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Evaluation of the Role of Some Cytokines and Biochemical Parameters in Patients With Uremic Pruritus in Diyala Governorate

A Thesis

Submitted to the College of Science, University of Diyala in Partial Fulfillment of the Requirements for the Master Degree of Science in Biology

By

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Dedication

To those who still give me positive energy,

patience, and diligence in all aspects of life ... my

softhearted ***mother*** *and beloved* ***father****.*

To those who have filled my life with their kindness and love,

My ***brothers*** *and* ***sisters***

To those who have provided me with continuous support,

My dear ***relatives*** *and* ***friends***

Safaa. S.A

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Safaa. S.A

Summary

Summary

Uremic pruritus (UP) is a common, bothersome, and in some cases, debilitating problem for patients with chronic kidney disease (CKD), end-stage renal disease (ESRD), and those undergoing hemodialysis (HD) or peritoneal dialysis (PD). The pathogenesis of UP is complex and not fully understood and many hypotheses have been proposed regarding the development of this disease.

This study aims to investigate the prevalence of hepatitis B and C viruses (HBV, HCV), together with human immunodeficiency virus (HIV), and to assess the levels of interleukin-6 (IL-6), interleukin-13 (IL-13), tumor necrosis factor-alpha (TNF-α), complement 3 (C3), and complement 4 (C4), as well as biochemical parameters, in hemodialysis patients with uremic pruritus.

The study was performed between **4th** of October 2021 – **5th** of March 2022 on 90 HD patients, who suffer from UP, and 30 healthy controls in Baqubah Teaching Hospital-Ibn Sina Dialysis Center. The severity of pruritus was evaluated and measured using a visual analogue scale (VAS). The patient was asked to mark his/her perceived pruritus severity along a 10 cm horizontal line (1-2 mild, 3-7 moderate, and 8-10 severe pruritus). Then, approximately 4 ml of blood was drawn from UP patients during the first 10 minutes of hemodialysis, and the same volume of blood was obtained from healthy controls under completely aseptic conditions. The samples were centrifuged, and the serums obtained for each sample were divided into four Eppendorf tubes and stored at −20°C to be analyzed later for detection of HBV, HCV, HIV, IL-6, IL-13, and TNF-α using Enzyme linked Immunosorbent assay (ELISA) kits, as well as C3 and C4 using Radial Immunodiffusion (RID) plates. The informed consent was taken from all hemodialysis patients.

A total of 90 HD patients, 19 (21.1%) were HCV-positive, 1 (1.1%) was HBV-positive, and 0 (0.0%) was HIV-positive. Patient samples who tested positive for HBV or HCV, as well as patients with cancer, active autoimmune disorders, skin diseases (e.g. dermatitis and psoriasis), and patients under 20 years old. These samples, numbering approximately 30, were excluded from further cytokine and complement protein tests.

The findings of our investigation revealed that the serum IL-6, IL-13, and TNF-α levels were significantly higher in HD patients with UP (95.67 ± 11.91 pg/ml, 16.11 ± 2.28 pg/ml, 148.16 ± 15.39 pg/ml, respectively) as compared with healthy controls (37.82 ± 2.38 pg/ml, 5.80 ± 0.26 pg/ml, 65.11 ± 2.98 pg/ml, respectively) (P = < 0.001). Furthermore, elevated IL-13 and TNF-α levels had a statistically significant correlation with pruritus severity (P = 0. 001 and 0. 003, respectively), whereas the relationship between IL-6 levels and pruritus severity was not statistically significant (P = 0.249).

According to the duration of HD (more than 1 year, less than 1 year, and less than 3 months), the levels of IL-6, IL-13, and TNF-α were found to be increased significantly with the increased period of HD (P = 0.026, 0.046, and 0.006, respectively).

Bivariate pearson correlation was used to test the correlation between IL-6, IL-13, and TNF-α. The results showed that IL-6 had direct correlation with IL-13 (r = 0.693**; P = < 0.001) and TNF-α (r = 0. 736**; P = < 0.001). Furthermore, there was also a statistically positive correlation between TNF-α and IL-13 (r = 0.875**; P = < 0.001).

The results of this study also indicate that serum levels of C3 and C4 were significantly decreased in HD patients with UP (57.83 ± 2.91 mg/dl and 14.59 ± 0.88 mg/dl, respectively) as compared to healthy controls (110.76 ± 2.20 mg/dl and 32.02 ± 1.09 mg/dl, respectively) (P = < 0.001).

Ultimately, our results showed that the serum levels of urea, creatinine, and phosphorus were significantly higher in HD patients with UP (120.95 ± 2.56 mg/dl, 5.27

± 0.18 mg/dl, 4.98 ± 0.15 mg/dl, respectively) when compared with healthy controls (22.16 ± 0.77 mg/dl, 0.46 ± 0.03 mg/dl, 3.21 ± 0.10 mg/dl, respectively) (P = < 0.001). In contrast, albumin was significantly decreased in HD patients with UP (36.98 ± 1.68 g/L) as compared to healthy controls (40.96 ± 0.67 g/L) (P = 0.03). Furthermore, high serum creatinine had a statistically significant correlation with the severity of pruritus (P= 0.029), whereas urea, phosphorus, and albumin did not (P = 0.546, 0.594, and 0.631, respectively).

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Abbreviations	Full Term
ACR	Albumin-to-Creatinine Ratio
AD	Atopic Dermatitis
AIDS	Acquired Immune Deficiency Syndrome
AP	Alternative Pathway
BDNF	Brain-Derived Nerve Growth Factor
C3	Complement 3
C4	Complement 4
CARPA	Complement Activation-Related Pseudoallergy
CI	Chronic Itch
CKD	Chronic Kidney Disease
CKD-aP	Chronic Kidney Disease-associated Pruritus
CP	Classical Pathway
CRP	C-Reactive Protein
CSFs	Colony Stimulating Factors
DM	Diabetes Mellitus
DOPPS	Dialysis Outcomes and Practice Patterns Study
DRG	Dorsal Root Ganglia
eGFR	Estimated Glomerular filtration rate
ELISA	Enzyme Linked Immunosorbent Assay
ESRD	End-Stage Renal Disease

Abbreviations

EUTOX	European Uremic Toxin
GFR	Glomerular Filtration Rate
HBsAg	Hepatitis B Surface Antigen
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HD	Hemodialysis
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
HT	Hypertension
IFNs	Interferons
IgAN	IgA Nephropathy
IL-4Rα	IL-4 Receptor subunit Alpha
IL-6R	IL-6 Receptor
ILs	Interleukins
iNKT cells	Invariant Natural Killer T Cells
KDIGO	Kidney Disease: Improving Global Outcomes
LN	Lupus Nephritis
LP	Lectin Pathway
LPS	Lipopolysaccharide
MPGN	Membranoproliferative Glomerulonephritis
NC	Negative Control
NK cells	Natural Killer Cells

NT-4	Neurotrophin-4
OD	Optical Density
PAMP	Pathogen-Associated Molecular Patterns
PARs	Proteinase-Activated Receptors
PBMCs	Peripheral Blood Mononuclear Cells
PCPs	Primary Care Providers
PD	Peritoneal Dialysis
PIGN	Postinfectious Glomerulonephritis
PN	Prurigo Nodularis
PROs	Patient-Reported Outcomes
PTH	Parathyroid Hormone
QOL	Quality of Life
r	Pearson Correlation
RID	Radial Immunodiffusion
ROS	Reactive Oxygen Species
RRT	Renal Replacement Therapy
SE	Standard Error
SFSN	Small Fiber Sensory Neuropathy
Sig	Significant
sIL-6R	Soluble IL-6 Receptor
sTNF-α	Soluble TNF-α
TGFs	Transforming Growth Factors

Abbreviations

Th-1	T Helper 1
Th-2	T Helper 2
tmTNF-α	Transmembrane TNF-α
TNFR1	Tumor Necrosis Factor Receptor 1
TNF-α	Tumor Necrosis Factor-alpha
UP	Uremic Pruritus
URS	Uremic Retention Solutes
UTs	Uremic Toxins
VAS	Visual Analogue Scale
WHO	World Health Organization
β2-M	*Beta2*-Microglobulin

Chapter One: Introduction

1.Introduction

Itch (also known as pruritus) is an irritating sensation that induces a desire to scratch. Itch is classified as acute or chronic based on its persistence, with the latter one lasting for more than six weeks in an affected individual (Weisshaar *et al*., 2019). Uremic pruritus (UP), also known as "chronic kidney disease-associated pruritus" (CKD-aP), is a common, troubling and in some cases debilitating problem for patients with chronic kidney disease (CKD), end-stage renal disease (ESRD) as well as those maintained on dialysis, including hemodialysis (HD) or peritoneal dialysis (PD) (Verduzco and Shirazian, 2020).

The prevalence of UP varies widely among research studies. It has been reported that it can range between 20% and 90% among CKD and ESRD patients and has a significant clinical impact associated with poor sleep, depression, reduced quality of life, and increased mortality among these patients (Pisoni *et al*., 2006; Rayner *et al*., 2017; Shirazian *et al*., 2017). CKD-aP can develop without any associated diagnosable skin disorders or primary skin lesions, although, over time, secondary skin alterations such as excoriations may occur as a result of intense scratching. Symptoms can be either localized, affecting symmetrical areas of the body, or generalized, affecting the whole body (Swarna *et al*., 2019; Kremer and Mettang, 2019; Ragazzo *et al*., 2020).

The pathogenesis of CKD-aP is complex and not fully understood, and many hypotheses have been proposed regarding its development. Multiple biomarkers have been reported to be associated with CKD-aP. Conventionally, it was assumed that efficiency of dialysis and metabolism biomarkers such as phosphorus, calcium, and parathyroid hormone are associated with an increased risk of UP (Ramakrishnan *et al*., 2013; Verduzco and Shirazian, 2020).

The involvement of immune system dysfunction in the pathogenesis of CKD-aP has been discussed in the past, but it remains unclear. In a study by Kimmel *et al*., (2006) an association was discovered between the serum interleukin-6 (IL-6) level and the occurrence of pruritus in 171 HD patients. Similar findings were recently reported by German researchers (N= 39 HD patients), who observed an increase in serum IL-6 level in patients with uremic pruritus compared to those who did not have pruritus (Schricker *et al*., 2019). In a recent study published in 2021, researchers discovered that serum levels of interleukin-31 (IL-31) were elevated in patients with UP compared to the control group. The same study found that interleukin-13 (IL-13) had a statistically significant correlation with severity of pruritus in UP patients (Oweis *et al*., 2021).

Tumor necrosis factor-alpha (TNF-α) is commonly elevated in patients with ESRD, and it is thought to play a key role in the pathophysiology of UP. TNF-α has been shown to sensitize the itch-recording nerve endings (c-fibers) in the skin, resulting in a more intense signal in response to a specific stimuli (Yosipovitch *et al*., 2003; Gupta *et al*., 2015). In a recent study published in 2020, the authors discovered that serum levels of interleukin-2 (IL-2) were elevated in patients with CKD-aP compared to those without CKD-aP (Rusyati *et al*., 2020).

It is important to highlight that complement activation can occur during hemodialysis (HD), which can lead to chronic inflammation in patients with ESRD (Melchior *et al*., 2021). Overactivation, deficiency, or abnormality of the control proteins of the complement system are often related to several skin diseases, such as psoriasis, lupus erythematosus, and urticaria (Giang *et al*., 2018). In HD patients, activation of the complement system may also induce an allergic response, hence many patients experience pruritus (Poppelaars *et al*., 2018a).

Since CKD is an immune-deficient disease, the prevalence of blood-borne viral infections among HD patients is considerably higher than in the general population. As

result, patients with CKD receiving HD are more susceptible to hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) (Ali *et al*., 2019; Kamal and Mahdi, 2018). The high incidence of these infections among HD patients is due to the existence of common risk factors such as improper or no vaccination, patients are not tested for HBV and HCV before starting HD therapy, inadequate disinfecting of dialysis machines, spreading the infection from one patient to another, and repeated blood transfusions (Roushan *et al*., 2016).

Aims of the study:

According to previous studies, the immunological and uremic toxin hypotheses may be the main contributors to chronic kidney disease-associated pruritus (CKD-aP). Furthermore, with an increasing number of supporters and opponents of these hypotheses, the high incidence of CKD-aP, the psychological and physical difficulties that CKD-aP patients face, and the high prevalence of blood-borne viral infections among these hemodialysis patients, we decided to investigate this disease and its immune hypothesis by measuring the following parameters:

1- Detection of the prevalence of hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) among hemodialysis patients attending Ibn Sina Dialysis Center in Diyala.
2- Evaluation of the serum interleukin-6 (IL-6), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α) levels among hemodialysis patients with uremic pruritus attending Ibn Sina Dialysis Center in Diyala.
3- Assessment of serum complement 3 (C3) and complement 4 (C4) levels in patients with chronic kidney disease-associated pruritus (CKD-aP).
4- Evaluation of urea, creatinine, phosphorus, and albumin levels in hemodialysis patients with uremic pruritus.

Chapter Two: Literature Review

2. Literature Review

2.1. Renal failure (kidney failure)

Renal failure is a medical condition in which one or both kidneys cannot maintain homeostasis, leading to the accumulation of nitrogenous metabolites (azotemia). Renal failure can be classified according to urine production: <50 ml for 24 hours is called anuric kidney failure, <500 ml over 24 hours is called oliguric kidney failure, and urine volumes between 500 ml and 6000 ml over 24 hours are called non-oliguric kidney failure. If the urine volume is over 6000 ml for 24 hours, this is called polyuric (Remer *et al.*, 2014).

Chronic kidney disease (CKD) is defined as the presence of kidney injury or decreased kidney function for at least three months, regardless of the cause (Levey *et al*., 2020). Kidney damage generally refers to pathological abnormalities in the native or transplanted kidney as detected by imaging, biopsy, or inferred from clinical markers such as increased albuminuria —that is, albumin-to-creatinine ratio (ACR) >30 mg/g — or urinary sediment alterations; decreased kidney function refers to a reduced glomerular filtration rate (GFR), which is usually estimated (eGFR) from the serum concentration of creatinine (Navaneethan *et al*., 2021).

In the western world, diabetes is the main risk factor for developing CKD, occurring in 30–50% of CKD patients. Hypertension and smoking are other powerful factors that increase the risk of CKD and the rate at which it progresses (Webster *et al.*, 2017). Instead, in India, Asia, and sub-Saharan Africa, glomerulonephritis is the leading cause of CKD, followed by CKD of unknown origin, likely triggered by soil pollution with heavy metals and pesticides and overuse of traditional herbal medications (Fitria *et al*., 2020). The Kidney Disease: Improving Global Outcomes (KDIGO) (2012) guidelines

recommend classifying individuals according to six GFR categories and three albuminuria categories, as shown in **Figure 2-1** (Levin and Stevens, 2014).

More than a quarter of CKD patients who develop end-stage renal disease (ESRD) are elderly (aged 75 and over) (Balogun *et al*., 2017). Patients with CKD experience significant lifestyle changes that seriously affect their physical or mental health. Older patients, in particular, have numerous comorbidities and complications. Their quality of life (QOL) decreases rapidly, and the burden of symptoms increases rapidly (Rosansky *et al*., 2017). The current treatment for patients with ESRD is renal replacement therapy (RRT), mainly dialysis, although the latter has not always been considered appropriate.

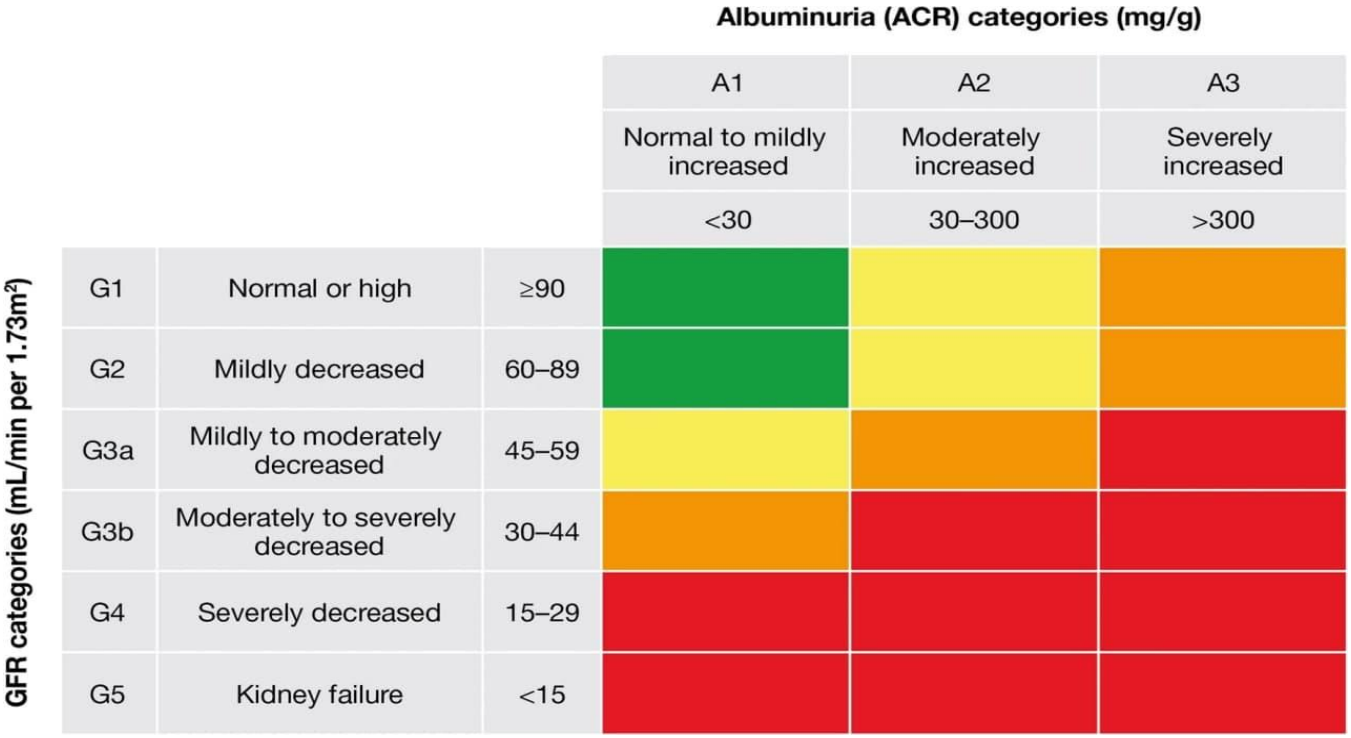

Figure 2-1: Prognosis of CKD by GFR and albuminuria categories. Green, low risk of disease progression; yellow, moderately increased risk of disease progression; orange, high risk of disease progression; red, very high risk of disease progression. Chronic kidney disease (CKD), glomerular filtration rate (GFR), albumin-to-creatinine ratio (ACR)(Levin and Stevens, 2014).

2.2. Itch (Pruritus)

Itch is a sensation that causes the desire to scratch. The terms "pruritus" and "itch" are used synonymously. Pruritus is the most common symptom in dermatology, which can occur with or without visible skin lesions and can be localized or generalized. It is important to differentiate between acute and chronic pruritus. Pruritus that persists for more than six weeks is defined as chronic pruritus (Weisshaar *et al*., 2019). Chronic itch (CI) or pruritus is mostly induced by skin disease (e.g. psoriasis, atopic dermatitis (AD), xerosis, and allergic contact dermatitis) and non-skin disease (e.g. CKD, malignant tumors, and chronic liver diseases). Non-skin or systemic diseases are defined as those originally without abnormalities in the skin, even though secondary lesions may develop due to intensive scratching (Irie and Kabashima, 2021). All of the skin disorders are induced by complex genetic and environmental factors, and they are distinguished by distinct changes in skin appearance, histology, and chemical content (Nattkemper *et al*., 2018).

In systemic skin disorders like chronic kidney disease-associated pruritus (CKD-aP), itch mediators that develop in damaged organs may be transported through the bloodstream to different areas of the body. As a consequence, patients with CKD often suffer from long-term pruritus without any identifiable primary skin lesions (Shirazian *et al*., 2017).

2.2.1. Clinical Classification of Pruritus

The exact mechanism of itch is poorly understood, and several theories have been proposed to explain it. According to the peripheral and central nervous systemic mechanisms, pruritus is divided into the following categories (Lee *et al*., 2016; Cevikbas and Lerner, 2020).

1) Skin-Derived Pruritus: arises from the skin and is caused by inflammation, dryness, or skin injury. Some common disorders include urticaria, scabies, and insect bite dermatitis.
2) Neuropathic Pruritus: is a type of itch that is caused by injured neurons, such as small fiber sensory neuropathy (SFSN).
3) Neurogenic Pruritus: it is caused by mediators, but without neuronal injury.
4) Psychogenic Pruritus: it has a mental origin, such as parasitosis delusions.

