كلية الطب

فرع الاحياء المجهرية

دور بعض المؤشرات الحيوية المناعية في التمييز بين العدوى البكتيرية والفيروسية بين الأطفال المصابين بأمراض الحمى الحادة

رسالة مقدمة إلى

مجلس كلية الطب وهي جزء من متطلبات نيل شهادة الماجستير في الأحياء المجهرية الطبية

من قبل الطالب

علي عباس ميران الكعبي

بكالوريوس علوم - علوم حياة (2008)

بإشراف

أ.د عبير ظاهر ناجي الحسناوي **أ.م.د مسار رياض رشيد الموسوي**

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