ACUTE TUBULO-INTERSTITIAL NEPHRITIS

Clinical Profile and Pathogenic Mechanisms

By

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ABSTRACT:

Acute tubulointerstitial nephritis (ATIN) is an important cause of renal morbidity. This study showed that it represents up to 8% of acute renal failure where biopsy material was available and accounted for 1% of all renal biopsy material. This study, which is believed to be the largest single retrospective study carried out to date, consists of 78 cases over nineteen years (1984-2002). Forty one cases were males and 37 were females.

Acute tubulointerstitial nephritis (ATIN) was divided into three groups according to the cause: drug-induced ATIN, idiopathic ATIN and TINU syndrome. Drug-induced ATIN has come to dominate this area of medicine and in this study it represented 85% of all the cases of ATIN. Comparing the creatinine level at different time points among the the diagnosis groups shows that the creatinine level at presentation was high in patients with drug-induced ATIN compared to patients with TINU syndrome or idiopathic ATIN and the P value was 0.020. Comparison of clinical features investigations with the reversibility of renal function (cr level < 150μmol/l) shows that patients with fever, normal or high haemoglobin level, normal or low potassium level, and normal or low level of phosphate tended to have reversible renal function with P values of 0.021, 0.018, 0.002 and 0.03 respectively. Comparing the result of investigations between the different diagnosis groups showed that the lymphocyte count tended to be lower in drug-induced ATIN and TINU syndrome than in idiopathic acute tubulointerstitial nephritis and this difference was statistically significant (P value = 0.026).

By one year follow-up, 75% of patients had an improvement in renal function (creatinine level < 150 μmol/l). Comparing the outcome for renal function among the different diagnostic groups showed a significant statistical difference (P values 0.017-

0.020). ATIN due to non-steroidal anti-inflammatory drugs carried a bad prognosis in comparison to other groups.

Histologically, sections of ATIN tissue biopsies showed a strong staining for CD3+ cells, CD4+ cells, CD8+ cells, CD68+ cells, eotaxin, CCR3, VCAM-1, IL-4 and eosinophil proteins. These results suggest that there is a Th 2 type of inflammatory and immune response in acute tubulointerstitial nephritis. Comparison between the infiltrating cells showed no significant difference (P values were between 0.549 and 1.00). Comparison between the infiltrating cells and the renal function outcome shows no relationship. On the other hand, there was a correlation between the CD68 positive cells and the creatinine level at presentation which indicated that there was a tendency for a greater CD68 positive cell infiltration to be associated with a higher creatinine level at presentation, and the P value was 0.003 ($r = 0.651$).

Comparison between the index of chronic damage and the reversibility of renal function shows a significant relationship at three months and one year time and the P values were 0.002 and 0.001 respectively (low index of chronic damage associated with low creatinine level).

This study also showed no relationship between idiopathic acute tubulointerstitial nephritis and Epstein-Barr virus.

Conclusion:

This study confirms that ATIN remains an important cause of acute renal failure, that is predominantly drug-related, and that renal biopsy has diagnostic and prognostic significance. Immunological mechanisms are important in pathogenesis with macrophage-dependent processes correlating with renal function at presentation. Prognosis is good providing diagnosis and therapeutic intervention occurs before irreversible chronic damage has developed.

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Chapter 1:

General introduction

1.1 Acute Tubulo-interstitial Nephritis

1.1.1 Definition:

Acute tubulo-interstitial nephritis (ATIN) is a disease characterised by predominant involvement of the renal interstitium and tubules by inflammatory cells, often associated with oedema or fibrosis and tubular atrophy. Interstitial nephritis is commonly accompanied by variable tubular damage, so the term tubulo-interstitial nephritis, or tubulo-interstitial nephropathy, is preferable and is often used interchangeably with interstitial nephritis. Tubulitis refers to infiltration of the tubular epithelium by leukocytes, usually lymphocytes.

Primary tubulo-interstitial nephritis denotes inflammation that is limited to tubules and interstitium: glomeruli and vessels are uninvolved or show minor changes.
Secondary tubulo-interstitial nephritis denotes tubulo-interstitial inflammation associated with a primary glomerular or vascular disease(1).

In the studies to be described here, all of the renal biopsies were reviewed by a single pathologist who applied definitions as described in the material and methods chapter.

1.1.2-Importance of interstitial changes in renal function:

From the literature, it is suggested that changes in the interstitium are a final common pathway in all types of end-stage renal disease and are the most common and pivotal lesion in nephrology, particularly as they can occur as a primary process and also as a secondary process following glomerular or vascular diseases (9).