2.2.2. Pathophysiology of Pruritus

The human skin is a stratified tissue that covering the entire body and acts as a protective barrier versus environmental irritants. The epidermis is the outer layer of the skin that is in direct contact with the external environment and consists mainly of keratinocytes (Joost *et al*., 2016). Under the epidermis is the dermis, which is separated from it by a basement membrane and contains blood and lymphatic vessels, immune cells, and skin appendages. Tissue resident mast cells coexist with immune cells, which are granulocytes with cytosolic granules storing leukotrienes, histamine, as well as other inflammatory mediators (Pasparakis *et al.*, 2014). The sensory ends of pruritus neurons terminate in the epidermis, creating branching free sensory neurons with a variety of receptor proteins for different mediators (Han *et al*., 2013).

During an incident of acute itch (elicited via biological, chemical, or physical stimulus), the epidermis frequently experiences a rupture or chemical damage (Green and Dong, 2016). As a consequence, immune cells and keratinocytes recognize the skin damage or pathogen-associated molecular patterns (PAMP) and produce a variety of pruritogens (itch-producing substances such as histamine, prostaglandins, cytokines, neuropeptides, and proteases) (Pasparakis *et al*., 2014). Furthermore, mast cells experience degranulation and discharge their cytosolic granule contents. These granules store mediators such as cytokines, chemokines, histamine, proteases, and serotonin (da

Silva *et al*., 2014). These mediators trigger local vasodilation and recruit circulatory immune cells, such as leukocytes and neutrophils, to the site of lesion in order to eliminate the possible pathogen and repair the damage (Pasparakis *et al*., 2014). The changes in the chemical environment of the affected skin area can activate primary afferent sensory neurons (unmyelinated C fibers) with cell bodies in the dorsal root ganglia (DRG) and trigeminal ganglia through G protein–coupled, toll-like, or interleukin receptors **(Figure 2-2)** (Schricker and Kimmel, 2021). These sensory neurons then activate secondary neurons in the dorsal horn of the spinal cord through itch-specific neurotransmitters and ultimately activate projection neurons that transmit the itch signal up the spinothalamic tract to the brain, causing a temporary itch sensation (Kamo *et al*., 2017).

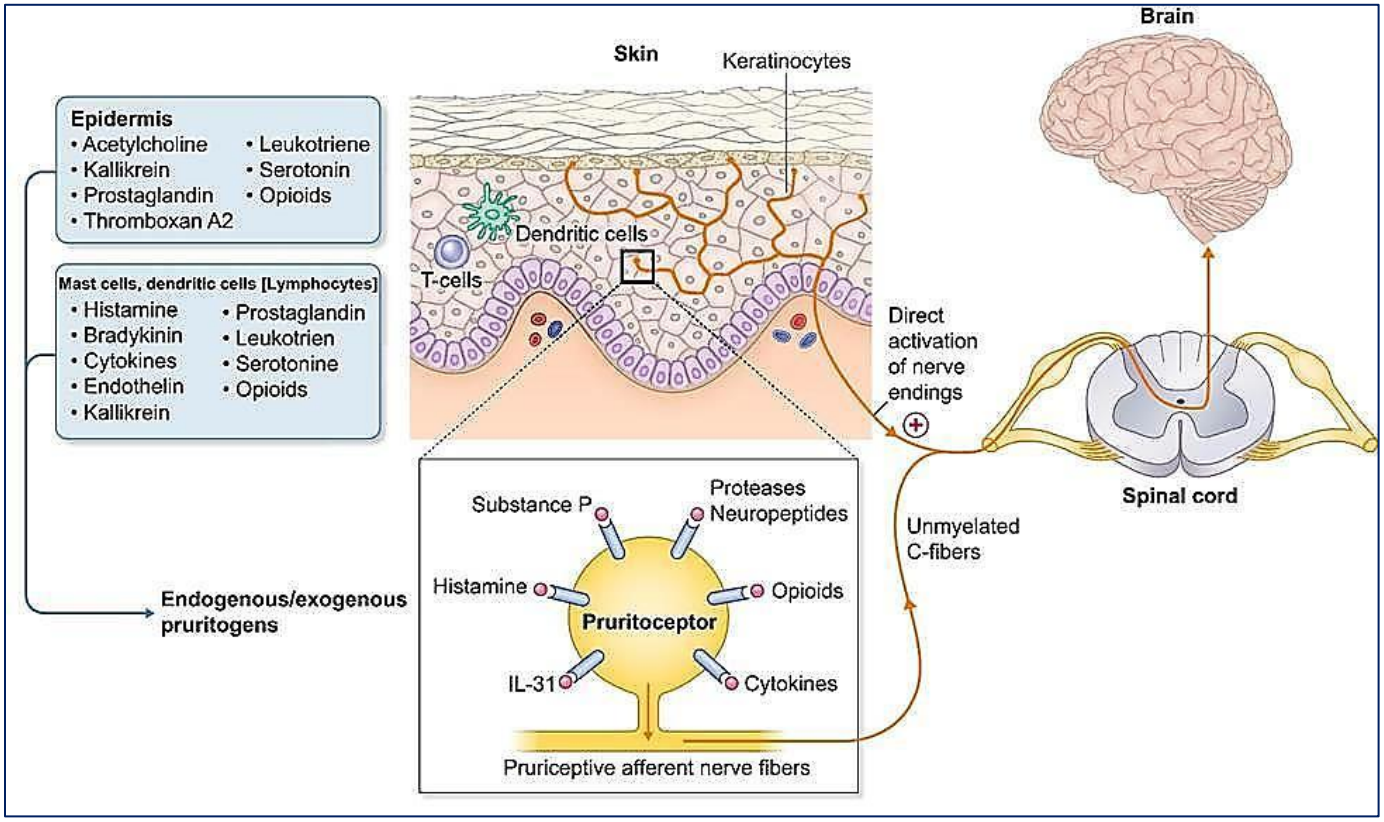

Figure 2-2: Neural pathway of itch originates in the skin and ends in the brain (Schricker and Kimmel, 2021).

2.3. Uremic Pruritus (UP)

Uremic pruritus (UP) or chronic kidney disease-associated pruritus (CKD-aP) was first reported by Chargin and Keil, (1932). This common and unpleasant symptom affects up to 50% of dialysis patients with end-stage renal disease (ESRD). Shirazian *et al*., (2017) described UP as the daily or near-daily occurrence of itching in the absence of primary dermatologic signs. CKD-aP is associated with poor quality of life, depression, worry, sleep disruption, and higher mortality in patients with advanced chronic renal disease. The pathogenesis of UP is unclear, but it is believed to be multifactorial (Shirazian *et al*., 2017).

2.3.1. Epidemiology of Uremic Pruritus (UP)

The prevalence of UP has differed significantly among scientific researches. It has been stated that it can vary anywhere from 20 % to 90 % (Pisoni *et al*., 2006; Rayner *et al*., 2017; Shirazian *et al*., 2017). According to recent studies, the prevalence of UP varies considerably between countries as well as various hemodialysis centers within one country (Kimata *et al*., 2014). However, because of improved dialysis facilities and various dialysis modalities, the occurrence of UP has decreased markedly in recent years. The prevalence is also affected by the type of dialysis used, such as hemodialysis or peritoneal dialysis (Agarwal *et al*., 2021). In a study conducted in Taiwan, Wu *et al*., (2016) found that peritoneal dialysis (PD) patients had less severe UP than hemodialysis (HD) patients, indicating that PD may provide better alleviation of pruritus symptoms. In contrast, Montesa *et al*., (2022) found that the prevalence and intensity of pruritus were similar in HD and PD patients. These data suggest that the type of dialysis techniques does not influence CKD-aP prevalence.

According to Weisshaa, (2016) CKD-aP affects 10% to 77% of HD patients worldwide. Regional variances and the irregular pattern of itch in HD patients may

explain why the disease is still underdiagnosed and underestimated in such patients. According to a meta-analysis of cross-sectional research, 55% of adult dialysis patients had CKD-aP (Hu *et al*., 2018). In terms of the severity of CKD-aP, the international Dialysis Outcomes and Practice Patterns Study (DOPPS) of 35,452 patients revealed that 18% of HD patients reported they were troubled by CKD-aP "very much" or "extremely" (Rayner *et al*., 2017).

Furthermore, in a study conducted in Diyala city, Iraq (n = 150 CKD patients on HD), Jasim, (2021) reported that 33.81% of HD patients were suffering from UP. CKD-aP remains largely underestimated, up to 18% of those with CKD-aP do not receive therapy, and 17% do not report itching to health-care professionals. Furthermore, 69% of medical directors underestimated the prevalence of pruritus in their unit (Rayner *et al*., 2017).

2.3.2. Pathogenicity of Uremic Pruritus

The pathophysiology of CKD-aP is unknown and many hypotheses have been offered to explain its occurrence. The most common of them is shown in **Figure 2-3** (Agarwal *et al*., 2021). In the pathogenesis of CKD-aP, it is postulated that the normal balance is disturbed by various dermatological (e.g. cutaneous barrier dysfunction and xerosis), systemic (e.g. immune system dysfunction), neurological (e.g. neuropathy, μ-opioid receptors overexpression and κ-opioid receptors down-regulation), and metabolic alterations (e.g. uremic toxins and metabolic byproducts accumulation, and changed concentrations of ions such as calcium and phosphate) that all promote the development of itching (Cevikbas and Lerner, 2020; Schricker and Kimmel, 2021).

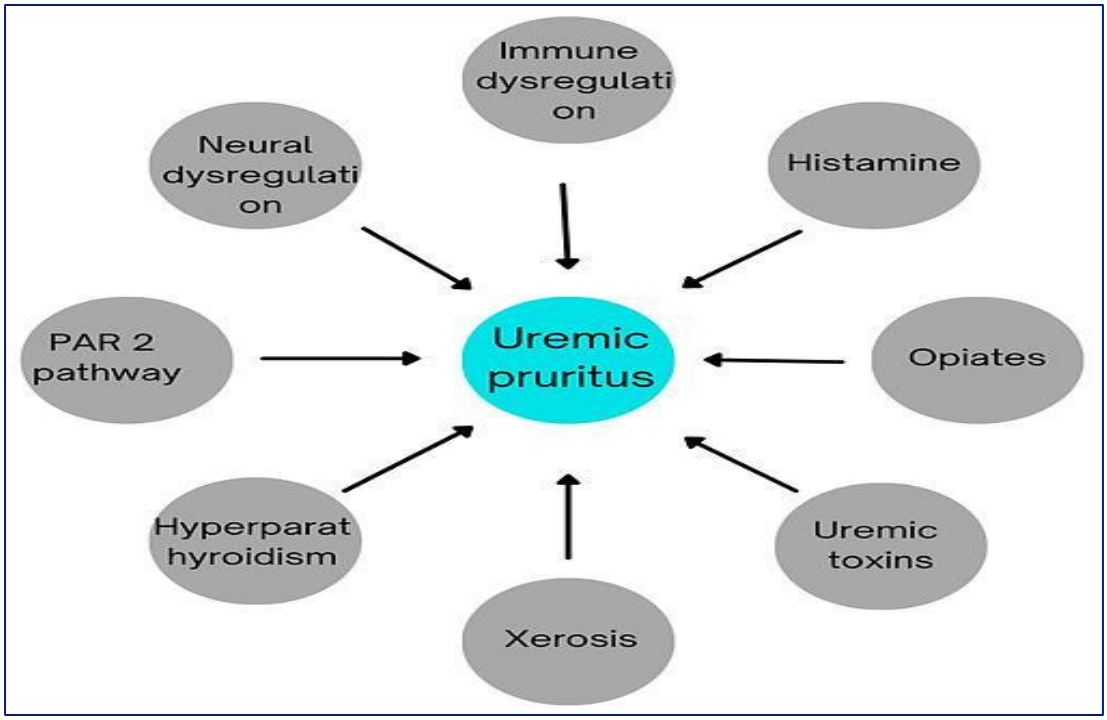

Figure 2-3: Multifactorial pathophysiology of uremic pruritus (Agarwal *et al*., 2021).

2.3.2.1. Immune-mediated hypothesis

It proposes that CKD-aP is caused by dysregulated systemic inflammation (Mettang and Kremer, 2015). This hypothesis is based on the elevated levels of inflammatory and pruritogenic parameters seen in patients with CKD-aP, including high levels of C-reactive protein (CRP) (Sarhan *et al*., 2020), interleukin-6 (IL-6), tumor necrosis factor (TNF-α) (Kimmel *et al*., 2006; Schricker *et al*., 2019), T-helper 1 cells, interleukin-2 (IL-2) (Rusyati *et al*., 2020), interleukin-31 (IL-31), and interleukin-13 (IL-13) (Oweis *et al*., 2021), and the association of CKD-aP with high white blood cell count, and low albumin levels (Pisoni *et al*., 2006). Synthetic dialysis membranes may induce uremic pruritus by stressing blood cells and stimulating the production of pruritogenic cytokines (Fallahzadeh *et al*., 2011). This hypothesis is further supported by the fact that immunosuppressive medications (such as calcineurin inhibitors) can alleviate uremic pruritus.

2.3.2.2. Xerosis hypothesis

Xerosis is the medical term used to describe excessively scaly and dry skin. It is considered a predominant feature in dialysis patients who suffer from UP (Hu *et al*., 2019). The major causes of xerosis are malfunctioning of the sebaceous glands and apocrine sweat glands. An increase in vitamin A and the pH of the stratum corneum contribute to xerosis. An elevation in skin pH may interfere with the action of proteases implicated in the desquamation of stratum corneum (Agarwal *et al*., 2021).

Thyroid gland hypoactivity and chronic inflammation mediated by mast cells may both play an important role in cutaneous xerosis (Szepietowski *et al*., 2004). Persistent xerosis compromises the skin barrier and induces uremic toxin accumulation in the skin. This, along with an increase in urea secretion in sweat, may elicit pruritus in CKD patients (Agarwal *et al*., 2021).

In a study conducted on 5658 non-dialysis CKD patients, Sukul *et al*., (2019) observed that patients with severe pruritus had drier skin as compared to patients without pruritus. Xerosis is regarded as a substantial factor in the severity of CKD-aP, but it is not the primary source of itching (Szepietowski *et al*., 2004).

2.3.2.3. Histamine hypothesis

Histamine, a well-known mediator of pruritus in dermatologic disease, is elevated in the plasma of patients with ESRD as a result of histamine retention in renal insufficiency. Those with CKD have more mast cells, and patients with severe UP have higher plasma levels of tryptase and histamine (Kuypers, 2009; Shafei and Nour, 2016). According to this theory, uremic pruritus is caused by mast cell, histamine, and tryptase release. The itch response is hypothesized to be influenced by mast cell proliferation, degranulation, and histamine release (Krystel-Whittemore *et al*., 2016).

2.3.2.4. Uremic toxin hypothesis

In individuals with CKD, a decrease in renal function leads to insufficient metabolite excretion. This results in the buildup of cytotoxic metabolites, which cause a variety of adverse effects. When the levels of serum phosphorus exceed the usual range, it mixes with serum calcium to produce calcium phosphate, which is accumulated in the skin as well as other organs. The accumulated calcium-phosphate compound stimulates local nerve endings, resulting in pruritus (Hu *et al*., 2019). According to Hsu *et al*., (2018) hemodialysis patients with CKD-aP have higher levels of serum aluminum than those without CKD-aP, emphasizing the necessity of limiting aluminum in CKD-aP patients. Vitamin A, aluminum, calcium, phosphorus, and magnesium have all been implicated as potential toxins and are related to UP (Kimata *et al*., 2014; Mettang and Kremer, 2015).

2.3.2.5. Opioid imbalance hypothesis

It is well known that opioid compounds block pain and are also known to induce itch. The opioid pathways, which include receptors on the brain, keratinocytes, melanocytes, peripheral nerves, immune cells, and hair follicles, have been increasingly recognized as an important regulator of itch (Bigliardi *et al*., 2009).

Endogenous opioid peptides, as well as the opioid system, play an important role in CKD-aP. The activation of the μ(*mu*)-opioid system has been shown to induce pruritus. It is confirmed by the observation that morphine (μ-opioid agonist) induces itching. Conversely, activating the κ(*kappa*)-opioid system minimizes itching. Furthermore, it was established that itch caused by substance P is reduced by activation of κ-opioid receptor and antagonism of μ-opioid receptors (Jaiswal *et al*., 2016; Shirazian *et al*., 2017). The opioid hypothesis has been supported by Wieczorek *et al.*, (2020) who observed that the expression of κ-opioid receptors was significantly decreased in the

skin of CKD-aP patients as compared to those without CKD-aP (Wieczorek *et al.*, 2020).

2.3.2.6. Hyperparathyroidism hypothesis

In patients undergoing hemodialysis, abnormalities of the parathyroid hormone (PTH) caused by secondary hyperparathyroidism are often detected. Low calcium levels in the blood can induce secondary hyperparathyroidism and, as a consequence, increase parathyroid hormone (PTH) secretion. PTH promotes phosphorus reabsorption in the renal tubules and controls blood phosphorus levels (Agarwal *et al*., 2021). According to Hu *et al*., (2019) the serum phosphorus, calcium, and parathyroid hormone levels were substantially higher in HD patients with itch as compared to those without itch. This indicates that PTH may play an important role in the pathophysiology of CKD-aP. This hypothesis is based on the fact that a complete parathyroidectomy can improve pruritus in individuals with secondary hyperparathyroidism. However, not all CKD-aP patients have hyperparathyroidism. In some people with high PTH levels, parathyroidectomy does not alleviate pruritus. Furthermore, injection of PTH does not elicit pruritus, indicating that it is not the only factor contributing to CKD-aP. As a result, parathyroidectomy as a therapy for pruritus is only effective in HD patients where hyperparathyroidism is a major risk factor for pruritus (Hu *et al*., 2019).

2.3.2.7. Neural dysfunction hypothesis

Deranged nervous system has also been proposed as a potential mechanism of uremic pruritus (UP). Generally, pruritus may be generated by centrally active mediators that do not harm the nervous system but instead activate the itch pathway. For instance, neurotropins (e.g. Brain-derived neurotrophic factor (BDNF), Neurotrophin-4 (NT-4)), a class of neurologic mediators that induce itching in patients with UP (Agarwal *et al*., 2021). According to Sorour *et al.*, (2019) the serum NT-4 levels were higher in HD

patients with UP as compared to those without UP, and there was a significant correlation between pruritus severity and high NT-4 levels. The same study found that the serum BDNF levels were significantly greater in UP patients as compared to healthy control group, but no significant differences were detected between HD patients with and without pruritus. As a consequence, the investigators suggested that these neurotrophins might improve the activity of other pruritus mediators (Sorour *et al*., 2019).

2.3.2.8. Other reported hypothesis

Proteinase-Activated Receptors (PARs) are a subfamily of G-Protein-Coupled Receptors that are triggered by specific proteinases. Four types of PAR have been identified by molecular cloning. PAR-2, which is cleaved by Trypsin-Like Serine Protease, is one of them, and it is associated with acute inflammation and considered a histamine-independent itch mediator (Heuberger and Schuepbach, 2019). According to Moon *et al*., (2014) the epidermal PAR-2 expression was significantly greater in patients with ESRD as compared with control group.

Furthermore, Huang *et al*., (2016) demonstrated that atmospheric carbon monoxide (Co) and nitrogen dioxide (No_2) levels are important contributors to uremic pruritus.

2.3.3. Diagnosis of Uremic Pruritus

Chronic kidney disease-associated pruritus (CKD-aP) is defined as itching that is directly related to kidney diseases and has no other underlying cause. CKD-aP has a varied clinical presentation, making diagnosis challenging (Mettang and Kremer, 2015). For example, its severity may vary over time from hardly appreciable to incessant and disturbing; it may be intermittent or persistent; and it could happen before, during, or after dialysis at any time (Rayner *et al*., 2017). CKD-aP tends to affect many areas of the body, such as the back, chest, face, scalp, and limbs, and can be generalized in up to

50% of patients and is most symptomatic at night (Mettang *et al*., 2002; Weiss *et al*., 2015). As previously stated, CKD-aP can develop without the presence of skin lesions. Symptoms can be generalized, affecting the whole body, or localized, affecting symmetrical parts of the body (Swarna *et al*., 2019; Ragazzo *et al*., 2020).

Further complicating the identification of CKD-aP is the fact that it can coexist with xerosis (dry skin) in 50% – 85% of patients (Szepietowski *et al*., 2004) and can occur with superimposed complications of itching, including impetigo, crusts, papules, ulcerations, erosions, and prurigo nodularis (**Figure 2-4**) (Suzuki *et al*., 2015; Verduzco and Shirazian, 2020). Based on this clinical heterogeneity and because CKD-aP is a prevalent disorder among dialysis patients, any itching in this population should be considered CKD-aP-related unless there is a convincing other explanation (Mettang and Kremer, 2015). Alternative explanations for itching include the following: comorbid liver, hematologic, and skin diseases; drugs such as opioids (Bautista *et al*., 2014; Mettang and Kremer, 2015).

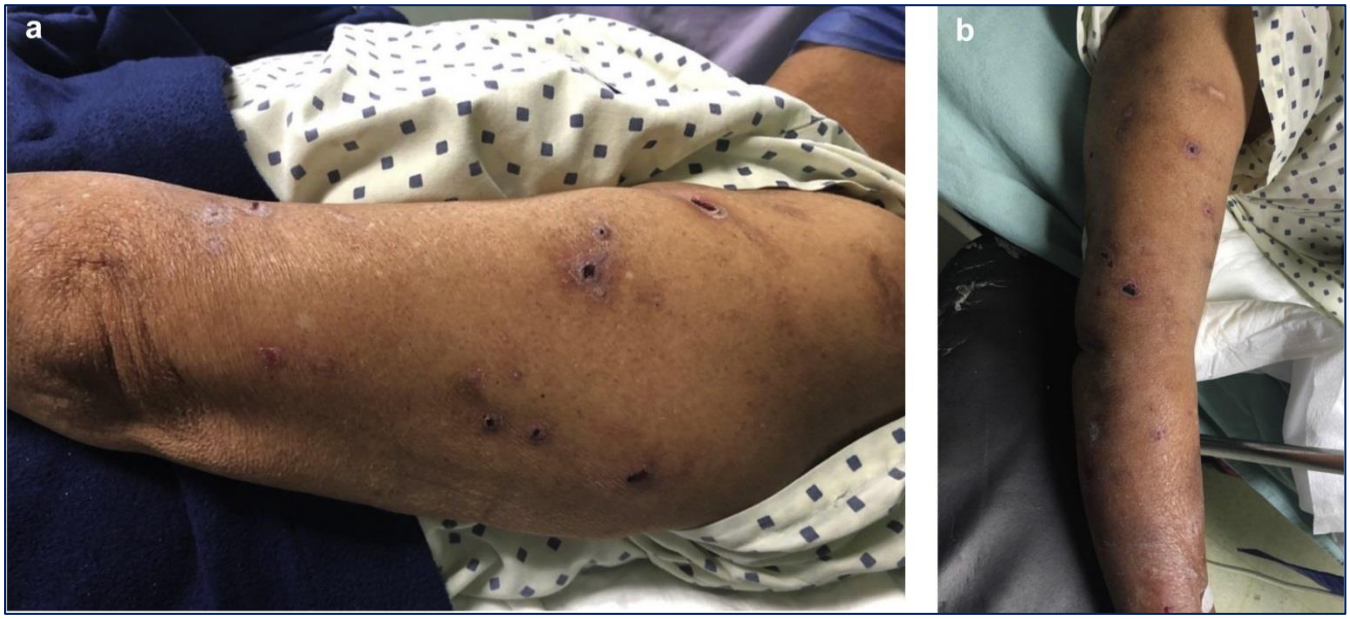

Figure 2-4: CKD-aP with xerosis and superimposed complications of itching including crust, erosions, and papules (Verduzco and Shirazian, 2020).

Itching is often quantified using patient-reported outcomes (PROs) since it is a sensory expression. Many PRO scales have been developed for measuring pruritus severity, which may be either unidimensional (measuring only the severity of pruritus) or multidimensional (assessing the intensity of pruritus as well as other characteristics such as duration, impact on the daily activities, degree, and position of pruritus) (Reszke and Szepietowski, 2018). In this study, we employed a visual analog scale (VAS) to assess the severity of uremic pruritus.

2.3.3.1. Visual Analogue Scale (VAS)

The visual analogue scale (VAS) is the most frequently utilized uremic pruritus severity scoring system. It is a method of transforming non-numerical values into numerical values. It is a scale from 1 to 10 (no pruritus (0), mild pruritus (1-2), moderate pruritus (3-7), severe pruritus (8-10)) (Reich *et al*., 2012; Haydek *et al*., 2017).

2.3.4. Treatment of Uremic Pruritus

Patients with UP are typically treated by nephrologists, dermatologists, as well as primary care providers (PCPs). In an international survey, over 50% of CKD-aP patients were handled by nephrologists, 24% by dermatologists, and just 19% by PCPs (Rayner *et al*., 2017). Since there are many factors associated with the pathogenicity of CKD-aP, and these factors differ significantly between patient groups, there is no one-size-fits-all therapy. Nevertheless, the most commonly used systemic and topical medications are summarized in **Table 2-1**.

Table 2-1: Topical and Systemic therapies for chronic kidney disease-associated pruritus (Bolognia *et al*., 2018; Swarna *et al*., 2019).

NO	Topical Medications (Creams or Ointments)	Systemic Medications	
		Moderate - Severe Pruritus	**Additional Options**
1.	Capsaicin	Aprepitant	Activated charcoal
2.	Cromolyn sodium	Butorphanol	Calcineurin Inhibitors
3.	Doxepin	Fluvoxamine	Erythropoietin
4.	Ketamine	Gabapentin	Ketotifen
5.	Lidocaine	Mirtazapine	Montelukast
6.	Menthol	Nalfurafine	Pentoxifylline
7.	Parmoxine	Naltrexone	Sertraline
8.	Pimecrolimus	Paroxetine	Thalidomide
9.	Tacrolimus	Pregabalin	
10.	γ-linolenic acid	Sertraline	

2.4. Cytokines

Cytokines are soluble glycoprotein molecules with a small molecular weight (6 – 70 kilodaltons) produced by a wide variety of cell types (mast cells, macrophages, natural killer (NK) cells, lymphocytes, stromal cells, and many others). They are mainly contributed in the immunological response and serve as essential mediators in the cellular communication network (Kulbe *et al*., 2012). Furthermore, cytokines are essential health determinants since they influence the maturation, proliferation, and responsiveness of immune and non-immune cells (Neurath, 2014).

Variability in the levels of cytokines in biological fluids such as serum, feces, sweat, and saliva is considered an important indicator of disease progression and diagnosis. Excessive or abnormal cytokine secretion, such as during a cytokine storm, can result in the failure of organs and death. As a result, cytokine levels are now considered a critical indicator for diagnosing clinical disorders.

In numerous disorders, such as cancer (Massagué, 2008), acquired immune deficiency syndrome (AIDS) (Brockman *et al*., 2009), renal diseases (Tinti *et al*., 2021), uremic pruritus (UP) (Kimmel *et al*., 2006), and other chronic diseases, accurate measurement of cytokines provides significant information for monitoring the immune state of patients.

2.4.1. Classification of Cytokines and Their Clinical Significance

Cytokines can be classified into a number of categories, including tumor necrosis factors (TNFs), interleukins (ILs), lymphokines, monokines, interferons (IFNs), colony stimulating factors (CSFs), and transforming growth factors (TGFs). Depending on their role, cytokines may also be classified as anti-inflammatory and pro-inflammatory (Sprague and Khalil, 2009).

2.4.1.1. Pro-Inflammatory Cytokines

The inflammatory response is mainly regulated by cytokines, which induce an acute phase response to defend the body from various disorders such as irritation, damage, and infection. This response begins with the release of pro-inflammatory cytokines from the same or different cells, including IL-12, IL-8, IL-6, IL-1, TNF-α, and IFN-γ. The primary functions of these cytokines are to alert surrounding tissues to the occurrence of infection or tissue damage. Furthermore, pro-inflammatory cytokines can enter the bloodstream, where they activate immune cells and cause substantial changes in the physiology of the host, such as acute-phase response and fever (Boshtam et *al*.,

2017). It is important to mention that excessive production of pro-inflammatory cytokines can induce chronic inflammation and impair biological homeostasis pathways, leading to health issues such as chronic kidney disease (CKD) (Mihai *et al*., 2018), cancer (Lin and Karin, 2007), diabetes (Pickup *et al*., 2000), and other chronic diseases.

2.4.1.2. Anti-inflammatory Cytokines

Anti-inflammatory cytokines are immunoregulatory molecules that inhibit the excessive inflammatory response induced by pro-inflammatory cytokines. They include IL-13, IL-11, IL-10, IL-6, IL-4, and TGF-β (Opal and DePalo, 2000). For example, IL-10 inhibits the synthesis of several pro-inflammatory cytokines and also has an anti-inflammatory impact on eosinophils, basophils, and mast cells, hence it plays an important role in allergic response (Al-Dabbagh *et al*., 2013). Some cytokines (e.g. IL-6) have both anti-inflammatory and pro-inflammatory properties.

Anti-inflammatory cytokines have recently been proven to be extremely useful in a number of clinical disorders related to excessive inflammation. For example, they can be utilized as medications to treat inflammatory diseases (Rider *et al*., 2016).

2.4.2. Cytokines as Uremic Toxins

Many metabolites accumulate in the body when kidney function declines progressively. These accumulating compounds, known as uremic toxins (UTs), might have negative pathophysiological consequences (Duranton *et al*., 2012). UTs can impact numerous organs and are primarily responsible for the progression of CKD (Lim *et al*., 2021), immune system impairment (Lau *et al*., 2018), uremic pruritus (Lu *et al*., 2021), and other chronic diseases. It is important to highlight that a molecule is classified as a UTs if two criteria are fulfilled, one relating to the process of its buildup in CKD and the other one concerning its role in CKD manifestations. Thus, UTs are defined as solutes typically eliminated by the kidney that persist in the state of CKD and interact adversely

with biological functions (Vanholder *et al*., 2003). It is critical to understand that uremic toxins are referred to as uremic retention solutes (URS) if no harmful effects are observed. According to the narrow definition of UTs, cytokines are not exactly uremic toxins, because they are not excreted by the kidney. However, as tiny proteins, they tend to be filtered by the renal corpuscle and degraded by the proximal convoluted tubules. Thus, cytokines may theoretically accumulate in kidney failure due to decreased degradation and may be encompassed by a wider definition of uremic toxins that involves defective molecule clearance in CKD. Furthermore, according to observational studies, high levels of different cytokines are associated with several clinical disorders. These findings are consistent with cell culture and preclinical studies of cytokine cytotoxicity in tissue damage, including renal or vascular diseases. In this context, numerous cytokines are listed among the 130 UTs and URS in the European Uremic Toxin (EUTOX) Working Group database, the most accurate source on the topic (Duranton *et al*., 2012).

In UTs and URS databases, a variety of cytokines were listed; they included inflammatory cytokines (e.g. TNF-α, IL-6, IL-18, IL-1β), chemokines (e.g. IL-8), adipokines (e.g. adiponectin, leptin), and anti-inflammatory cytokines (e.g. IL-10) (Castillo-Rodríguez *et al*., 2017). According to the uremic toxin hypothesis, CKD-aP is caused by an accumulation of UTs. Therefore, these cytokines may play a critical role in the pathogenicity of uremic pruritus.

In vitro and in vivo research supports the theory that cytokine production during hemodialysis is predominantly mediated by (1) direct contact between peripheral blood mononuclear cells (PBMCs) and the dialysis membranes; (2) activation of the complement system (C3a, C5a, and C5b-9 formation); and (3) transmission of bacterial-derived compounds like lipopolysaccharide (LPS) from dialysis fluid to the blood stream (Pertosa *et al*., 2000).

2.4.3. Role of Cytokines in Pruritus

Mast cells, eosinophils, and basophils are all capable of secreting T helper 2 (TH2) -type cytokines such as IL-4, IL-13, and IL-31 (Steinhoff *et al*., 2018). According to recent research, IL-4 and IL-13 directly stimulate sensory neurons, and the expression of IL-4 receptor subunit alpha (IL-4Rα) and IL-13Rα1 was detected in the DRG in mice and humans (Oetjen *et al*., 2017). Both IL-4 and IL-13 produce pruritus by exerting direct neurological effects. In acute and chronic pruritus, the branching ends of afferent neurons present in the epidermis sense exogenous molecules (e.g. irritants, pruritogens, and allergens). This sensation induces recruitment of immune cells such as CD4+ T cells, mast cells, and basophils. These cells produce mediators like IL-4, IL-13, and IL-31, which activate cognate receptors on sensory neurons, causing neuropeptides to be released and contributing to the pruritus-scratch cycle. Nerve impulses are transmitted from afferent neurons to the cell bodies in the dorsal root ganglia, where they synapse with second neurons in spinal cord. The thalamus then assists in the processing of itch signals **(Figure 2-5)** (Nakagawa *et al*., 2018; Cevikbas and Lerner, 2020).

TNF-α is an essential pro-inflammatory cytokine that has been proven to sensitize the itch-recording nerve endings (c-fibers) in the epidermis, resulting in a more apparent signal in response to a specific stimulus (Yosipovitch *et al*., 2003). In UP patients, skin microinflammation, CRP, and the production of pro-inflammatory cytokines such as IL-2, IL-6, and TNF-α have all been identified (Chen *et al*., 2010; Schricker *et al*., 2019; Sarhan *et al*., 2020).

According to Fallahzadeh *et al*., (2011), pro-inflammatory cytokine levels in HD patients with itching are greater than in HD patients without itching. In a recent study published in 2022 in Poland, researchers discovered that serum levels of interleukin-31 were significantly higher in HD patients with pruritus as compared to those without

pruritus (Świerczyńska *et al*., 2022). These findings confirmed the immunological hypothesis of uremic pruritus.

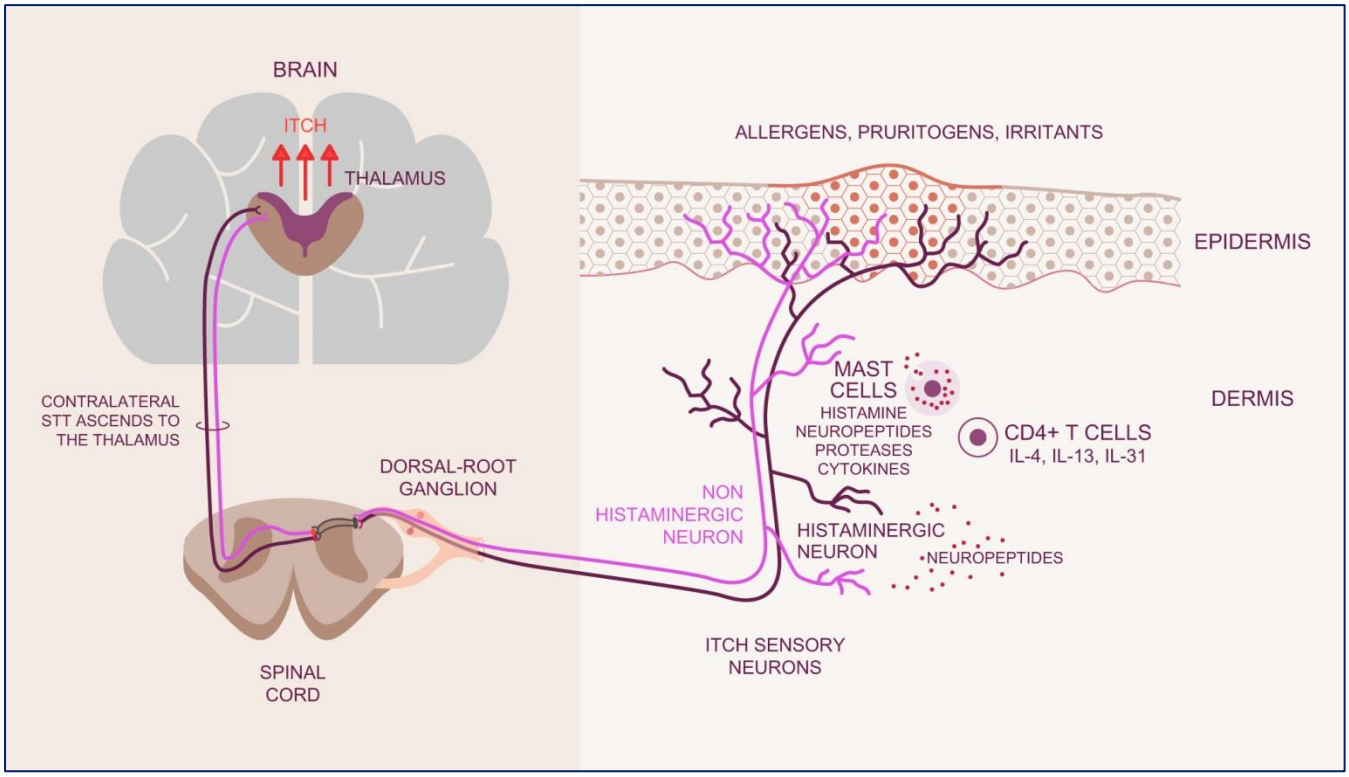

Figure 2-5: The initiation of pruritus (Cevikbas and Lerner, 2020).

2.4.3.1. Interleuki-6 (IL-6)

Interleukin-6 (IL-6) is a member of the pro-inflammatory cytokine family, induces the expression of a variety of proteins responsible for acute inflammation, and plays an important role in the proliferation and differentiation of cells in humans (Uciechowski and Dempke, 2020). It is produced by various types of cells, including T cells, B cells, monocytes, keratinocytes, endothelial cells, mesangial cells, adipocytes, and some tumor cells (Mihara *et al*., 2012). IL-6, like other members in the cytokine family (e.g. IL-27, IL-11, and leukaemia inhibitory factor), communicates via a similar signaling pathway, mediated by signal-transducing receptor subunit gp130 (Rose-John, 2018). In the classical signaling, IL-6 binds to a specific membrane-associated receptor, the IL-6

receptor (IL-6R), which is expressed on several cell types (e.g. leukocytes, megakaryocytes, and hepatocytes), and then forms IL-6R complex with a gp130 homodimer (Ren *et al*., 2016; Wolf *et al*., 2014). In addition, all cells that lack membrane-bound IL-6R are still involved in the so-called trans-signaling process, which occurs as a result of interaction between IL-6, soluble IL-6 receptor (sIL-6R), and gp130.

IL-6 has been implicated in numerous of biological processes, including inflammatory responses, lymphocyte activation, and glucose metabolism regulation (Jones and Vignali, 2011; Castillo-Rodrguez *et al*., 2017). According to several studies, IL-6 has been associated with the pathophysiology of a number of disorders, including chronic kidney disease (CKD) and end-stage renal disease (ESRD). Gupta *et al*., (2012) and Amdur *et al*., (2016) found a correlation between high serum proinflammatory cytokine levels (e.g. IL-6) and impaired renal function during and rapid progression of CKD. Furthermore, the high IL-6 levels have been linked to mortality in dialysis patients with ESRD (Sun *et al*., 2016). Patients with ESRD showed a rapid decrease in blood IL-6 concentrations after renal transplantation (Simmons *et al*., 2005).

Several studies have found a probable relationship between IL-6 and the occurrence of pruritus as a result of chronic sulphur mustard exposure (Panahi *et al*., 2013), as well as prurigo nodularis (PN) (Konda *et al*., 2015). According to Kimmel *et al*., (2006) the high serum IL-6 level has a correlation with the occurrence of uremic pruritus in HD patients with CKD (P = 0.019), but IL-6 had no significant correlation with the severity of pruritus. Similar findings were reported by German researchers (N= 39 HD patients), who observed an increase in serum IL-6 levels in patients with uremic pruritus compared to those who did not have pruritus (Schricker *et al*., 2019). It should be noted that some studies have found a correlation between inflammatory cytokines (e.g. IL-6, TNF-α) and depression in HD patients (Taraz *et al*., 2015). Simultaneously, depression

in HD patients is an indicator of future CKD-aP incidence (Yamamoto *et al*., 2009), whereas coexistence of depressive symptoms in the children with CKD population occurs (Kogon *et al*., 2019).

2.4.3.2. Tumor Necrosis Factor-Alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that has pleiotropic effects on a variety of cell types. It has been implicated as a significant regulator of inflammatory responses and is known to have a role in the etiology of various inflammatory and autoimmune disorders (Bradley, 2008). TNF-α is a 157-amino acid homotrimer protein that is predominantly produced by activated macrophages, T lymphocytes, mast cells, and natural killer cells (Horiuchi *et al*., 2010). It is known to induce a variety of inflammatory molecules, including other cytokines and chemokines.

TNF-α exists in two forms: transmembrane (tmTNF-α) and soluble (sTNF-α). sTNF-α is generated via the enzymatic cleavage of tmTNF-α (Jiang *et al*., 2017). tmTNF-α is present mostly on monocytes and macrophages, where it interacts with tissue receptors via cell-to-cell contact (Qu *et al*., 2017). sTNF-α specifically binds to tumor necrosis factor receptor 1 (TNFR1), whereas tmTNF-α binds to both TNFR1 and TNFR2 (Probert, 2015). TNFR1 is expressed in all human tissues and is the primary TNF-signaling receptor. TNFR2 is widely expressed in immune cells and aids in just a few biological responses (Morita *et al.*, 2001). In summary, TNF-α binds to its receptors, predominantly TNFR1 and TNFR2, and then transmits chemical signals that regulate biological activities such as inflammation and cell death.

TNF-α is typically raised (4–5) folds in patients with ESKD, and it is associated with left ventricular hypertrophy in dialysis patients (Gupta *et al*., 2015).The soluble TNF receptors are also significantly elevated in ESKD (Wolley and Hutchison, 2018). In a study conducted on 1773 HD patients, Narita *et al*., (2006) found that the serum β2-

Microglobulin (*β2*-M) levels increased significantly in HD patients with severe pruritus (N = 473 patients) as compared to the mild and moderate itch patient groups (*P* = 0.0006). Furthermore, a significant correlation was found between severe pruritus and the occurrence of mortality among HD patients. The same study hypothesized that the high *β2*-M levels may stimulate TNF-α or IL-2 production, resulting in the activation of CD4 T cells, thereby increasing the risk of uremic pruritus.