Different morphologic observations strongly suggest that changes in tubular function and glomerular filtration correlate significantly with the presence of progressive deterioration of the interstitial tissues, rather than with changes in glomerular tuft integrity

(2-5). Many explanations have been proposed to describe this structure-function correlation: the “clogged drain” theory, capillary bed changes, and the vascular tone hypotheses. First, filtrate delivered by an intact glomerular tuft cannot pass into a collecting apparatus through tubules that are disrupted by inflammatory infiltrates, thus the name “clogged drain”. Second, a continuous decline in tubulo-vascular capillary surface area, as might occur in tubulointerstitial nephritis, might provoke an increase in resistance in the efferent arterioles. This could lead to outflow restriction from the glomeruli by decreasing circulatory capacity, such that filtration would reduce (2, 6, 7). Third, in the vascular tone hypothesis, with moderate inflammation in the interstitium and some tubular atrophy, a minimum amount of sodium might be actively pumped out of the proximal tubule and thick ascending limb, and so the osmotic gradient in the interstitium may not adequately build-up. Therefore, very little water would be removed from the tubular fluid, polyuria would develop, and the increase in tubular flow rate would reduce the secretion of renin from the juxtaglomerular apparatus. Thus, the content of angiotensin 2 would decrease in the efferent arteriole. The net effect of these events would be that the tone in the efferent arteriole would reduce, with a consequent fall in the glomerular filtration rate. This outcome is known as a modified Thurau mechanism, whereby glomerular filtration adjusts to insufficient tubular function (2, 8).

1.1.3 History:

Councilman was the first to describe acute non purulent tubulointerstitial nephritis (ATIN) in 1898. Volhard and Fahr included tubulointerstitial lesions in their classification of renal diseases in 1914. In the pre-antibiotic era, ATIN was observed in connection with scarlet fever or diphtheria. In 1946, More et al, described tubulointerstitial lesions from

pharmaceutical drugs. Between 1971 and 1974, Klassen, Mc Cluskey, Milgrom, Steblay, Andres, Wilson and Rudofsky reported that tubulointerstitial nephritis could be mediated by the immune system. At the present time, there is a growing knowledge of tubulointerstitial antigens, nephrogenic T-cells and fibrogenic processes (9).

1.1.4 Epidemiology:

As the clinical features of ATIN are not specific and there is a need for renal biopsy for diagnosis, the precise assessment of incidence and prevalence is complex. In the literature, the only study to look at the prevalence of ATIN was reported by Pattersson et al (10). Examining 314,000 military recruits in the Finnish army who had haematuria and /or proteinuria, 174 underwent a renal biopsy. However, only two were found to have ATIN, giving a prevalence of 7 per million in this cohort (10).

In any renal unit, ATIN is present in 1% to 3% of all renal biopsies (11-13), with drug-related ATIN being found in nearly 50% these cases.

In groups of patients being biopsed to determine a cause for their acute renal failure, the incidence of ATIN was 8% to 14% (14-16), and in the majority the diagnosis was not suspected before biopsy**.**

1.1.5 Aetiology of acute tubulo- interstitial nephritis:

The commonest cause of ATIN in most series is drugs. Tinu syndrome (the tubulointerstitial nephritis and uveitis syndrome) is a well-recognized but uncommon cause of ATIN. Infectious agents have sporadically been associated with ATIN (see table.1) (17) but whether these are true associations, or related to therapy, is uncertain. Of note, the Epstein Barr virus is given as a cause of ATIN but, as will be described in the present study, it has not been substantiated as a cause of ATIN. Systemic diseases such as systemic

lupus erythematosus, vasculitis and acute post-infectious glomerulonephritis may be associated with ATIN but as these conditions occur with a well-defined glomerular lesion, they have not been included in the present study. Occasionally, there is no overt reason for development of ATIN and when a cause cannot be clearly attributed, the term "idiopathic" (i.e unknown aetiology) is used.