2.4.3.3. Interleukin-13 (IL-13)

Interleukin-13 (IL-13) is a cytokine with a molecular weight of 12 kDa that is expressed by CD4 T helper 2 (Th2) cells, basophils, eosinophils, mast cells, invariant natural killer T (iNKT) cells, and type 2 innate lymphoid cells (Sampson, 2017). The transcription factor (GATA3) is primarily responsible for regulating of IL-13 transcription. IL-13 has around 25% sequence similarity with IL-4 and is found on human chromosome 5q31 (Mannon and Reinisch, 2012). IL-13 mediates various essential biological processes, including airway hyperresponsiveness, allergic inflammation, mastocytosis, tissue eosinophilia, IgE production, and intracellular parasitism (Jain *et al*., 2021).

The cytokines IL-13 and IL-4, which are produced by T helper 2 (TH2) cells, are important players in atopic dermatitis (AD). Both cytokines were found in high concentrations in the serum and epidermis of patients with AD. IL-4 is produced during the early stages of AD, while IL-13 is associated with disease chronicity (Oetjen *et al*., 2017). A variety of effector cytokines, including IL-4, IL-13, and IL-31, have been found to be significantly expressed downstream of the skin barriers in AD (Yang and Kim, 2019). In addition to AD, IL-13 and IL-4 have a role in the chronic pruritus of different skin diseases.

According to Oweis *et al*., (2021) serum IL-31 level was significantly greater in UP patients as compared to the control group (P = 0.0001), while the difference in the levels of IL-13 and IL-33 between the two groups was not statistically significant (P = 0.41 and 0.18, respectively). Furthermore, IL-13 had a statistically significant relationship with the itch score (P = 0.014) and the severity of itch (P = 0.03), while IL-31 and IL-33 were not statistically significant.

2.5. Role of Complement System in the Pathogenicity of Uremic Pruritus

The complement system is an effector component of the innate immune system that is involved in tissue inflammation, pathogen elimination, and the clearance of dead cells and cell debris. It is made up of more than 50 secreted and cell membrane-bound proteins that interact in highly organized ways to perform their tasks. Complement proteins in the blood are mostly produced by liver cells (Pekna and Pekny, 2021). The complement system plays a significant role in the opsonization, activation of several inflammatory pathways, and osmolytic destruction of microbes and injured cells in a variety of disorders, particularly inflammatory renal diseases (Couser, 2012). The system is composed of three pathways: (1) classical pathway (CP), which is triggered by any molecule that recognized by complement component 1q (C1q); (2) lectin pathway (LP), which is triggered when pathogen associated molecular patterns (e.g. mannose present on microbial surfaces) are recognized by pattern recognition molecules (e.g. lectin); and (3) alternative pathway (AP) is activated when complement 3 (C3) is hydrolyzed spontaneously (Bajic *et al*., 2015).

The role of the complement system in the pathophysiology of numerous systemic and renal disorders such as postinfectious glomerulonephritis (PIGN), lupus nephritis (LN), IgA nephropathy (IgAN), membranoproliferative glomerulonephritis (MPGN), and C3 glomerulopathies has been extensively studied during the last two decades (Ricklin *et al*., 2010; Couser, 2012).

As previously stated, chronic kidney disease (CKD) is defined as a decrease in kidney function for at least three months. It is caused by hypertension, infection, diabetes, and excessive complement activation (Reindl *et al*., 2019). Complement activation prompts the progression of kidney disease by stimulating the production and release of inflammatory cytokines (e.g. IL-6, TNF-α), as well as reactive oxygen species (ROS) and matrix protein synthesis (Vikulova *et al*., 2018; Poppelaars *et al*., 2018b).

Several studies have been conducted to investigate the complement activation, the complement pathways implicated, and other factors that contribute to complement activation during hemodialysis (HD). Previously, the first-use syndrome was a significant problem in dialysis patients, termed after the fact that these immune reactions were most acute in new hemodialysis patients. These incompatible reactions occur as a result of complement activation during blood-membrane interaction and they are similar to complement activation-related pseudoallergy (CARPA) (Hempel *et al*., 2017). These findings were recently demonstrated by Melchior *et al*., (2021), who reported that complement activation occurs during the interactions between blood and synthetic HD membranes, resulting in systemic inflammation in CKD patients. In hemodialysis patients, activation of the complement system may also induce an allergic response, hence many patients experience pruritus (Poppelaars *et al*., 2018a).

According to Al-dulaimy *et al*., (2018) a descriptive study was conducted to evaluate the immune response of patients with renal failure by determining the levels of complement 3 (C3) and complement 4 (C4). The authors found that the levels of C3 and C4 were significantly decreased in the patient group as compared to healthy controls.

2.6. Prevalence of Blood-borne Viral Infections among Hemodialysis Patients

Blood-borne viral infections are a public health concern, particularly among high-risk patients, such as those with renal failure. The high incidence of these infections in hemodialysis patients is due to the existence of common risk factors such as a high number of blood transfusions, prolonged vascular access, high exposure to infected patients and contaminated equipment, and cross-contamination from circuits (Patil *et al*., 2018). Hemodialysis is a reliable alternative method for treatment of patients with chronic kidney disease (CKD). As a result of CKD being an immune deficient state, blood-borne viral infections, notably hepatitis B virus (HBV) and hepatitis C virus (HCV), are a significant cause of morbidity and mortality among hemodialysis patients (Bhaumik and Debnath, 2012). An increase in blood transfusions has been associated with HBV infection. Long-term hemodialysis patients were more likely to be infected than short-term hemodialysis patients (Ingin *et al*., 2021). According to Bhaumik and Debnath, (2012) after one month of hemodialysis, a patient's chances of being HBV positive rise 1.47 times.

Regular viral tests and monitoring of hemodialysis patients may significantly minimize the prevalence of HBV and HCV infections. Individuals with pre-end-stage renal disease should also be immunized before undergoing dialysis (Chi, 2012).

According to Kamal and Mahdi, (2018) hemodialysis (HD) patients are at greater risk of the hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) than healthy individuals.

The prevalence of HCV among patients undergoing hemodialysis is significantly higher than in the general population, ranging from 10–50% depending on geographical location (Park *et al*., 2018). Sero-prevalence rates of HCV infection among HD patients

in the Middle East have been reported to be 35% in Jordan, 12% in Iran, 19% in Saudi Arabia, and 23% in Turkey (Ashkani-Esfahani *et al*., 2017). While the prevalence of HBV infection ranges from 0–58% in Tehran (Mohammed *et al*., 2015), and it ranges from 7.21–50% in Turkey (Daglar *et al*., 2014).

In Iraq, HCV is the most common blood-borne viral infection among hemodialysis patients. Hence, several studies have been conducted in various Iraqi cities about this virus, including the studies of Athbi and Jasim, (2015), Ibrahim *et al*., (2018), Muhrath, (2018), Sinjari and Bakr, (2018), Abdilazeem and Nasir, (2019), and Al-Taan and Khalid, (2020), which reported that the infectious rate of HCV among hemodialysis patients was 6.6%, 4.3%, 5.66%, 9.2%, 46.36%, and 20%, respectively.

Chapter Three:
Materials & Methods

3. Materials and Methods:

3.1. Study groups

3.1.1. Study Design

This cross-sectional comparative study was performed between 4th of October 2021 – 5th of March 2022 on hemodialysis (HD) patients, who suffer from uremic pruritus (UP), and healthy controls in Baqubah Teaching Hospital-Ibn Sina Dialysis Center.

3.1.2. Patients and Controls

The study conducted on 90 chronic HD patients with UP. Their ages ranged between 21 and 83 years. A secondary control group of 30 healthy adults was recruited from outpatient clinic visitors, colleagues, and workers. Their ages ranged between 25 and 55 years. The control groups were chosen based on two criteria: first, that they did not suffer from any chronic skin disorders (e.g. eczema, psoriasis, or others); and second, that they did not have any other chronic diseases (e.g. diabetes, malignancy, autoimmune disease, and others).

3.1.3. Exclusion criteria

Patient samples who tested positive for HBV, HCV, or HIV, as well as patients with cancer, active autoimmune disorders, skin diseases (e.g. dermatitis and psoriasis), and patients under 20 years old, numbering around 30 patients, were excluded.

3.1.4. Hemodialysis Characteristics

A questionnaire was used to collect data from each hemodialysis patient, which included age, gender, chronic disease, dialysis duration, number of dialysis sessions per week, number of hours per day, risk factor for CKD, risk factor for itching, distribution of itching, duration of itching, severity of itching, and type of medication used (**appendix-1**).

3.1.5. Pruritus Severity Assessment

The severity of itch or pruritus was evaluated and measured using a visual analogue scale (VAS). It is a scale of 1 to 10 (1-2 mild pruritus, 3-7 moderate pruritus, and 8-10 severe pruritus, while 0 no pruritus).

3.1.6. Blood Collection and Preparation

Approximately 4 ml of blood was drawn from UP patients during the first 10 minutes of hemodialysis, and the same volume of blood was obtained from healthy controls under completely aseptic conditions. The blood was then put into gel tubes and allowed to clot at room temperature. The samples were centrifuged at 3000 rpm for 15 minutes, and the serums were separated into four Eppendorf tubes and stored at −20°C to be analyzed later for detection of HBV, HCV, HIV, IL-6, IL-13, and TNF-α using ELISA kits, as well as C3 and C4 using RID plates. Kidney function biochemical parameter tests (urea, creatinine, phosphorus, and albumin) were obtained from the records of the Ibn-Sina laboratory. The informed consent was taken from all hemodialysis patients (**Figure 3-1**) (**appendix-2**).

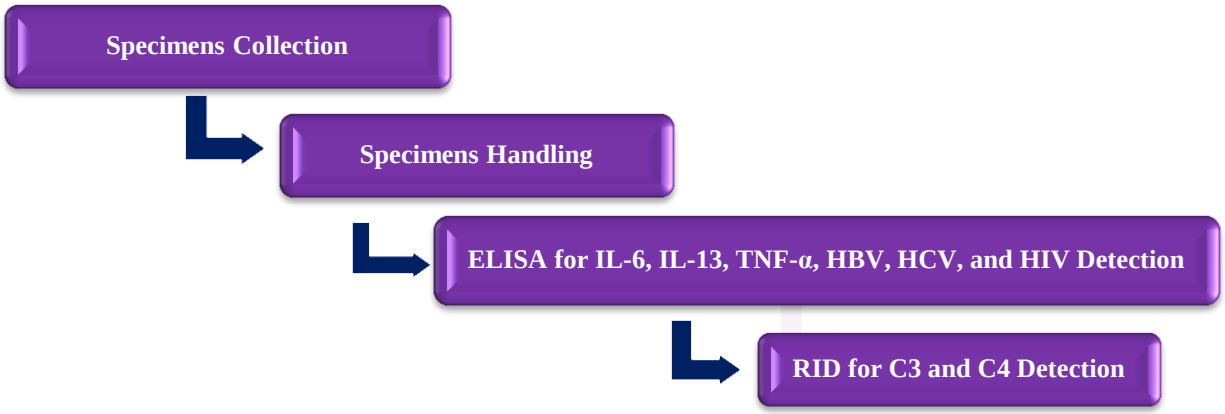

Figure 3-1: The main steps of the current study

3.2. Materials

3.2.1. Laboratory Apparatus and Instruments

Laboratory apparatus and instruments used in this study are shown in **Table (3-1)**.

Table 3-1: Devices and Instruments.

ID	Apparatus and equipment	Company	Origin
1.	Centrifuge	Camero	Germany
2.	Cotton	Gelson	France
3.	Deep freezer	Beko	Turkey
4.	Disposable gloves	Broche	Turkey
5.	Disposable syringes	Medeco	UAE
6.	Disposable tips (blue 1000 µl)	Broche	Turkey
7.	Disposable tips (yellow 100, 200µl)	Biobasic	Canada
8.	Distilled water	Nova	Turkey
9.	ELISA reader and washer	DIALAB	Austria
10.	Eppendorf tube (1.5 ml)	Broche	Turkey
11.	Eppendorf tube Rack	Thermo Fisher Scientific	USA
12.	Gel tube	Broche	Turkey
13.	Graduated cylinder	Pyrex	China
14.	Incubator	Memmert	Germany
15.	Lab Blotting Paper	EMI Specialty Papers	USA
16.	Micropipette	Brand	Germany
17.	Multi-channel pipette	Human	Germany
18.	printer	Seiko Epson	Japan
19.	Rack tube	Thermo Fisher Scientific	USA
20.	Ruler	Partner	China
21.	Stopwatch	Termaks	Germany
22.	Tourniquet	Philippine Medical Supplies	Philippine

3.2.2. Enzyme linked Immunosorbent assay (ELISA) kits and Radial Immunodiffusion (RID) plates

ELISA kits for the diagnosis of blood-borne viral infections and for determining cytokine levels are shown in **Tables (3-2)** and **(3-3)**. **Table (3-4)** shows the RID assay that is used for determining the complement proteins (C3, C4).

Table 3-2: Components of HBV, HCV, and HIV ELISA Kits.

	HBV (HbsAg) Kit		**HIV (Ag/Ab) Kit**		**HCV (Anti-HCV) Kit**	
	Company- Fortress England		**Company- Fortress England**		**Company- Fortress England**	
NO	**Components**	**Volume**	**Components**	**Volume**	**Components**	**Volume**
1.	Microwell Plate 96 Tests	12x8 well strips	Microwell Plate 96 Tests	12x8 well strips	Microwell Plate 96 Tests	12x8 well strips
2.	Negative Control	1ml	Negative Control	1ml	Negative Control	1ml
3.	Positive Control	1ml	Positive Control	1ml	Positive Control	1ml
4.	HRP Conjugate Reagent	7ml	HRP Conjugate Reagent	12ml	HRP Conjugate Reagent	12ml
5.	Stock Wash Buffer	30ml	Biotin Conjugate	3.5ml	Biotin Conjugate	6ml
6.	Chromogen Solution A	7ml	Stock Wash Buffer	50ml	Stock Wash Buffer	50ml
7.	Chromogen Solution B	7ml	Chromogen Solution A	6ml	Chromogen Solution A	6ml
8.	Stop Solution	7ml	Chromogen Solution B	6ml	Chromogen Solution B	6ml
9.	Plate Cover	1 Sheet	Stop Solution	6ml	Stop Solution	6ml
10.			Plate Cover	2 Sheets	Plate Cover	2 Sheets

Table 3-3: Components of IL-6, IL-13, and TNF-α ELISA Kits.

Components				Quantity
Company – Bioassay Technology Laboratory - England				
NO	**IL-6 (20 – 320) ng/L**	**TNF-α (30 – 480) ng/L**	**Il-13 (4 – 64) ng/L**	
1.	Standard solution (640ng/L)	Standard solution (940ng/L)	Standard solution (128ng/L)	0.5ml
2.	Pre-coated ELISA plate	Pre-coated ELISA plate	Pre-coated ELISA plate	12 * 8 well strips
3.	Standard diluent	Standard diluent	Standard diluent	3ml
4.	Streptavidin-HRP	Streptavidin-HRP	Streptavidin-HRP	6ml
5.	Stop solution	Stop solution	Stop solution	6ml
6.	Substrate solution A	Substrate solution A	Substrate solution A	6ml
7.	Substrate solution B	Substrate solution B	Substrate solution B	6ml
8.	Wash buffer	Wash buffer	Wash buffer	20ml
9.	Biotinylated Human IL-6 antibody	Biotinylated Human TNF-α antibody	Biotinylated Human IL-13 antibody	1ml
10.	Plate sealer	Plate sealer	Plate sealer	2 pics
11.	User instruction	User instruction	User instruction	1

Table 3-4: Components of C3 and C4 RID plate.

NO	Features	Complement (C3)	Complement 4 (C4)
1.	**Normal Value**	91 – 156 mg/dl	20 – 50 mg/dl
2.	**Components**	Agarose gel containing the goat antiserum C3	Agarose gel containing the goat antiserum C4
3.	**Company**	LTA srl	LTA srl
4.	**Origin**	Italy	Italy

3.3. Methods

3.3.1. Enzyme linked Immunosorbent Assay (ELISA)

3.3.1.1. ELISA kits for the diagnosis of blood-borne viral infections

These tests were carried out on 90 hemodialysis patients. All patients who tested positive for HBV, HCV, or HIV were excluded from further cytokine and complement protein testing.

3.3.1.1.1. Hepatitis B surface antigen (HBsAg) ELISA Kit

3.3.1.1.1.1. Assay Principle

This test is an enzyme immunoassay that employs the sandwich principle. A microplate containing microtiter wells has been coated with monoclonal Anti-HBs antibodies (antibody against HBsAg), which represents the solid-phase antibody. The serum samples are added to each well of the microplate. If hepatitis B surface antigens (HBsAg) are present in the samples, they will bind to solid-phase antibodies. Following that, guinea-pig anti-HBs labeled with the horseradish peroxidase (HRP) enzyme is

added. In a state of positive reaction, the labelled antibody binds to any previously created solid phase antibody-HBsAg complex. When the test-well is incubated with enzyme substrate, it becomes blue, then yellow when the reaction is stopped using sulphuric acid. If there is no HBsAg in the sample, the labeled antibody cannot be bound precisely, and merely a low background color emerges (Stevens *et al*., 1988; Stevens *et al.*, 1987).

3.3.1.1.1.2. Assay Procedure

Step 1» Reagents preparation: The reagents and samples were allowed to reach room temperature (18–30 °C) for at least 15 minutes. Then, distilled water was used to dilute 30 ml of wash buffer, yielding 600 ml of diluted wash buffer.

Step 2» Numbering wells: The strips required were placed in the strip-holder along with a sufficient number of wells, which included three negative controls (e.g. B1, C1, D1), two positive controls (e.g. E1, F1), and one blank.

Step 3» Adding Sample and HRP-Conjugate: 50µl of positive control, negative control, HRP-Conjugate, and specimen were added into their respective wells except the Blank. Disposable pipette tips were used for each specimen to avoid cross-contamination.

Step 4» Incubating: The plate was covered by the plate cover and incubated for 60 minutes at 37°C.

Step 5» Washing: At the end of the incubation, the plate cover was removed and the plate was automatically washed 5 times with the wash buffer in the ELISA washer. Following the wash, the plate was turned down onto blotting paper, and tapped to remove any remaining residues.

Step 6» Coloring: Then, 50 µl of chromogen solution A and chromogen solution B were added to each well, including the blank, and mixed gently. Then the plate was covered with a new cover and incubated for 15 minutes at 37°C in the dark. The enzymatic interaction between the chromogen solutions and the HRP conjugate yields blue color in the positive controls and HBsAg-positive sample wells.

Step 7» Stopping reaction: A multichannel pipette was used to add 50µl of stop solution into each well and mixed gently. An intensive yellow color is produced in the HBsAg-positive sample wells and positive controls.

Step 8» Measuring the absorbance: After ten minutes of adding the stop solution, the optical density (OD value) of each well was assessed using a microplate reader set at 450 nm.

Step 9» Calculate the Cut-off Value and Evaluate the Results: The cut-off value was determined by this equation [Cut-off = Negative Control (NC) × 2.1]. Note: NC is the average absorbance value of three negative controls.

3.3.1.1.2. Hepatitis C virus antibody (Anti-HCV) ELISA Kit

3.3.1.1.2.1. Assay principle

This test is a two-step incubation enzyme immunoassay that employs the sandwich principle. A microplate containing microtiter wells has been coated with recombinant hepatitis C virus (HCV) antigens expressed in *Escherichia coli* (recombinant core and NS3/4/5). The serum samples are added along with Biotin-Conjugated HCV antigens to each well of the microplate. In the first incubation period, if HCV antibodies are present in the samples, they will bind to coated recombinant antigens from one side and to biotin-conjugated HCV antigens from the other side. After that, the microwells are washed to remove any unattached serum proteins. The bound HCV antibodies are identified during the second incubation period by adding HRP-Conjugate. Following

that, the microwells are washed to eliminate unbound HRP-conjugates and Chromogen solutions are added to each respective wells. The enzymatic reaction between the chromogen solutions and the HRP-Conjugate produces a blue color in both the positive control and HCV antibody-positive sample wells and remains colorless in negative control and HCV antibody-negative sample wells. When the process is stopped using sulfuric acid, the blue color turns yellow. The color intensity is directly proportional to the amount of captured antibodies in the wells (Choo *et al*., 1990; Alter, 1987).

3.3.1.1.2.2. Assay Procedure

Step 1» Reagents preparation: The reagents and samples were allowed to reach room temperature (18–30 °C) for at least 15 minutes. Then distilled water was used to dilute 50 ml of wash buffer, yielding 1000 ml of diluted wash buffer.

Step 2» Numbering wells: The strips required were placed in the strip-holder along with a sufficient number of wells, which included one blank, two positive controls (e.g. E1, F1), and three negative controls (e.g. B1, C1, D1).

Step 3» Adding Biotin-conjugate reagent: Initially, 50µl of Biotin-conjugate was added into their respective wells, except the blank.

Step 4» Adding samples: Then, 50µl of positive and negative controls, and specimens were added into their respective wells, except the blank. Disposable pipette tips were used for each specimen to avoid cross-contamination.

Step 5» Incubating: The plate was covered by the plate cover and incubated for 60 minutes at 37°C.

Step 6» Washing: At the end of the incubation, the plate cover was removed and the plate was automatically washed 5 times with the wash buffer in the ELISA washer.

After the washing, the plate was turned down onto blotting paper, and tapped to remove any remaining residues.

Step 7» Adding HRP-conjugate: Then, 100µl HRP-conjugate was added into each well, except the blank, and gently mixed by tapping the plate.

Step 8» Incubating: The plate was covered and incubated for 30 minutes at 37°C.

Step 9» Washing: At the end of the incubation, the plate cover was removed and the plate was automatically washed 5 times with the wash buffer in the ELISA washer. Following the wash, the plate was turned down onto blotting paper, and tapped to remove any remaining residues.

Step 10» Coloring: Then, 50 µl chromogen solution A and chromogen solution B were added to each well, including the blank, and gently mixed. Then the plate was covered with a new sealer and incubated for 30 minutes at 37°C in the dark. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in positive control and HCV antibody positive sample wells.

Step 11» Stopping Reaction: Using a multichannel pipette, 50µl of stop solution was added into each well and mixed gently. An intense yellow color develops in the positive control and HCV antibody positive sample wells.

Step 12» Measuring the Absorbance: After ten minutes of adding the stop solution, the optical density (OD value) of each well was assessed using a microplate reader set at 450 nm.

Step 13» Calculate the Cut-off Value and Evaluate the Results: The cut-off value was determined by this equation [Cut-off = Negative Control (NC) + 0.12]. Note: NC is the mean absorbance value for three negative controls.

3.3.1.1.3. Human Immunodeficiency Virus (HIV) ELISA Kits

3.3.1.1.3.1. Assay Principle

This antigen (Ag)/Antibody (Ab) (1+2) ELISA kit is a two-steps incubation enzyme immunoassay that employs the "sandwich" principle. A microplate containing microtiter wells has been coated with recombinant human immunodeficiency virus (HIV) antigens (HIV-1*gp*41, *gp*120, and HIV-2 *gp*36) and anti-HIV (*p*24) antibodies. The plasma or serum samples are added together with biotinylated anti-HIV (*p*24) antibodies to each well of the microplate. During the first incubation period, if HIV1/2 antibodies are present in the samples, they will bind to coated recombinant antigens in the wells. Similarly, if HIV *p*24 antigens are present in the samples, they will be captured as a double antibody "sandwich" combination made up of coated antibodies-*p*24-biotinylated antibodies (Barbe *et al*., 1994).

Following that, the microwells are washed to eliminate any unattached serum proteins. During the second incubation period, the identification of captured HIV *p*24 antigen-biotinylated antibody complex or HIV 1/2 antibodies is accomplished through the addition of the enzyme horseradish peroxidase (HRP), which has been coupled to avidin and second HIV 1+2 recombinant antigens.

- **Detection of *p*24 antigens:** When *p*24 antigens have been captured in the wells, avidin will interact with the biotin and bind HRP enzyme to the Ab-*p*24-Ab complex.
- **Detection of HIV 1/2 antibodies:** Once HIV 1/2 antibodies are captured in the wells, the HRP-conjugated antigens bind to the captured antibodies, producing an Ag-Ab-Ag (HRP) complex (Barbe *et al*., 1994).

Following that, the microwells are washed to eliminate unbound HRP-conjugates and Chromogen solutions are added to each respective wells. The enzymatic reaction between the chromogen solutions and the HRP-Conjugate produces a blue color in both

positive control and HIV1/2 antibodies or *p*24 antigens-positive sample wells and remains colorless in negative control and HIV1/2 antibodies or *p*24 antigens-negative sample wells. When the process is stopped using sulfuric acid, the blue color turns yellow. The color intensity is directly proportional to the amount of captured antibodies or *p*24 antigens in the wells (Barré-Sinoussi *et al*., 1983; Barbe *et al*., 1994).

3.3.1.1.3.2. Assay Procedure

Step 1» Reagents preparation: The reagents and samples were allowed to reach room temperature (18–30 °C) for 15 minutes. Then distilled water was used to dilute 50 ml of wash buffer, yielding 1000 ml of diluted wash buffer.

Step 2» Numbering Wells: The strips required were placed in the strip-holder along with a sufficient number of wells, which included two Negative controls (e.g. B1, C1), three Positive controls (one for HIV1 Ab, one for HIV2 Ab and one for HIV Ag controls - e.g. E1, F1, G1), and one blank.

Step 3» Adding Biotin-Conjugate reagent: Initially, 20µl of biotinylated anti-HIV *p*24 antibodies were added into their respective wells, except the blank.

Step 4» Adding Sample: Then, 100µl of positive and negative controls, and specimens were added into their respective wells, except the blank. Disposable pipette tips were used for each specimen to avoid cross-contamination.

Step 5» Incubating: The plate was covered by the plate cover and incubated for 60 minutes at 37°C.

Step 6» Washing: At the end of the incubation, the plate cover was removed and the plate was automatically washed 5 times with the wash buffer in the ELISA washer. After the washing, the plate was turned down onto blotting paper, and tapped to remove any remaining residues.

Step 7» Adding of HRP-conjugate: 100µl HRP-conjugate was added into each well, except the blank and gently mixed by tapping the plate.

Step 8» Incubating: The plate was covered by the plate cover and incubated for 30 minutes at 37°C.

Step 9» Washing: At the end of the incubation, the plate cover was removed and the plate was automatically washed 5 times with the wash buffer in the ELISA washer. Following the wash, the plate was turned down onto blotting paper, and tapped to remove any remaining residues**.**

Step 10» Coloring: Then, 50 µl chromogen solution A and chromogen solution B were added to each well, including the blank, and gently mixed. Then the plate was covered with a new sealer and incubated for 15 minutes at 37°C in the dark. The enzymatic reaction between the Chromogen solutions and the HRP-Conjugate produces blue color in positive control and HIV 1/2 positive for antigens/antibodies sample wells.

Step 11» Stopping Reaction: Using a multichannel pipette, 50µl of stop solution was added into each well and mixed gently. An intense yellow color develops in the positive control and HIV 1/2 positive for antigens/antibodies sample wells.

Step 12» Measuring the Absorbance: After fifteen minutes of adding the stop solution, the optical density (OD value) of each well was assessed using a microplate reader set at 450 nm.

Step 13» Calculate the Cut-Off value and Evaluate the Results: The cut-off value was determined by this equation [Cut-off = Negative Control (NC) + 0.12]. Note: NC is the mean absorbance value for three negative controls.

3.3.1.2. Human Interleukin-6 (IL-6), Interleukin-13 (IL-13), Tumor necrosis factor-alpha (TNF-α) ELISA Kits

Patient samples who tested positive for HBV or HCV, as well as patients with cancer, active autoimmune disorders, skin diseases, and patients under 20 years old, numbering around 30 patients, were excluded. These cytokine tests were carried out on 60 hemodialysis patients with uremic pruritus (UP) and 30 healthy controls. The process is the same in all three kits, with a slight difference in the preparation of the standards. As a consequence, the assay principle and procedure were the same.

3.3.1.2.1. Assay Principle

This kit is an enzyme-linked immunosorbent assay (ELISA). The plate has been pre-coated with the human (IL-6 or IL-13 or TNF-α) antibody. IL-6, IL-13 or TNF-α present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human (IL-6 or IL-13 or TNF-α) antibody is added and binds to (IL-6 or IL-13 or TNF-α) in the sample. Then Streptavidin-HRP is added and binds to the biotinylated (IL-6 or IL-13 or TNF-α) 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 (IL-6 or IL-13 or TNF-α). The reaction is terminated by the addition of an acidic stop solution, and absorbance is measured at 450 nm.

3.3.1.2.2. Reagent Preparation

All reagents are brought to room temperature before use.

3.3.1.2.2.1. Standards Preparation

- **IL-6 ELISA Kit:** Firstly, 120µl of standard (640 ng/L) was combined with 120ul of standard diluent and generated a 320 ng/L standard stock solution. Then, the standard

stock solution was allowed to settle for 15 minutes with gentle mixing before preparing dilutions. Following that, duplicates were prepared by serially diluting the standard stock solution (320 ng/L) with standard diluent, yielding 160 ng/L, 80 ng/L, 40 ng/L, and 20 ng/L solutions **(Appendix 3).**

- **IL-13 ELISA Kit:** Firstly, 120µl of standard (128 ng/L) was combined with 120ul of standard diluent and generated a 64 ng/L standard stock solution. Then, the standard stock solution was allowed to settle for 15 minutes with gentle mixing before preparing dilutions. Following that, duplicates were prepared by serially diluting the standard stock solution (64 ng/L) with standard diluent, yielding 32 ng/L, 16 ng/L, 8 ng/L, and 4 ng/L solutions **(Appendix 4).**
- **TNF-α ELISA Kit:** Firstly, 120µl of standard (960ng/L) was combined with 120ul of standard diluent and generated a 480 ng/L standard stock solution. Then, the standard stock solution was allowed to settle for 15 minutes with gentle mixing before preparing dilutions. Following that, duplicates were prepared by serially diluting the standard stock solution (480 ng/L) with standard diluent, yielding 240 ng/L, 120 ng/L, 60 ng/L, and 30 ng/L solutions **(Appendix 5)**. **Note: Standard diluent serves as the zero standard (0 ng/L).**

3.3.1.2.2.2. Wash Buffer Preparation

Distilled water was used to dilute 20 ml of wash buffer, yielding 500 ml of diluted wash buffer.

3.3.1.2.3. Assay Procedure

1. As instructed, all reagents, standard solutions, and samples were prepared and placed at room temperature. The assay is also performed at room temperature.
2. Firstly, 50 µl of standard solutions were added to standard wells.

3. Then, 40 µl of samples were added to each sample well, and then 10 µl of biotinylated human (IL-6 or IL-13 or TNF-α) antibody was added to the sample wells. Also, 50 µl of streptavidin-HRP was added to sample wells and standard wells (but not to blank control well). The plate was mixed gently, then covered with a sealer and incubated for 60 minutes at 37°C.
4. The sealer was removed and the plate was automatically washed 5 times with the wash buffer in the ELISA washer. Following the wash, the plate was turned down onto blotting paper, and tapped to remove any remaining residues.
5. Following that, 50µl of substrate solutions A and B were also added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in the dark.
6. Ultimately, 50µl of stop solution was added to each well, the blue color changed to yellow immediately.
7. The Optical Density (OD value) was determined for each well after five minutes of adding the stop solution using a microplate reader set to 450 nm.

3.3.2. Determination of complement proteins (C3, C4) by Radial Immunodiffusion (RID) plates

RID tests were carried out on 60 hemodialysis patients with uremic pruritus (UP) and 30 healthy controls.

3.3.2.1. Assay Principle

The quantitation of serum complement components is usually performed by radial immunodiffusion (RID). RID assays are based on an antigen-antibody precipitation reaction. When a serum sample (antigen) is added to wells cut in an agarose gel containing a particular antibody, it forms an immune-complex that is visible as a ring

around the well. The ring diameter is directly proportional to the complement protein concentration of the sample (Mancini *et al*., 1965; Recasens *et al*., 2005).

3.3.2.2. Assay Procedure

1. The samples were allowed to reach room temperature (18–30 °C) for 15 minutes.
2. The plate was removed from its envelope and left at room temperature for a few minutes to ensure any condensed water in the wells could evaporate.
3. Then, 5µl of samples were added to each well, and the plate was left to absorb completely before being handled.
4. The plate was covered and placed in a moist chamber for 72 hours.
5. After incubation, the precipitating rings around the wells were measured using a measuring ruler and compared with the conversion table provided with the kit. The normal values of C3 and C4 according to WHO are 91–156 mg/dl and 20–50 mg/dl, respectively (Mancini *et al*., 1965).

3.4. Statistical analysis

Statistical Package for Social Science (SPSS) version 26 software was used to analyze the data. The data were presented as mean ± standard error (M ± SE). Independent-samples T test was used to find a significant difference in the levels of study parameters between UP patients and healthy controls. Furthermore, the values of these parameters in correlation to itch severity were statistically analyzed using a one-way ANOVA test. Statistically significant differences were defined as those with a *P* value less than 0.05. On the other hand, Bivariate Pearson Correlation was used to test the correlation between IL-6, IL-13, and TNF-α, and the correlation was significant (**) at the 0.01 level (2-tailed).

Chapter Four: Results & Discussion

4. Results and Discussion:

4.1. Infection rate of hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) among hemodialysis (HD) patients

A total of 90 hemodialysis patients (55 males and 35 females) with a mean age of 48.81 ± 1.55 years were recruited. According to the Enzyme-Linked Immunosorbent assay (ELISA) results, 19 (21.1%) patients were HCV-positive, 1 (1.1%) patient was HBV-positive, and 0 (0.0%) was HIV-positive **(Table 4-1)**. As previously mentioned, all patients who tested positive for HBV, HCV, or HIV were excluded from further cytokine and complement protein tests.

Table 4-1: The infection rate of HBV, HCV, and HIV among hemodialysis patients.

Study Group		**Hemodialysis patients**	**HCV Positive (+)**	**HBV Positive (+)**	**HIV Positive (+)**
Gender	Total (%)	90 (100%)	19 (21.1%)	1 (1.1%)	0 (0.0%)
	Male (%)	55 (61.1%)	11 (12.2%)	1 (1.1%)	0 (0.0%)
	Female (%)	35 (38.9%)	8 (8.9%)	0 (0%)	0 (0.0%)
Age (Mean ± Standard Error)		(48.81 ± 1.55)			

The prevalence of these blood-borne viruses has been observed to vary greatly between countries and HD centers within a country, as well as between different research studies. These results were partially in agreement with several recent Iraqi studies. For instance, Ibrahim *et al.*, (2018) observed that 3 (3.2%) of HD patients were

HBV-positive and all recruited patients were HIV-negative in a study done in Duhok City on 94 HD patients. In addition, relatively similar findings were recently reported in Karbala city by Athbi and Jasim, (2015) (n = 165 patients on HD), who observed that 1 (0.6%) was HBV-positive and 11 (6.6%) were HCV-positive. Furthermore, in a study of 306 HD patients conducted in Diyala city, Khalaf and Hussein, (2021) reported that 24 (7.8%) of the patients were HCV-positive.

Furthermore, our results are relatively comparable to several studies conducted in different countries, such as a study conducted on 140 HD patients in North Cyprus by Güvenir *et al*., (2019), who reported that 5 (3.6 %) patients were HCV-positive, 1 (0.7 %) patient was HBV-positive, and 1 (0.7 %) patient was HIV-positive.

The high incidence of these blood-borne viruses among hemodialysis patients is due to the existence of common risk factors such as improper or no vaccination, patients are not tested for HBV and HCV before starting HD therapy, inadequate disinfecting of dialysis machines, spreading the infection from one patient to another, and repeated blood transfusions (Roushan *et al*., 2016). Furthermore, the majority of people with chronic kidney disease (CKD), particularly those on hemodialysis (HD), are elderly, frail, and have multiple comorbidities, putting them at high risk for viral infection complications (Wang, 2020).

4.2. Demographic characteristics of the study groups

A total of 90 individuals participated in this study, including 60 hemodialysis (HD) patients with uremic pruritus (UP) and 30 healthy controls. The mean ages for HD patients and healthy controls were 48.03 ± 1.84 and 36.33 ± 2.32 years, respectively. **Table (4-2)** shows a significant difference in ages and genders among patients with uremic pruritus and controls (P = 0.001).

Table 4-2: The mean age of study groups.

Age & Gender			
Study Group		**HD Patients with Uremic Pruritus**	**Control**
Gender	Total (%)	60 (100%)	30 (100%)
	Male	34 (56.7%)	22 (73.3%)
	Female	26 (43.3%)	8 (26.7%)
Age (Mean ± Standard Error)		(48.03 ± 1.84)	(36.33 ± 2.32)
***P*-value (Patients VS Controls)** ***P* -value <0.05**		0.001	

These findings were somewhat consistent with the findings of Oweis *et al*., (2021), who showed that the mean age of HD patients was 43.4 years. In a recent Iraqi research, Jasim, (2021) revealed that the average age of HD patients with UP was 51 years. Similar findings were reported by Kumar *et al*., (2020), who found that the mean age for HD patients was 55.8 years in a research done in Nepal.

4.3 Characteristics of hemodialysis (HD) patients who suffer from uremic pruritus (UP)

According to the hemodialysis period findings, 41 (68.3%) of patients were maintained on HD for more than 1 year, 16 (26.7%) for less than 1 year, and 3 (5%) for less than 3 months **(Table 4-3)**. Based on the visual analogue scale (VAS), 24 (40%) of patients had severe pruritus, 23 (38.3%) had moderate pruritus, and 13 (21.7%) experienced mild pruritus **(Table 4-3)**. The risk factor for CKD was hypertension in 20 (33.3%) of HD patients, diabetes mellitus in 13 (21.7%), hypertension and diabetes mellitus in 11 (18.3%), polycystic kidney disease in 4 (6.7%), unknown in 4 (6.7%),

interstitial nephritis in 3 (5%), kidney stone in 3 (5%), and renal genesis in 2 (3.3%) **(Table 4-3).**

Table 4-3: The general characteristics of hemodialysis patients with uremic pruritus.

Patients characteristics		Number (60)	Percentage (100%)
Duration of Hemodialysis	1. More than 1 year	41	68.3%
	2. Less than 1 year	16	26.7%
	3. Less than 3 months	3	5%
Pruritus severity (VAS)	1. Severe	24	40%
	2. Moderate	23	38.3%
	3. Mild	13	21.7%
Risk factors of CKD	1. Hypertension	20	33.3%
	2. Diabetes Mellitus	13	21.7%
	3. Hypertension & Diabetes Mellitus	11	18.3%
	4. Polycystic Kidney Disease	4	6.7%
	5. Unknown	4	6.7%
	6. Interstitial Nephritis	3	5%
	7. Kidney Stone	3	5%
	8. Renal Agenesis	2	3.3%

In the current results shown in **Table (4-3)**, we observed that 68.3% of patients were maintained on HD for more than a year, 26.7% for less than a year, and 5% for less than three months. These findings vary considerably between research papers. Moreover, based on the visual analogue scale (VAS), our findings revealed that 40% of patients had severe pruritus, 38.3% had moderate pruritus, and 21.7% experienced mild

pruritus **(Table 4-3)**. These results have differed significantly between research studies**.** For instance, Jasim, (2021) (n = 75 UP patients on HD) reported that 26.7% of patients had severe pruritus, 56% had moderate pruritus, and 17.3% experienced mild pruritus in a study done in Iraq. In addition, relatively similar findings were recently reported in Jordan by Oweis *et al*., (2021) (n = 65 UP patients on HD), who observed 6.2% of patients had severe pruritus, 43.8% had moderate pruritus, and 50% experienced mild pruritus. As previously stated, the mechanism of uremic pruritus (UP) is unknown, and several hypotheses have been suggested to explain it (Agarwal *et al*., 2021).

Finally, our results demonstrated that the risk factor for CKD was hypertension in 33.3% of HD patients, diabetes mellitus in 21.7%, hypertension and diabetes mellitus in 18.3%, polycystic kidney disease in 6.7%, unknown in 6.7%, interstitial nephritis in 5%, kidney stone in 5%, and renal genesis in 3.3% **(Table 4-3).** These findings were consistent with the results of Albayati, (2015), Jasim, (2021), and Abraham *et al*., (2016), who found that hypertension and diabetes mellitus, as well as polycystic kidney disease, interstitial nephritis, and kidney stones, are major risk factors for chronic kidney disease. Furthermore, Webster *et al*., (2017) reported that the main causes of CKD in all high-income and middle-income countries are diabetes mellitus (DM) and arterial hypertension (AH).

Hypertension is highly prevalent in CKD and increases progressively as kidney function declines. The pathogenesis of hypertension in CKD is complex and multifactorial. Chronic hypertension may induce renal arterial stiffness (arteriosclerosis), sodium and fluid retention, inflammation, and abnormalities in endothelial function (e.g. mesangial matrix expansion, thickening of the glomerular basement membrane, and fusion of podocyte foot processes), which decreases the surface area available within the glomerulus for filtration and causes the glomerular filtration system to be leaky. Over time, these processes result in ischemia, or cell death, and atrophy of the vasculature that

supports the glomerulus and the tubules. Hence, this reduces the ability of the kidneys to filter the blood, and eventually leads to kidney failure (Huan *et al*., 2015).

Diabetic kidney disease develops in approximately 40% of patients who are diabetic and is the leading cause of CKD worldwide. Although ESRD may be the most recognizable consequence of diabetic kidney disease. The natural history of diabetic kidney disease includes glomerular hyperfiltration, progressive albuminuria, declining GFR, and ultimately, ESRD. Metabolic changes associated with diabetes (e.g. hyperglycemia) lead to glomerular hypertrophy, glomerulosclerosis, and tubulointerstitial inflammation and fibrosis (Alicic *et al*., 2017).