Table 1-Infections associated with acute tubulo- interstitial nephritis- adapted from (17)

Bacteria	Spirochaetes	Rickettsia	Viruses	Others
-Streptococci -Diphtheria -Pneumococci -Brucellosis -Legionella -Tuberculosis -Typhoid fever -Yersinia pseudo-tuberculosis Enterobacteriaceae	-Syphilis -Leptospirosis	-Rocky Mountain fever -Mediterranean spotted fever	-Human immunodeficiency virus -Cytomegalovirus -Epstein-Barr virus -Hantavirus -Measles -Echovirus -Coxsackie virus -Adenovirus -Mumps -Influenza -Herpes simplex -Hepatitis A -Hepatitis B	Toxoplasma Mycoplasma Leishmania Chlamydia infection

1.1.6:-Clinical presentation and diagnosis of ATIN:

ATIN is a heterogeneous disorder not only in aetiology, but also in presentation, laboratory findings and outcomes. The diagnosis is most commonly considered in hospitalized patients who experience a progressive rise in serum creatinine. The aetiology of acute renal failure in such patients is frequently unclear, especially if they are infected, receiving multiple medications, undergoing diuresis, and/or exhibiting haemodynamic instability. In such complex settings, ATIN is frequently placed low in the differential diagnosis of acute renal failure if there is no concomitant fever, skin rash, eosinophilia, or eosinophiluria. Although these accompanying signs suggest ATIN when present, their absence is not helpful in excluding the diagnosis.

The triad of fever, rash, and eosinophilia is generally found in less than 30% of patients with ATIN (18). These allergic manifestations are commonest in beta-lactam associated ATIN than in other drug-related causes of ATIN (16, 19).The reduced output of urine is an inconstant finding and urinalysis commonly shows mild to moderate proteinuria with an increased number of red and white blood cells. Nephrotic range of proteinuria is rare and is only found in ATIN due to non-steroidal anti-inflammatory drugs, when it is usually associated with minimal change glomerulonephropathy (20, 21). Eosinophilia and eosinophiluria are variable findings and they tend to support the diagnosis of allergic, drug-induced ATIN, when present. The published frequency of eosinophiluria (it is not often tested for) varies according to the criteria used for the definition of eosinophiluria and the method used for staining of urinary leukocytes, as it ranges from 40% to 100% (22). Hansel's stain is approximately four times more sensitive than Wright's stain (23). However, it has been suggested that the presence of eosinophiluria is relatively specific and may be diagnostic for ATIN (23). In one study where patients with a

confirmed diagnosis of ATIN were compared to those with other causes of renal disease, eosinophiluria was found to have a sensitivity of 40%, specificity of 72%, but a positive predictive value of only 30% (24). In fact eosinophiluria can be found in chronic tubulointerstitial nephritis of different aetiologies, in transplant rejection, prostatitis and in eosinophilic cystitis (23, 25), so it is not specific for ATIN and its absence does not reject the probability of this type of nephritis. All the evidence considered, eosinophiluria is not a definitive test for the diagnosis of ATIN (24).

Ultrasound imaging of patients with ATIN shows a normal to enlarged kidney with increased cortical echogenicity similar to, or higher than, that of the liver (22) and corresponds with the extent of interstitial infiltration in the biopsy (26). There are no specific characteristics on ultrasonic imaging that differentiate ATIN from other causes of acute renal failure. The renal biopsy (preferably done under ultrasound guidance) is the only tool for a firm diagnosis.

1.2 Anatomy of the renal interstitium

The renal intertitium is scant in the cortex and outer stripe of the outer medulla but becomes more abundant in the inner stripe and inner medulla. The cortical and medullary interstitium can be considered separately. In the cortex, the peritubular interstitium must be distinguished from the peri-arterial spaces, in which lymphatics are present (27).

1.2.1 Cortical Interstitium:

The fractional volume of the cortical interstitium in human kidneys has been calculated to range from 5% to 37%, with a tendency to increase with age (28). A subdivision is generally made between the peri-tubular interstitium surrounding the tubules

and capillaries and the peri-arterial interstitium, which accompanies the intra-renal arteries and contains the lymphatics. The two are continuous with each other. In the peri-tubular interstitium of healthy kidneys, the majority of interstitial cells are fibroblasts and dendritic cells, where macrophages and lymphocytes are almost absent. The close spatial association of fibroblasts and dendritic cells is striking so that portions of dendritic cells may be almost completely enclosed by fibroblasts. The two cell types may be distinguished at the light microscopic level by immunocytochemistry.

The peri-arterial interstitium is a loose layer of connective tissue surrounding the intra-renal arteries to their termination as afferent arterioles at the glomerulus. The renal veins are next to this sheath but not included in it (29). The scaffold of the peri-arterial connective tissue sheath is constituted of fibroblasts with extremely attenuated cell processes that form large, loose meshes filled with bundles of collagenous fibres and large quantities of ground substance. The peri-arterial fibroblasts may contain α-smooth-muscle actin and intermediate filaments of the vimentin type (30), but they lack the enzyme ecto-5'-nucleotidase (5'NT) that is important metabolizing cAMP to adenosine (31).