4.4. Cytokine levels in hemodialysis (HD) patients who suffer from uremic pruritus (UP) and healthy controls

The results showed that the serum interleukin-6 (IL-6), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α) levels in HD patients with UP were (95.67 ± 11.91, 16.11 ± 2.28 pg/ml, 148.16 ± 15.39 pg/ml, respectively) as compared with healthy controls (37.82 ± 2.38 pg/ml, 5.80 ± 0.26 pg/ml, 65.11 ± 2.98 pg/ml, respectively). From these results, it is clear that serum IL-6, IL-13, and TNF-α levels were significantly higher in HD patients with UP as compared to healthy controls (P = < 0.001), as shown in **Table 4-4**.

Table 4-4: The serum interleukin-6 (IL-6), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α) levels in hemodialysis (HD) patients with uremic pruritus (UP) and healthy controls.

Groups	N (90)	IL-6 (*pg/ml*) (*Mean ± SE*) *	IL-13 (*pg/ml*) (*Mean ± SE*) *	TNF-α (*pg/ml*) (*Mean ± SE*) *
HD Patients with UP	**60**	**95.67 ± 11.91**	**16.11 ± 2.28**	**148.16 ± 15.39**
Controls	**30**	**37.82 ± 2.38**	**5.80 ± 0.26**	**65.11 ± 2.98**
P*-Value		**0.001**	**0.001**	**0.001**
P value < 0.05 ***** *Values are expressed as mean ± standard error (SE)*			* *Independent-samples T test*	

There is growing evidence that immune dysfunction is a major contributor to UP. Kimmel *et al*., (2006) observed that patients with UP exhibited a higher ratio of T helper type 1 (Th1) cells, as determined by intracellular cytokines and chemokine receptors

expression. Evidence also showed that the serum histamine, IL-2, IL-6, and IL-31 levels were elevated in HD patients with UP (Schricker *et al*., 2019; Rusyati *et al*., 2020; Oweis *et al*., 2021). The discovery that several immunosuppressive drugs, such as thalidomide (a primarily Th1-inhibiting drug), alleviate UP is the most convincing argument in support of this immune hypothesis (Sharma and Kwatra, 2016).

The findings of our investigation revealed that the serum IL-6 levels were significantly higher in HD patients with UP as compared to healthy controls **(Table 4-4)**. These findings were in agreement with a study by Kimmel *et al*., (2006) who found an association between the serum IL-6 level and the occurrence of pruritus in 171 hemodialysis patients (*P*= 0.019). Similar findings were reported by German researchers (N= 39 HD patients), who observed an increase in serum IL-6 levels in patients with uremic pruritus compared to those who did not have pruritus (Schricker *et al*., 2019). Turkish researchers (n = 249 HD patients) also discovered that the levels of serum pro-inflammatory cytokines (such as IL-6) and CRP were higher in UP patients than in those without UP (Ozen *et al*., 2018). Furthermore, Shafei and Nour, (2016) reported that serum IL-6 levels were significantly higher in HD patients as compared to healthy controls and IL-6 was significantly increased in patients with moderate pruritus as compared to other hemodialysis groups. In contrast, Ko *et al*., (2021) discovered no significant variation in IL-6 levels between HD patients with and without pruritus.

Interleukin-6 (IL-6) promotes inflammatory responses by activation and proliferation of lymphocytes, differentiation of B cells, leukocyte recruitment, and an indication of liver acute-phase proteins (Stenvinkel *et al*., 2005). The main reasons behind the elevation of serum IL-6 levels in non-dialysis CKD and ESRD patients may be associated with a variety of factors, including reduced kidney function, retention of uremic toxins, persistent infections, and genetic factors, as well as old age (Stenvinkel *et al*., 2005). On the other hand, it is logical to suggest that the dialysis technique itself

represents an additional stimulus to the inflammatory response. This was demonstrated by Takahashi *et al*., (2000) who found that both peritoneal dialysis (PD) and hemodialysis (HD) procedures cause an elevation in serum IL-6 levels and increase the expression of mononuclear cell IL-6 mRNA. Furthermore, Caglar *et al*., (2002) found that serum IL-6 levels were elevated after hemodialysis, indicating a hemodialysis-related inflammatory response. Several dialysis-related factors, including the use of bioincompatible synthetic HD membranes, catheters for vascular access, and non-sterile dialysate, as well as peritonitis and catheter infections in peritoneal dialysis, have been reported to contribute to the production of IL-6 and/or promote the inflammatory response (Stenvinkel and Alvestrand, 2002; Ekdahl *et al*., 2017; Dai *et al*., 2017).

Another explanation for elevated serum IL-6 and TNF-α levels in HD patients is the complement system. This was recently demonstrated by Melchior *et al*., (2021), who reported that complement activation occurs during the interactions between blood and HD membranes, resulting in systemic inflammation in CKD patients. The complement system promotes recruitment and activation of leukocytes, resulting in an oxidative burst and the release of pro-inflammatory cytokines (e.g. IL-6, IL-1, and TNF-α) and chemokines (Rysz *et al*., 2006; Poppelaars *et al*., 2018b).

Finally, since many cytokine-encoding genes are expressed in adipocytes, it has been suggested that adipose tissue might contribute up to 20% of systemic IL-6 levels. As a result, visceral obesity might be another explanation for high IL-6 levels in ESRD patients (Axelsson *et al*., 2004).

Tumor necrosis factor- alpha (TNF-α) is a pro-inflammatory cytokine produced by a variety of cells, mainly activated macrophages, mast cells, and T lymphocytes. Our research found that serum TNF-α levels were significantly greater in hemodialysis patients as compared to healthy control group **(Table 4-4)**. These results were partially in agreement with findings of Gupta *et al*., (2015), who demonstrated that TNF-α is

typically raised (4–5) folds in patients with ESRD. Similar findings were reported by Rysz *et al*., (2006) and Lee *et al*., (2015), who observed an increase in serum levels of TNF-α in hemodialysis patients as compared to healthy control group. In addition, the soluble TNF receptors are also significantly elevated in ESRD patients (Wolley and Hutchison, 2018).

Kimmel *et al*., (2006) showed that the level of TNF-α was significantly increased in hemodialysis patients compared to healthy control group. Nonetheless, the increase in TNF-α serum concentrations in UP patients did not reach statistical significance. In contrast, in a study of 175 HD patients conducted in Taiwan, Ko *et al*., (2021) found that the level of TNF-α was not significantly different between HD patients with and without pruritus.

Yosipovitch *et al*., (2003) demonstrated that TNF-α induces the itch-nerve endings (c-fibers) in the skin, resulting in a more intense signal in response to a specific stimuli, suggesting that TNF-α may play an important role in the pathophysiology of CKD-aP. Furthermore, abnormalities interactions between dermal mast cells and the distal ends of non-myelinated C fibers may play a role in UP through the release of various triggering substances from mast cells, including histamine and TNF-α (Lugon, 2005).

Interleukin-13 (IL-13) is a pleiotropic cytokine that plays an important role in inflammatory and immunological responses. It is primarily released by T helper type 2 (Th2) cells and contributes to a variety of human disorders such as bronchial asthma and multiple skin diseases (Seyfizadeh *et al*., 2015). Furue *et al*., (2020) found that the serum IL-13 levels were elevated in atopic dermatitis (AD) patients, and it is associated with itch severity. In this study, we observed that IL-13 levels in HD patients with UP were significantly higher than in healthy controls **(Table 4-4)**. Our results relatively disagreed with the findings of Oweis *et al*., (2021), who discovered no significant variation in IL-13 levels between UP patients and healthy controls. The same study

found that IL-13 had a significant correlation with the severity of itch in UP patients. The level of IL-13 in plasma and the expression of IL-13 mRNA in peripheral blood mononuclear cells (PBMCs) were significantly higher in lupus nephritis patients than those in the controls ($P < 0.001$) (Chen *et al*., 2001).

Cytokines are important mediators of inflammatory and immune responses. Patients with CKD or ESRD usually present with abnormalities of the immune system due to reduced kidney function and the buildup of uremic toxins, as well as dialysis membrane bioincompatibility. During HD sessions, cytokines (e.g. IL-6 and TNF-α) are produced mostly by monocytes stimulated by dialyzer fluids, complement mediators, and blood-membrane interactions (Rysz *et al*., 2006; Sharif *et al*., 2015).

4.5. Complement protein levels in hemodialysis (HD) patients who suffer from uremic pruritus (UP) and healthy controls

The results revealed that the serum levels of complement 3 (C3) and complement 4 (C4) in hemodialysis patients with uremic pruritus were (57.83 ± 2.91 mg/dl and 14.59 ± 0.88 mg/dl, respectively) as compared to healthy controls (110.76 ± 2.20 mg/dl and 32.02 ± 1.09 mg/dl, respectively). According to these findings, serum levels of C3 and C4 were significantly decreased in HD patients with UP compared to healthy controls ($P = < 0.001$), as shown in **Figure 4-1.**

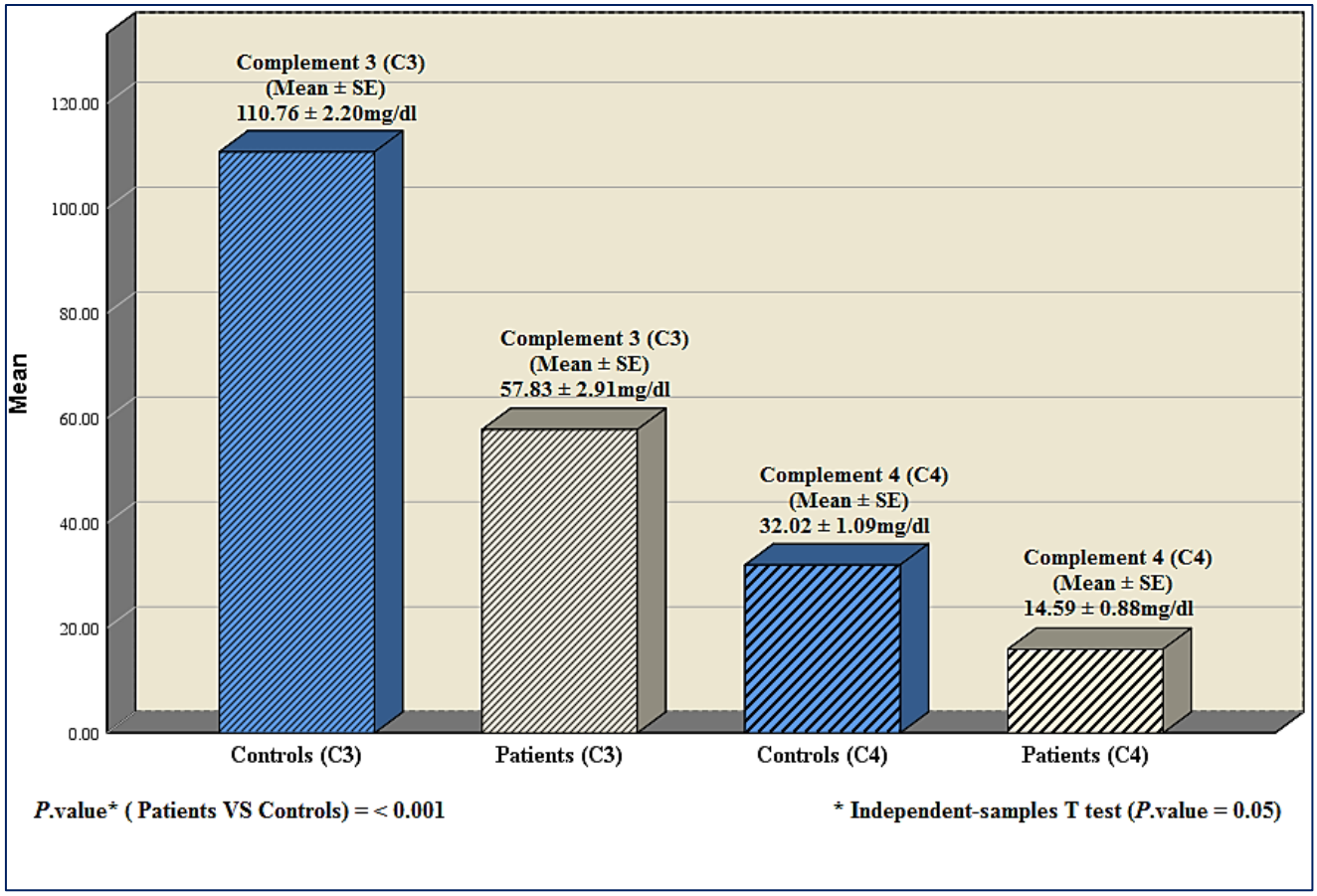

Figure 4-1: The serum levels of complement 3 (C3) and complement 4 (C4) in hemodialysis patients with uremic pruritus and healthy controls.

These findings were consistent with a study conducted in Spain by Rodríguez-Sanz *et al*., (2017), which found that serum C3 and C4 levels were decreased in polysulfone-allergic HD patients as compared to non-allergic HD patients (control). Furthermore, these results were partially in agreement with the findings of Al-dulaimy *et al*., (2018), who demonstrated that C3 and C4 were typically decreased in the general population of HD patients with CKD. Similar findings were reported by Albayati, (2015), who observed a decrease in serum C3 and C4 levels in HD patients as compared to healthy controls.

On the other hand, our findings relatively disagreed with the findings of Elia and Mustafa, (2019), who observed that there was a significant increase in C3 levels for

patients with renal failure compared to the control group, while C4 levels for renal failure patients showed no significant change compared to the control group. C3 is an acute-phase protein that is required for all complement activation pathways (Sahu and Lambris, 2001). Hence, the high serum C3 level indicates a low-grade of systemic inflammation. In contrast, the low C3 level may indicate chronic inflammation and excessive complement activation (Thurman, 2015). Moreover, chronic renal disease and long-term dialysis may have a direct correlation with chronic inflammation. As a result, the discrepancies across studies may be explained by the stages of renal disease and the length of the dialysis period.

Our results of lower serum complement proteins (C3, C4) in hemodialysis patients who suffer from uremic pruritus suggest a high rate of complement activation and proteolytic degradation, resulting in the release of chemotactic molecules known as anaphylatoxins. Anaphylatoxins (C3a and C5a) can induce activation and degranulation of mast cells. The latter releases mediators like tryptase, causing systemic symptoms such as itching in HD patients. In a study conducted in Germany, Dugas-Breit *et al*., (2005) found that the mast cell tryptase levels were elevated in UP patients, and the severity of pruritus in these patients was correlated significantly with tryptase levels. Moreover, C5a may induce the release of histamine from human skin mast cells and peripheral blood basophils via C5a receptors without antigens and IgE interaction. Notably, C5aRs are expressed on the surfaces of skin mast cells but not of other kinds of human mast cells (Yanase *et al.*, 2021). This may explain the itch-related complement activation in hemodialysis patients.

The mechanism of complement activation during hemodialysis may involve binding of ficolin-2 to synthetic hemodialysis membranes, which leads to lectin pathway (LP) activation. On the other hand, the binding of C3b or properdin to synthetic HD membranes may induce alternative pathway (AP) activation. The end results of

complement activation involve membrane attack complex (MAC), opsonins (C3b, iC3b), and anaphylatoxins (C3a, C5a) formation (Poppelaars *et al*., 2018a). Activation of this system promotes recruitment of leukocytes, resulting in an oxidative burst and the release of pro-inflammatory cytokines (e.g. IL-6, IL-1, and TNF-α) and chemokines (Rysz *et al*., 2006; Poppelaars *et al*., 2018b). Additionally, in HD patients with CKD-aP, overactivation of the complement system may lead to a high rate of C3 and C4 consumption, which might explain our findings.

4.6. Biochemical parameter levels in hemodialysis (HD) patients who suffer from uremic pruritus (UP) and healthy controls

The results showed that the serum levels of urea, creatinine, phosphorus, and albumin in hemodialysis patients with uremic pruritus were (120.95 ± 2.56, 5.27 ± 0.18 mg/dl, 4.98 ± 0.15 mg/dl, and 36.98 ± 1.68 g/L, respectively) when compared with healthy controls (22.16 ± 0.77 mg/dl, 0.46 ± 0.03 mg/dl, 3.21 ± 0.10 mg/dl, and 40.96 ± 0.67 g/L, respectively). From these results, it is clear that serum levels of urea, creatinine, and phosphorus were significantly higher in HD patients as compared to healthy controls (P = < 0.001), whereas albumin was significantly decreased in HD patients compared to healthy controls (P = 0.03) as shown in **Table 4-5.**

Table 4-5: The level of biochemical parameters in patients with uremic pruritus and healthy controls.

Groups	N (90)	Urea (15 – 39) *mg/dl* (*Mean* ± *SE*)*	Creatinine (0.1 – 1.2) *mg/dl* (*Mean* ± *SE*)*	Phosphorus (2.8 to 4.5) *mg/dl* (*Mean* ± *SE*)*	Albumin (36 - 52) *g/L* (*Mean* ± *SE*)*
HD Patients with UP	**60**	**120.95 ± 2.56**	**5.27 ± 0.18**	**4.98 ± 0.15**	**36.98 ± 1.68**
Controls	**30**	**22.16 ± 0.77**	**0.46 ± 0.03**	**3.21 ± 0.10**	**40.96 ± 0.67**
P*-Value		**0.001**	**0.001**	**0.001**	**0.03**
P value <0.05 ***** *Values are expressed as mean ± standard error (SE)*				* *Independent-samples T test*	

Creatinine is a byproduct of muscle creatine phosphate, whereas blood urea is a low-molecular-weight nitrogenous waste product produced mostly from dietary protein catabolism and cellular protein turnover (Pandya *et al*., 2016; Gounden *et al.*, 2020). The results of this study indicate that serum levels of creatinine and urea were significantly higher in HD patients with UP as compared to healthy controls (P = < 0.001) (**Table 4-5**). These findings were consistent with several recent Iraqi studies, including Ghassan *et al*., (2015), Albayati, (2015), and Jasim, (2021), who observed an increase in serum creatinine and urea levels in HD patients as compared to healthy control group. However, no significant differences were detected between HD patients with and without pruritus in any of these studies.

Furthermore, our results are comparable to several studies conducted in different countries, such as a study of 175 HD patients conducted in Taiwan by Ko *et al*., (2021), which found that the serum levels of urea and creatinine were significantly higher in HD

patients with UP as compared to healthy controls. Similar findings were observed by Alghazal *et al*., (2020) in Libya. However, there was no significant difference in urea and creatinine levels between HD patients with and without pruritus.

The kidneys are the primary organs in charge of eliminating blood urea and creatinine. In CKD, decreased blood flow to the glomerulus and reduced glomerular filtration rate (GFR) lead to a lower distal tubular flow rate and increase the reabsorption of urea and decrease its excretion, which may explain the increased serum urea concentration (Isra'a, 2010). Moreover, the elevation of serum creatinine levels may be attributed to the decrease in creatinine clearance due to the decrease in the GFR (Sarkar *et al*., 2006).

The results of this study also indicate that serum levels of phosphorus were significantly greater in HD patients with UP as compared to healthy control group (P= < 0.001) (**Table 4-5**). These findings were consistent with a cross-sectional study conducted on 382 hemodialysis and peritoneal dialysis patients in China, which reported that serum phosphorus levels were significantly higher in HD patients with UP as compared to those without UP (Hu *et al*., 2019). Similar results were reported by Gatmiri *et al*., (2013) and Schricker *et al*., (2019), who found that the frequency of pruritus and its severity were significantly higher in patients with a higher serum phosphorus level. On the other hand, a cross-sectional study conducted on 175 hemodialysis patients in Taiwan found no significant differences in the serum phosphorus levels between HD patients with and without pruritus (Ko *et al*., 2021).

Phosphorus is an essential mineral necessary for bone health, vascular function, energy generation, and intracellular signaling (Zhou *et al*., 2021). During CKD, the kidneys become unable to remove extra phosphorus from the body. When the levels of serum phosphorus exceed the usual range, it mixes with serum calcium to produce calcium phosphate, which is accumulated in the skin as well as other organs. The

accumulated calcium-phosphate compound stimulates local nerve endings, resulting in pruritus (Hu *et al*., 2019).

Moreover, the findings of our investigation revealed that the serum levels of albumin were significantly decreased in HD patients with UP as compared to healthy controls (P= < 0.003) (**Table 4-5**). These results were in agreement with the findings of Schricker *et al*., (2019), Chen *et al*., (2010), and Kimmel *et al*., (2006), who found that blood albumin levels were lower in the general population of HD patients with pruritus compared to those who did not have pruritus. On the other hand, Alghazal *et al*., (2020) and Ko *et al*., (2021) did not find any differences in the serum albumin levels between HD patients with and without pruritus. The discrepancies among these studies may be explained by the stages of renal disease, duration of dialysis, type of synthetic dialysis membrane, chronic comorbidities, and the complex pathogenesis of CKD-aP.

Albumin (69 kDa) is one of the most abundant proteins in the blood, comprising 75-80% of the blood plasma (Levitt and Levitt, 2016). In individuals with CKD and ESRD, hypoalbuminemia is very common. Decreased albumin synthesis along with increased clearance and degradation are major contributing factors (Haller, 2005). Furthermore, hemodialysis procedures, especially the dialyzer membranes, expose patients to foreign bodies, which trigger an inflammatory response that is a known cause of low albumin levels. Hemodialysis also induces protein adsorption on the synthetic membranes and tubes, resulting in a decrease in albumin levels (Ward *et al*., 2019; Kalantar-Zadeh *et al*., 2021). In conclusion, the altered albumin homeostasis in ESRD patients is mainly caused by a systemic inflammation, which is a known cause of uremic pruritus.

4.7. Results according to the pruritus severity (mild, moderate, and severe)

4.7.1. Correlation between pruritus severity and cytokines

The results showed that elevated IL-13 and TNF-α levels had statistically significant correlation with pruritus severity (*P* = 0. 001 and 0. 003, respectively). On the other hand, the relationship between IL-6 levels and pruritus severity was not statistically significant (*P* = 0.249), as shown in **Table 4-6**.

Table 4-6: The correlation between pruritus severity and cytokine levels.

Patients Groups		N (60)	IL-6 (*pg/ml*) (*Mean ± SE*)*	IL-13 (*pg/ml*) (*Mean ± SE*)*	TNF-α (*pg/ml*) (*Mean ± SE*)*
1. Severe Pruritus		24	119.65 ± 20.06	25.61 ± 4.43	210.25 ± 29.95
2. Moderate Pruritus		23	83.54 ± 18.51	11.48 ± 2.70	113.40 ± 14.96
3. Mild Pruritus		13	72.87± 23.03	6.78 ± 0.62	95.02 ± 21.65
P-Value*	Severe vs Moderate		0.182	0. 004	0. 004
	Severe vs Mild		0.144	0. 001	0. 003
	Moderate vs Mild		0.738	0.401	0.630
	Difference Between Groups		0.249	0.001	0.003

P value <0.05 — * *One-way ANOVA*

* *Values are expressed as mean ± standard error (SE)*

The findings demonstrated that elevated IL-13 levels had statistically significant correlation with pruritus severity (*P* = 0. 001) (**Table 4-6**). These findings were consistent with a cross-sectional comparative study conducted on 65 HD patients in Jordan, which reported that IL-13 had a statistically significant correlation with the severity of pruritus in UP patients (Oweis *et al*., 2021). There are not many studies about the serum levels of IL-13 in patients with UP. However, many studies have found a relationship between IL-13 and different skin diseases. For instance, Oetjen *et al*., (2017) observed that the levels of IL-13 were elevated in the epidermis and serum of atopic dermatitis (AD) patients. Furthermore, IL-13 along with IL-4 play an important role in the Th2-mediated inflammatory response. IL-13, which is produced by Th2 cells, has a role in B-cell maturation and differentiation, eosinophil chemotaxis, and is a key mediator of allergic asthma (Gandhi *et al*., 2017). IL-13 along with IL-4 also downregulate the expression of fundamental genes involved in skin barrier function and integrity (Brunner *et al*., 2017).

The results of this study also indicate that elevated TNF-α levels had statistically significant correlation with pruritus severity (*P* = 0. 003) (**Table 4-6**). These findings were contrary to previous studies by Chen *et al*., (2010) and Kimmel *et al*., (2006), which found no significant correlation between itch severity and TNF-α levels among UP patients. One possible explanation for the discrepancy is chronic diseases, duration of dialysis, type of dialysis membranes used during HD, and age of patients under study, as well as cigarette smoking, since all of these may enhance the status of inflammation. In addition, individual genetic polymorphisms and the complex pathogenesis of uremic pruritus could also explain the discrepancies. Moreover, the findings of our investigation revealed that the difference in serum IL-6 levels among the severe, moderate, and mild itch patient groups was statistically insignificant (*P* = 0.249). These findings were consistent with study of Kimmel *et al*., (2006), which found no significant correlation

between itch severity and IL-6 levels among UP patients. In contrast, Schricker *et al*., (2019) found a significant correlation between the visual analogue scale (VAS) for pruritus severity and IL-6 levels among UP patients.

4.7.2. Correlation between pruritus severity and biochemical parameters

The findings demonstrated that the levels of creatinine among severe, moderate, and mild itch patient groups differed significantly (P = 0. 029). The difference in urea, phosphorus, and albumin levels, on the other hand, were statistically insignificant among these patient groups (P = 0.546, 0.594, and 0.631, respectively) as shown in **Table 4-7**.

Table 4-7: The correlation between pruritus severity and biochemical parameter levels.

Patients Groups		N (60)	Urea (*mg/dl*) (*Mean ± SE*)*	Creatinine (*mg/dl*) (*Mean ± SE*)	Phosphorus (*mg/dl*) (*Mean ± SE*)	Albumin (*g/L*) (*Mean ± SE*)*
1. Severe Pruritus		24	124.25 ± 4.56	5.85 ± 0.31	4.83 ± 0.26	38.16 ± 2.99
2. Moderate Pruritus		23	117.82 ± 3.76	4.96 ± 0.26	5.18 ± 0.25	37.47 ± 2.72
3. Mild Pruritus		13	120.38 ± 5.18	4.75 ± 0. 32	4.90 ± 0.25	33.92 ± 2.71
P-Value*	Severe vs Moderate		0.276	0. 029	0. 325	0. 858
	Severe vs Mild		0.577	0. 022	0. 859	0. 353
	Moderate vs Mild		0.714	0.649	0.514	0.439
	Difference Between Groups		0.546	0.029	0.594	0.631

P value <0.05 * *One-way ANOVA*

***** *Values are expressed as mean* ± *standard error*

The results of this study demonstrated that high serum creatinine had a statistically significant correlation with the severity of pruritus (**Table 4-7)**. These findings were consistent with Hu *et al*., (2019) and Gobo-Oliveira *et al.*, (2017), who reported that the serum creatinine levels were significantly increased in HD patients with severe pruritus. Similar findings were reported by Narita *et al*., (2006), who found that the group with severe pruritus had significantly higher levels of serum creatinine. Furthermore, Shafei and Nour, (2016) reported that serum creatinine levels were significantly higher in patients with severe pruritus as compared to those with moderate and mild pruritus. In contrast, Ko *et al*., (2021) and Alghazal *et al*., (2020) did not find any significant differences in the serum creatinine levels among HD patients with and without pruritus.

A recent study conducted in north India by Pratyusha *et al*., (2021) reported that high creatinine levels were significantly associated with skin xerosis in hemodialysis patients. Persistent xerosis compromises the skin barrier and induces uremic toxin accumulation in the skin. This, along with an increase in urea secretion in sweat, may elicit pruritus in CKD patients (Agarwal *et al*., 2021). This may explain why patients with severe pruritus have higher levels of serum creatinine as compared to other patient groups. Another explanation of the elevation of creatinine in these patient groups may be associated with factors outside of kidney function, which include consuming large amounts of protein, the muscle bulk of patients, and taking certain medicines (e.g. trimethoprim and cimetidine), as well as some health conditions such as diabetes, high blood pressure, and heart disease (Yadav, 2016; Bamanikar *et al*., 2016; Gounden *et al.*, 2020).

The results of this study also indicate that serum levels of urea among severe, moderate, and mild itch patient groups differed insignificantly (P = 0.546) (**Table 4-7)**. These findings were consistent with the results of Ko *et al*., (2021) and Alghazal *et al*., (2020), which did not find any significant differences in the serum urea levels among

HD patients with severe, moderate, and mild itch. Similar findings were reported in a recent study conducted in Iraq by Jasim, (2021), which revealed that there were no significant differences in the level of urea between patients with mild, moderate, and severe pruritus. In contrast, Hu *et al*., (2019) and Gobo-Oliveira *et al.,* (2017) reported that the serum urea levels were significantly increased in HD patients with severe pruritus.

In addition, our results showed that there were no significant differences in the serum phosphorus levels among patients with mild, moderate, and severe pruritus (**Table 4-7)**. These findings were in agreement with different studies, including Alghazal *et al*., (2020), Ko *et al*., (2021), Gobo-Oliveira *et al.*, (2017), Chen et al., (2010), and Kimmel *et al*., (2006), who found that there were no statistically significant differences in the serum phosphorus levels among mild, moderate, and severe itch patient groups. In contrast, Hu *et al*., (2019), Schricker *et al*., (2019), and Gatmiri *et al*., (2013) reported that the serum phosphorus levels increase as the severity of pruritus increases in HD patients.

Furthermore, our results showed that there were no significant differences in the serum albumin levels among patients with mild, moderate, and severe pruritus (**Table 4-7)**. These findings were consistent with those of Alghazal *et al*., (2020), Ko *et al*., (2021), Gobo-Oliveira *et al.,* (2017), Chen *et al*., (2010), and Narita *et al*., (2006), who found that there were no statistically significant differences in the serum albumin levels between mild, moderate, and severe itch patient groups. In contrast, Schricker *et al*., (2019) and Kimmel *et al*., (2006) found that blood albumin levels were lower in the general population of HD patients with severe pruritus. As we previously mentioned, one possible explanation for the discrepancy among these different studies is chronic diseases, duration of dialysis, type of dialysis membrane used during HD, and age of patients under study, as well as cigarette smoking, since all of these may enhance the

status of inflammation. In addition, individual genetic polymorphisms and the complex pathogenesis of uremic pruritus could also explain the discrepancies.

4.8. Results according to the duration of hemodialysis (HD) (more than 1 year, less than 1 year, and less than 3 months)

4.8.1. Correlation between hemodialysis period and cytokine levels

The levels of IL-6, IL-13, and TNF-α were found to be significantly different between HD periods of more than 1 year, less than 1 year, and less than 3 months (*P* = 0.026, 0.046, and 0.006, respectively) as shown in **Table 4-8**. From these results, it is clear that elevated IL-6, IL-13, and TNF-α had a statistically significant correlation with the increase in the HD period.

Table 4-8: The correlation between hemodialysis period and cytokine levels.

Patients Groups		N (60)	IL-6 (*pg/ml*) (*Mean ± SE*) *	IL-13 (*pg/ml*) (*Mean ± SE*) *	TNF-α (*pg/ml*) (*Mean ± SE*) *
More than 1 year (> 1 Y)		**41**	**117.07 ± 16.06**	**19.92 ± 3.16**	**180.91 ± 20.34**
Less than 1 year (< 1 Y)		**16**	**52.69 ± 8.80**	**8.18 ± 0. 54**	**75.30 ± 9.22**
Less than 3 months (< 3 M)		**3**	**32.48 ± 4.14**	**6.32 ± 1.33**	**89.04 ± 4.17**
P*-Value	**(> 1 Y) vs (< 1 Y)**		**0. 016**	**0. 023**	**0. 002**
	(> 1 Y) vs (< 3 M)		**0. 114**	**0. 863**	**0. 171**
	(< 1 Y) vs (< 3 M)		**0. 717**	**0.401**	**0.844**
	Difference Between Groups		**0. 026**	**0. 046**	**0. 006**
P value <0.05 ***** *Values are expressed as mean* ± *standard error (SE)*				* *One-way ANOVA*	

In the current data presented in **Table 4-8**, we noticed that IL-6, IL-13, and TNF-α had a direct correlation with the increase in HD period. These findings were partially in agreement with those of Kimmel *et al*., (2006), who demonstrated that patients with uremic pruritus were maintained on hemodialysis therapy for a significantly longer period and had higher levels of inflammatory biomarkers (e.g. IL-6, TNF-α, and CRP) as compared to those without uremic pruritus.

Long-term hemodialysis may induce a chronic inflammatory state which subsequently increases inflammatory cytokine production such as IL-6 and TNF-α and this may explain our results.

4.8.2. Correlation between hemodialysis period and complement protein levels

The results revealed that the levels of C3 and C4 were insignificantly different between HD periods of more than 1year, less than 1 year, and less than 3 months (P = 0.626 and 0.859, respectively) as shown in **Table 4-9**. In conclusion, the correlation between complement protein (C3 and C4) levels and the HD period was not statistically significant.

Table 4-9: The correlation between hemodialysis period and complement protein levels.

Patients Groups		N (60)	Complement (C3) (*mg/dl*) (*Mean ± SE*) *	Complement (C4) (*mg/dl*) (*Mean ± SE*) *
More than 1 year (> 1 Y)		**41**	**55.91 ± 3.64**	**14.72 ± 1.05**
Less than 1 year (< 1 Y)		**16**	**62.30 ± 5.64**	**14.66 ± 1.91**
Less than 3 months (< 3 M)		**3**	**60.30 ± 2.70**	**12.43 ± 3.37**
P*-Value	**(> 1 Y) vs (< 1 Y)**		**0. 345**	**0. 974**
	(> 1 Y) vs (< 3 M)		**0. 748**	**0. 585**
	(< 1 Y) vs (< 3 M)		**0. 889**	**0. 614**
	Difference Between Groups		**0.626**	**0.859**
P value <0.05 ***** *Values are expressed as mean ± standard error (SE)*			* *One-way ANOVA*	

In the current results shown in **Table (4-9)**, we observed that the relationship between complement proteins (C3 and C4) and the HD period was not statistically significant. However, the lack of significant differences in serum complement protein levels between long-term dialysis patients and those who just started dialysis suggests that other factors such as accumulation of uremic toxins, comorbidities, genetic predisposition, lifestyle factors, and kidney disease per se may be major contributors to complement activation in ESRD patients.

4.8.3. Correlation between hemodialysis period and biochemical parameter levels

The results revealed that the levels of phosphorus were significantly different between HD periods of more than 1 year, less than 1 year, and less than 3 months (P = 0.007). The difference in urea, creatinine, and albumin levels, on the other hand, were statistically insignificant (P = 0.337, 0.218, and 0.342, respectively) as shown in **Table 4-10**. From these results, it is clear that the levels of phosphorus increase significantly in HD patients who have been on HD for less than a year.

Table 4-10: The correlation between hemodialysis period and biochemical parameter levels.

Patients Groups (N=60)		N (60)	Urea (*mg/dl*) (*Mean ± SE*)*	Creatinine (*mg/dl*) (*Mean ± SE*)	Phosphorus (*mg/dl*) (*Mean ± SE*)	Albumin (*g/L*) (*Mean ± SE*)
More than 1 year (> 1 Y)		41	119.87 ± 3.02	5.27± 0. 20	4.83 ± 0.17	38.51 ± 2.38
Less than 1 year (< 1 Y)		16	125.93 ± 5.56	5.53 ± 0. 40	5.61 ± 0.27	34.50 ± 1.12
Less than 3 months (< 3 M)		3	109.00 ± 1.00	3.96 ± 0. 52	3.56 ± 0.58	29.33 ± 2.72
P-Value*	(> 1 Y) vs (< 1 Y)		0. 305	0. 540	0. 021	0. 300
	(> 1 Y) vs (< 3 M)		0. 364	0. 125	0. 062	0. 243
	(< 1 Y) vs (< 3 M)		0. 181	0.082	0.005	0.531
	Difference Between Groups		0.337	0.218	0.007	0.342

P value <0.05 * *One-way ANOVA*

**Values are expressed as mean ± standard error (SE)*

The results of this study, shown in **Table (4-10)**, indicate that serum levels of urea creatinine were insignificantly different between HD periods. These findings were somewhat consistent with a recent Iraqi study by Jasim, (2021), who observed no significant differences in levels of urea and creatinine between pruritus patients who have been on HD for more than six months and those who have been on HD for less than six months. The kidneys are the primary organs in charge of eliminating urea, creatinine, and phosphorus from the blood. Regardless of the duration of hemodialysis, decreased glomerular filtration rate (GFR) in CKD leads to accumulation of these uremic toxins in the human body.

4.9.Results According to the prevalence of hypertension and diabetes mellitus among patients with uremic pruritus (UP)

4.9.1. Cytokine levels in UP patients based on hypertension and diabetes mellitus

The levels of IL-6, IL-13, and TNF-α were found to be insignificantly different between patients with hypertension and diabetes mellitus (P = 0.342, 0.576, and 0.588, respectively), as shown in **Table 4-11**.

Table 4-11: The level cytokines in patients with uremic pruritus depending on hypertension and diabetes mellitus.

Patients Groups (N=60)		N (60)	IL-6 (*pg/ml*) (*Mean ± SE*)	IL-13 (*pg/ml*) (*Mean ± SE*)	TNF-α (*pg/ml*) (*Mean ± SE*)
Hypertension (HT)		**20**	**110.11 ± 23.18**	**18.95 ± 4.55**	**160.36 ± 30.42**
Diabetes Mellitus (DM)		**13**	**75.83 ± 24.27**	**15.06 ± 4.86**	**133.31 ± 39.49**
P*-Value	**HT vs DM**		**0.342**	**0.576**	**0.588**
P value <0.05 *Values are expressed as mean ± standard error (SE)*				* *Independent-samples T test*	

We found that the levels of IL-6, IL-13, and TNF-α were not significantly different between patients with hypertension and those with diabetes mellitus, as shown in **Table 4-11**. In an Iraqi study, Jasim (2021) also found no significant difference in the serum level of an inflammatory cytokine (IL-31) between HD patients with diabetes and those with other conditions such as hypertension.

CKD is an inflammatory condition that causes an increase in inflammatory cytokines regardless of the presence of other related conditions such as diabetes or hypertension. As a result, no statistically significant differences were identified.

4.9.2. Complement protein levels in UP patients based on hypertension and diabetes mellitus

The levels of C3 and C4 were found to be insignificantly different between patients with hypertension and diabetes mellitus (P = 0.504 and 0.427, respectively) as shown in **Table 4-12.**

Table 4-12: The level of complement proteins in patients with uremic pruritus depending on hypertension and diabetes mellitus.

Patients Groups (N=60)		N (60)	Complement (C3) (*mg/dl*) (*Mean ± SE*)	Complement (C4) (*mg/dl*) (*Mean ± SE*)
Hypertension (HT)		**20**	**55.30 ± 5.63**	**13.79 ± 1.62**
Diabetes Mellitus (DM)		**13**	**61.13 ± 6.28**	**15.96 ± 2.22**
P*-Value	**HT vs DM**		**0.504**	**0.427**
P value <0.05 *Values are expressed as mean ± standard error (SE)*			* *Independent-samples T test*	

In the current results shown in **Table (4-12)**, we observed that the levels of C3 and C4 were insignificantly different between patients with hypertension and diabetes mellitus. As we mentioned above, CKD is an inflammatory condition that causes an increase in inflammatory biomarkers regardless of the presence of other related conditions such as diabetes or hypertension. As a result, no statistically significant differences were identified.

4.9.3. Biochemical parameter levels in UP patients based on hypertension and diabetes mellitus

The levels of urea and creatinine were found to be significantly different between patients with hypertension and diabetes mellitus (P = 0. 035 and 0. 014, respectively). The difference in phosphorus and albumin levels, on the other hand, were statistically insignificant (P = 0. 366 and 0. 672, respectively) as shown in **Table 4-13.**

Table 4-13: The level of biochemical parameters in patients with uremic pruritus depending on hypertension and diabetes mellitus.

Patients Groups (N=60)		N (60)	Urea (*mg/dl*) *(Mean ± SE)*	Creatinine (*mg/dl*) *(Mean ± SE)*	Phosphorus (*mg/dl*) *(Mean ± SE)*	Albumin (*g/L*) *(Mean ± SE)*
Hypertension (HT)		**20**	**129.80 ± 5.45**	**6.26 ± 0.34**	**5.01 ± 0.30**	**36.85 ± 2.64**
Diabetes Mellitus (DM)		**13**	**113.15 ± 4.08**	**5.00 ± 0.26**	**5.39 ± 0.21**	**35.00 ± 3.52**
P*-Value	**HT vs DM**		**0. 035**	**0. 014**	**0. 366**	**0. 672**
P value <0.05 *Values are expressed as mean ± standard error (SE)*				* *Independent-samples T test*		

The results of this study indicate that serum urea and creatinine levels were significantly increased in patients with hypertension as compared to those with diabetes mellitus **Table 4-13.** These findings were partially in agreement with Joshi *et al*., (2016), AL-Hamdani, (2010), and Jabary *et al*., (2006), who found there was a significant increase in the mean values of serum urea and creatinine in hypertensive patients as compared with control. In contrast, Jasim (2021) discovered no significant variation in urea and creatinine levels between HD patients with diabetes and those with other conditions such as hypertension.

Chronic hypertension may induce renal arterial stiffness (arteriosclerosis), sodium and fluid retention, inflammation, and abnormalities in endothelial function (e.g. mesangial expansion), which restrict the available filtration area within the glomerulus (Huan *et al*., 2015). This results in decreased blood flow to the glomerulus and reduced glomerular filtration rate (GFR), which subsequently leads to a lower distal tubular flow

rate and increases the reabsorption of urea and decreases its excretion, that might explain the higher serum urea concentration (Isra'a, 2010). Furthermore, an increase in serum creatinine levels may be associated with a reduction in creatinine clearance caused by a decrease in GFR (Sarkar *et al*., 2006).

As we previously mentioned, the quantity of creatinine produced each day is also influenced by the muscle mass. Thus, creatinine levels differ significantly between females and males, with lower creatinine levels in individuals with less muscle mass. The serum urea also increases in a state of dehydration, high dietary protein, as well as upper GI hemorrhage (Gounden *et al*., 2020; Pandya *et al*., 2016). This may explain the variance in urea and creatinine levels among research studies.