1.2.1.1 Fibroblasts:

Fibroblasts are the central cells in the renal interstitium. They are interconnected by morphologically specialized contacts (32) and they adhere to the basement membranes that surround the tubules, the glomeruli, and the capillaries. They are in close touch with lymphatics, nerve terminals, and all types of migrating interstitial cells.

Renal fibroblasts are similar in shape and ultra structure to fibroblasts in the interstitium of other organs (33).Their nuclear profiles are often stellate, surrounded by a thin rim of cytoplasm devoid of cell organelles. The attenuated cytoplasmic processes extend far from the perikaryon and often penetrate the narrow space between adjacent basement

membranes of tubules and capillaries. Fibroblasts have an extensive protein synthesis apparatus, with abundant rough endoplasmic reticulum (ER) and free ribosomes. The presence of the golgi complex in the processes points to the release of procollagen into the extracellular space at these sites (34). Lysosomes are rarely observed under control conditions. Phenotypic changes in fibroblasts, which may be reversible, can occur *in vivo* under the influence of environmental factors, such as altered extracellular matrix composition, cytokines, and growth factors (34-38).

Cortical (peri-tubular) fibroblasts exhibit the enzyme ecto-5'-nucleotidase (5'NT). The activity of this enzyme in cortical fibroblasts shows regional variation. Normally, the activity of the enzyme is highest in fibroblasts in the deep cortex (39, 40), but under anaemic conditions, the activity of the enzyme is also strongly up-regulated in fibroblasts in the superficial cortex and, to a lesser extent, in the medullary rays (41-43). This finding has suggested a link between the expression of 5' NT and the synthesis of erythropoietin (40, 44). Indeed, co-localization studies of 5'NT and erythropoietin mRNA have demonstrated that the 5'NT-positive fibroblasts synthesize renal erythropoietin (45, 46).

Myofibroblasts abound under pathologic conditions, for example, in inflammation and interstitial fibrosis (34, 47). Myofibroblasts display an increase in α-smooth-muscle actin, a nucleus with numerous indentations, and an increased amount of cytoplasm around the nucleus containing many organelles (35); they are coupled by gap junctions (37). The most evident function of fibroblasts is the production and modelling of extracellular material, such as collagenous and non collagenous fibres and ground substance (34). The modulation of that synthetic function by cytokines, secreted by immune, epithelial, and endothelial cells in the local environment of fibroblasts, accounts in part for the increase in deposition of matrix during inflammatory processes (34, 48-50).

Several fibres make up the interstitial reticulum, among them, typical interstitial collagenous fibres (types I, III, VI) (51). Type III fibres correspond to the classically described reticular fibres that form a network enveloping individual tubules. In addition, microfibrils clearly identified by an electron-lucent core and a diameter of ~15 to 30 nm are found in the renal interstitium (52).

Proteoglycans are an important component of the interstitial matrix in the kidney, especially in the medulla. As elsewhere in the body, various glycoproteins (fibronectin, laminin, and others) are found in association with tubular basement membranes, connecting them to cell membranes as well as to fibrillar structures of the interstitial matrix.

1.2.1.2 Cells of the immune system and lymphatic system within the normal tubulointerstitium:

Monocytes/macrophages, dendritic cells and lymphocytes are known to be present in the interstitium of healthy kidneys (28, 53, 54).

Macrophages differ from dendritic cells (31, 55, 56). Macrophages have a marked capacity for phagocytosis, which is the basis for their most important and most specific functions: antimicrobial activity, clearance of damaged host cells, and tumouricidal activity. Major histocompatibility complex (MHC) class II, which is not constitutively expressed by macrophages, may be up-regulated by pro-inflammatory cytokines. Activated macrophages have a large cell body with plump, short processes and numerous surface folds. Their prominent lysosomal apparatus distinguishes them from other immune cells.

There is usually a relatively large number of macrophages around the intra-renal arteries which may be due to the fact that the peri-arterial interstitium is linked to the connective tissue of the pelvic wall. This continuity establishes a pathway for retrograde

infection of the renal cortex. Intra-renal nerve fibres and lymphatics are also embedded in the peri-arterial tissue (29, 57, 58). Lymphatics begin near to the afferent arteriole and leave the kidney running within the peri-arterial tissue sheath toward the hilum. Interstitial fluid can also drain through the peri-arterial or peri-tubular tissues to eventually reach the lymphatics (29). These pathways may be important to the systemic distribution of substances that are released into the peri-tubular interstitium, such as renin (29), or other hormones, such as erythropoietin, as well as to a variety of vasoactive substances.