The results of this study also indicate that serum of phosphorus and albumin levels were insignificantly different between patients with hypertension and diabetes mellitus(P = 0. 366 and 0. 672, respectively) as shown in **Table 4-13**.

4.10. Correlation between interleukin-6 (IL-6), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α)

Bivariate pearson correlation was used to test the correlation between IL-6, IL-13, and TNF-α. The results showed that IL-6 had a direct correlation with IL-13 (r = 0.693**; P = < 0.001) and TNF-α (r = 0. 736**; P = < 0.001). Furthermore, there was also a statistically positive correlation between TNF-α and IL-13 (r = 0.875**; P = < 0.001), as shown in **Table 4-14.**

Table 4-14: Correlation between IL-6, IL-13, and TNF-α.

Correlations		IL-6	IL-13	TNF-α
IL-6	***Pearson Correlation (r)***	**1**	**0.693****	**0. 736****
	Sig. (2-tailed)		**0.001**	**0.001**
IL-13	***Pearson Correlation***	**0.693****	**1**	**0.875****
	Sig. (2-tailed)	**0.001**		**0.001**
TNF-α	***Pearson Correlation***	**0. 736****	**0.875****	**1**
	Sig. (2-tailed)	**0.001**	**0.001**	

*** Correlation is significant at the 0.01 level (2-tailed).*

These findings were consistent with those of Robak *et al*., (1996), who discovered a positive relationship between TNF-α and IL-6 in a study conducted in Poland. Lee *et al*., (2015) found that both TNF-α and IL-6 were elevated in patients with CKD. Furthermore, IL-6 production during inflammatory conditions and infections is induced

via stimulation of cells by IL-1 or TNF-α (Kang *et al*., 2019). Elevated levels of the two cytokines (TNF-α and IL-6) in CKD patients, as well as induction of IL-6 by TNF-α, might explain the correlation between these two cytokines.

As previously stated, IL-13 plays a critical role in immune and inflammatory responses, and it is mostly secreted by Th2 cells (Seyfizadeh *et al*., 2015). According to Derocq *et al*., (1994), IL-13 stimulates IL-6 production by human keratinocytes, although several reports suggest an association of IL-13 with proinflammatory and anti-inflammatory immune responses (Darkhal *et al*., 2015; Chalubinski *et al*., 2016; Chang *et al*., 2017). This might explain the correlation between IL-13 and both IL-6 and TNFα.

Martnez-Reyes *et al*., (2018), on the other hand, discovered no significant correlation between IL-13 and TNF-α in insulin-resistant patients.

Conclusions & Recommendations

6. Conclusions and Recommendations:

6.1. Conclusions

1. The viral infection rates of hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) among hemodialysis patients were as follows: 19 (21.1%) patients were HCV-positive, 1 (1.1%) patient was HBV-positive, and 0 (0.0%) was HIV-positive.

2. The serum interleukin-6 (IL-6), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α) levels increase significantly in hemodialysis patients with uremic pruritus as compared to healthy controls.

3. High IL-13 and TNF-α levels have a statistically significant correlation with the severity of pruritus.

4. IL-6 has a positive statistical relationship with both IL-13 and TNF-α, and there is also a direct relationship between TNF-α and IL-13.

5. The serum IL-6, IL-13, and TNF-α levels increase significantly with the increased period of hemodialysis.

6. The serum levels of complement 3 (C3) and complement 4 (C4) decrease significantly in hemodialysis patients with uremic pruritus as compared to healthy controls.

7. The serum levels of urea, creatinine, and phosphorus increase significantly in hemodialysis patients as compared to healthy controls, whereas albumin decreases in HD patients.

8. High serum creatinine has a statistically significant correlation with the severity of pruritus.

6.2. Recommendations

1. Further studies are needed to compare the serum level of interleukin-13 (IL-13), tumor necrosis factor-alpha (TNF-α), complement proteins, and kidney function parameters between hemodialysis patients with and without pruritus.

2. More investigations are necessary to evaluate the involvement of histamine, tryptase, mast cells, and basophils in uremic pruritus development.

3. Additional research is needed to assess serum interleukin-4 (IL-4), interleukin-10 (IL-10), and interferon gamma (IFN-γ) levels, as well as inflammatory biomarkers (C-reactive protein) and *β2*-microglobulin levels in patients with chronic kidney disease-associated pruritus (CKD-aP).

4. Evaluation of the possible role of anti-interleukin-6 (IL-6), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α) receptor antibodies in the treatment of severe pruritus.

5. Evaluation of the serum interleukin-6 (IL-6), interleukin-13 (IL-13), and tumor necrosis factor-alpha (TNF-α) levels, as well as complement 3 (C3) and complement 4 (C4) levels in non-hemodialysis chronic kidney disease (CKD) patients.

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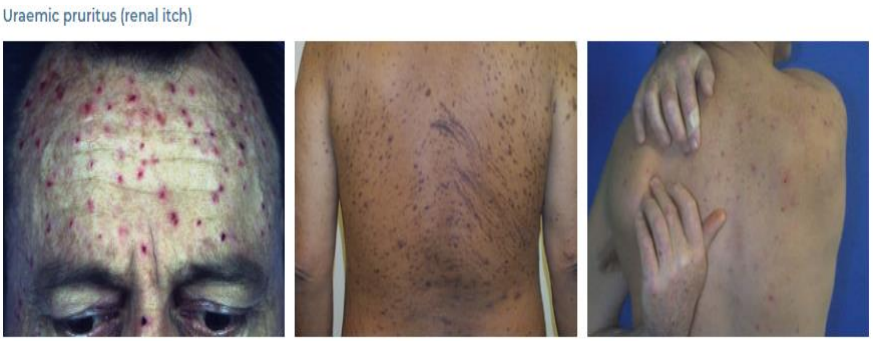
Appendices

Appendix (1): Questionnaire for Hemodialysis patients information.

- Case Number : Date: Name :
- Gender : ☐Male ☐Female Age :
- Chronic Diseases : ☐Heart Diseases ☐Diabetes ☐Others ☐None
- Duration of Dialysis: ☐ < 3 M ☐ < 1 Y ☐More than 1 Y
- How many sessions per weak do you receive haemodialysis ? …….
- How many hours are you treated for each session of haemodialysis ?.....
- Risk factor for Chronic Kidney Disease (CKD)?

☐ Hypertension (HT)
☐ Chronic Glomerulonephritis
☐ Interstitial Nephritis
☐ Polycystic Kidney disease
☐ Kidney Stone
☐ Diabetes Mellitus (DM)
☐ None



- Do you have itchy skin ? ☐Yes ☐No
- Risk Factor for Itching ?

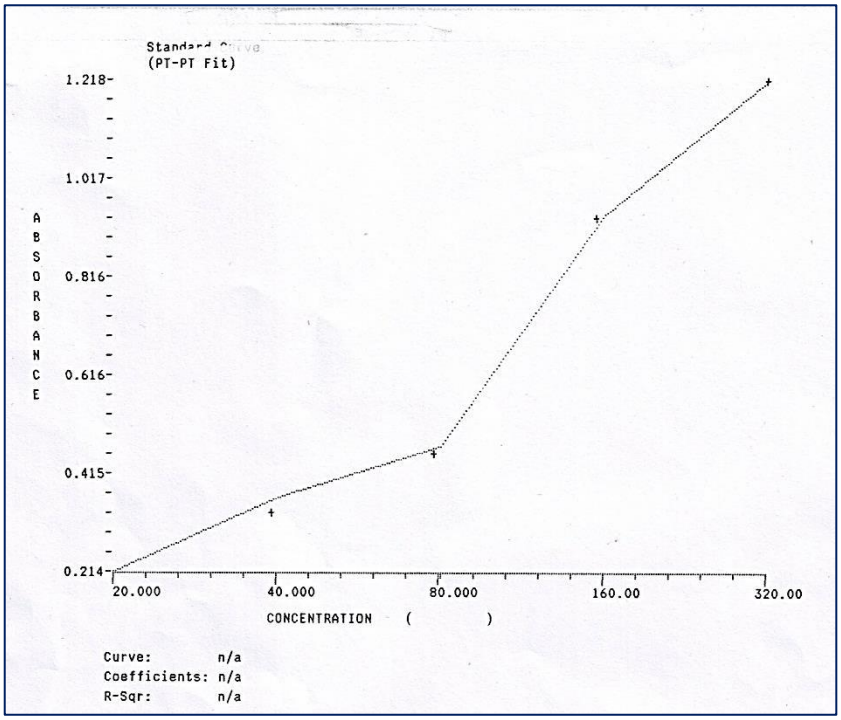
☐ Diabetes Mellitus (DM) ☐ Psoriasis ☐Hepatitis ☐ None

- When did the itching start ? ☐Pre-dialysis ☐Post-dialysis
- What Type of itching ? ☐Localize ☐Generalize
- Severity of itching ? ☐Mild ☐Moderate ☐Sever
- Type of medications that use? ☐Drug ☐Ointment ☐None
- Dermatological opinions ? ☐Uremic related ☐Non-Uremic related

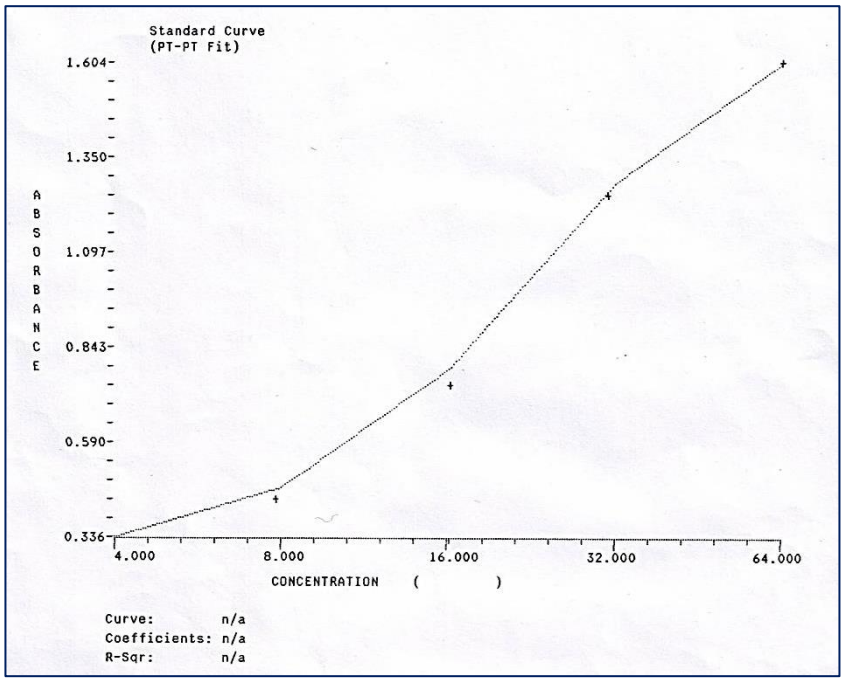
Appendix (2): Ethical Approval.

The hospital administrators have given their valid consent. All participants and health-care providers have also been informed about the study's purpose. The participants' participation in the study was entirely voluntary, and they have the right to withdraw at any time, for any reason, and without affecting their health or care. Data was coded to ensure confidentiality, and participants were informed that the information collected would only be used for research purposes, and oral consent was obtained.

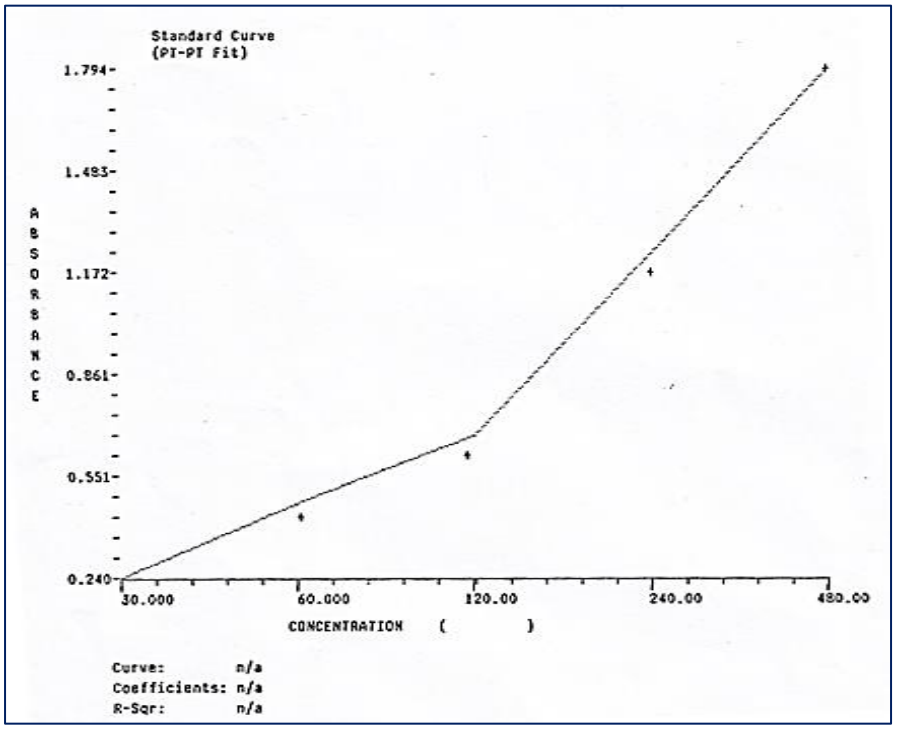
Appendix (3): Interleukin 6 (IL-6) standard curve.



Appendix (4): Interleukin 13 (IL-13) standard curve.



Appendix (5): Tumor necrosis factor-alpha (TNF-α) standard curve.



الخلاصة

الخـــلاصـــة

الحكة اليوريمية (UP) هي مشكلة شائعة، مزعجة، وفي بعض الأحيان تكون منهكة للأشخاص الذين يعانون من مرض الكلى المزمن (CKD) والفشل الكلوي في مراحله النهائية (ESRD) وكذلك أولئك الذين يخضعون لغسيل الكلى الدموي (Hemodialysis) أو البريتوني (Peritoneal Dialysis). آلية نشوء وتطور الحكة اليوريمية غير مفهومة تماما الى الان وقد تم اقتراح العديد من الفرضيات فيما يتعلق بتطور هذا المرض.

تهدف هذه الدراسة للتحري عن انتشار فيروسات التهاب الكبد B و C (HBV, HCV)، الى جانب فيروس نقص المناعة البشرية (HIV)، و لتقييم مستوى الـ إنترلوكين 6 (IL- 6)، إنترلوكين 13 (IL- 13)، عامل نخر الورم ألفا (TNF-α)، المتمم 3 (C3)، والمتمم 4 (C4)، بالإضافة إلى بعض المؤشرات الكيموحيوية لدى المرضى الخاضعين للغسيل الكلوي والمصابين بالحكة اليوريمية.

أجريت هذه الدراسة في الفترة ما بين 4 أكتوبر 2021 و 5 مارس 2022 على 90 شخصا من المرضى الخاضعين للغسيل الكلوي الدموي والذين يعانون من الحكة اليوريمية وعلى 30 من الأشخاص الأصحاء (السيطرة) في مركز ابن سينا للغسيل الكلوي في مستشفى بعقوبة التعليمي. تم تقييم شدة الحكة وقياسها باستخدام مقياس تناظري بصري (VAS). حيث، طُلب من المريض تحديد شدة الحكة التي يشعر بها على خط أفقي بطول 10 سم (1-2 حكة خفيفة، 3-7 حكة متوسطة، 8-10 حكة شديدة). بعد ذلك، تم سحب ما يقرب الـ 4 مل من دم المرضى الذين يعانون الحكة اليوريمية خلال الدقائق العشر الأولى من بدأ عملية الغسيل الكلوي، وتم الحصول على نفس كمية الدم من الأشخاص الأصحاء في ظل ظروف معقمة تمامًا. تم فصل عينات الدم باستخدام جهاز الطرد المركزي وقسمت الأمصال التي تم الحصول عليها من كل عينة إلى أربع أنابيب إيبندورف وخزنت عند درجة حرارة (20−) درجة مئوية لتحليلها لاحقًا للكشف عن الـ HBV، HCV، وHIV بالإضافة إلى قياس مستوى الـ IL- 6، IL- 13،

TNF- α، C3، و C4 باستخدام تقنيتين الممتز المناعي المرتبط بالأنزيم (ELISA) والانتشار المناعي الشعاعي (RID). تم أخذ الموافقة المسبقة من جميع المرضى الخاضعين للغسيل الكلوي قبل إجراء الدراسة.

من مجموع 90 مريضا خاضعا للغسل الكلوي، 19 (21.1 %) أعطوا نتائج إيجابية لفيروس التهاب الكبد C (HCV)، 1 (1.1%) أعطوا نتائج إيجابية لفيروس التهاب الكبد B (HBV)، و0 (0.0 %) أعطوا نتيجة إيجابية لفيروس نقص المناعة البشرية (HIV). تم استبعاد عينات المرضى الذين ثبتت إصابتهم بفيروس التهاب الكبد B (HBV) و فيروس التهاب الكبد C (HCV) وكذلك مرضى السرطان واضطرابات المناعة الذاتية النشطة وأمراض الجلد (مثل التهاب الجلد والصدفية) والمرضى الذين تقل أعمارهم عن 20 عامًا، البالغ عددهم حوالي 30 مريضًا من اختبارات السيتوكينات وبروتينات المتمم الإضافية.

كشفت نتائج دراستنا أن مستويات الـ IL-6، IL-13، و TNF-α في الدم كانت أعلى بشكل ملحوظ لدى المرضى الخاضعين للغسيل الكلوي الذين يعانون من الحكة اليوريمية (95.67 ± 11.91، 16.11 ± 2.28، 148.16 ± 15.39 بيكوغرام/ مل لتر، على التوالي) مقارنة بالأشخاص الأصحاء (Control) (37.82 ± 2.38، 5.80 ± 0.26، 65.11 ± 2.98 بيكوغرام/ مل لتر، على التوالي) (*P* تساوي 0.001) . علاوة على ذلك، كان لارتفاع المستوى المصلي لـ IL-13 و TNF-α علاقة ذات دلالة إحصائية مع شدة الحكة (P تساوي 0.001، 0.003 على التوالي)، بينما لم يكن لمستويات الـ IL-6 أي علاقة ذات دلالة إحصائية مع شدة الحكة (*P* تساوي 0.249).

وفقا لفترة الغسل الكلوي (أكثر من سنة، أقل من سنة، أقل من ثلاثة أشهر)، وجد أن مستويات الـ IL-6، IL-13، و TNF-α تزداد بشكل ملحوظ مع زيادة فترة الغسل الكلوي (*P* تساوي 0.026، 0.046، 0.006 على التوالي).

تم استخدام ارتباط بيرسون ثنائي المتغير لاختبار الارتباط بين الـ IL-6، IL-13، و TNF-α. أظهرت النتائج بأن الـ IL-6 يمتلك علاقة طردية مع IL-13 (r = **0.693: P تساوي < 0.001) و TNF-α (r = 0. **736: P تساوي < 0.001). علاوة على ذلك، كان هناك ايضا علاقة ايجابية ذات دلالة إحصائية بين الـ TNF-α و الـ IL-13 (r = **0. 875: P تساوي < 0.001).

تشير نتائج هذه الدراسة أيضًا إلى أن مستويات الـ C3 و C4 في المصل كانت منخفضة بشكل ملحوظ لدى المرضى الخاضعين للغسيل الكلوي الذين يعانون من الحكة اليوريمية (57.83 ± 2.91، 14.59 ± 0.88 مليغرام/ديسيلتر، على التوالي) مقارنة بالأصحاء (Control) (110.76 ± 2.20، 32.02 ± 1.09 مليغرام/ديسيلتر، على التوالي) (P تساوي 0.001).

في الختام، أظهرت نتائجنا ان مستويات الـ يوريا، كرياتينين، والفسفور ازدادت بشكل ملحوظ في دم مرضى الغسيل الكلوي الذين يعانون من الحكة اليوريمية (120.95 ± 2.56، 5.27 ± 0.18، 4.98 ± 0.15 مليغرام/ديسيلتر، على التوالي) مقارنة بالأصحاء (Control) (22.16 ± 0.77، 0.46 ± 0.03، 3.21 ± 0.10 مليغرام/ديسيلتر، على التوالي) (P تساوي 0.001). في المقابل، انخفض مستوى الالبومين بشكل ملحوظ لدى المرضى (36.98 ± 1.68 غرام/لتر) مقارنة بالأصحاء (40.96 ± 0.67 غرام/لتر) (P تساوي 0.03). علاوة على ذلك، كان لارتفاع الكرياتينين في الدم علاقة ذات دلالة إحصائية مع شدة الحكة (P تساوي 0.029)، بينما لم يكن لليوريا والفوسفور والألبومين أي علاقة ذات دلالة إحصائية مع شدة الحكة (P تساوي 0.546، 0.594، 0.631).

جمهورية العراق

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قسم علوم الحياة

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صفاء شهاب احمد الزيـــدي

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