Dendritic cells share several functional characteristics with macrophages. However, they constitute a distinctive cell lineage of bone marrow origin (59, 60). They are "professional" antigen-presenting cells and are able to take up antigens and to process them to short peptides, which can be presented to T cells in the context of MHC class Π. Their constitutively high expression of MHC class Π, their expression of co-stimulatory molecules, and their strong tendency for recruitment to lymphatic organs explain why dendritic cells are potent stimulators of naïve T cells (61). As in other peripheral tissues, dendritic cells turn over rapidly in the kidney (62). They exit the organs mainly via lymphatics and enter lymphatic organs, where antigen presentation takes place (61).

In the healthy kidney, there are usually many more dendritic cells than macrophages (63) and, as previously discussed, they are mainly in the peri-tubular interstitium in close proximity to fibroblasts. In marked contrast to macrophages, dendritic cells in healthy renal interstitium lack the prominent lysosomal apparatus. Renal dendritic cells and fibroblasts are difficult to distinguish on a morphological basis, because both may show a stellate cellular shape and display substantial amounts of mitochondria and endoplasmic reticulum. The nuclei of dendritic cells are rounded, often with a deep indentation. In contrast to fibroblasts, the major cell organelles in dendritic cells are

located in the cell cytoplasm around the nucleus, and the cell processes are highly branched. Also in contrast to fibroblasts, dendritic cells lack a layer of actin filaments beneath the plasma membrane and do not have structurally defined attachments to the basement membranes of tubules and vessels or junctional complexes. However, contacts to fibroblasts and other interstitial cells do exist.

Lymphocytes make up several functionally well-defined classes, such as cytotoxic killer cells and T helper cells. In the interstitium of healthy kidneys, lymphocytes are rare. They can generally be distinguished on the basis of their usually round nuclei, displaying extensive heterochromation condensation and a low number of cell organelles.

1.2.2 Medullary interstitium:

The inner medullary interstitium is rich in acidic mucosubstances, which stain with cationic dyes and lectins in a characteristic manner with variations observed in different states of hydration (64). The cellular composition of the medullary interstitium is similar to that in the cortex. The number of dendritic cells that express MHC class Π shows regional variations and it is the highest in the inner stripe; at deeper levels of the inner medulla, immune cells are no longer detectable. Fibroblasts in the entire medulla show the same basic characteristics as in the renal cortex, namely, attachment of their processes to the basement membrane of tubules and vessels, interconnections by specific junctions (65), high quantities of rough ER, bundles of actin filaments (α-smooth-muscle actin) under the plasma membrane, and intermediate filaments. In contrast to cortical peri-tubular fibroblasts, medullary fibroblasts do not express 5'NT. Within the inner stripe and the inner medulla, actin and vimentin filaments in fibroblasts become increasing prominent. The cisterns of the rough ER and the peri-nuclear cisterns become very widened and densely filled with flocculent material. Within the inner medulla, the interstitial cells are

oriented strictly perpendicular to the longitudinal axis of the tubules and vessels. They anchor to the basement membrane of loops of Henle and vasa recta. Because of their conspicuous numbers of lipid droplets, the inner medullary interstitial fibroblasts can usually be distinguished from those in the other renal zones; these lipid-laden interstitial cells (54, 66-68) are a specific population of fibroblasts (28, 69). Fibroblasts in the inner medulla produce large amounts of glycosaminoglycans, which are particularly abundant in the inner medullary interstitium (68). They also produce vasoactive lipids, in particular prostaglandin (PG) E_2 (70-72). These may affect not only the renomedullary hemodynamics but also, in an autocrine pathway, the contractile tone of the interstitial cells themselves (73).

1.3 Animal experimental models of acute tubulointerstitial nephritis

Most of what we know about the pathogenesis of ATIN has come from experimental work in animal model systems (74, 75).

Various experimental models, especially in rodents, have been devised to induce tubulointerstitial damage, but only a few mimic human ATIN. The available models have been divided based on immunofluorescence findings as this corresponds to some extent with what is observed in the human disease. The use of immunofluorescence allows greater discrimination, whatever the histological appearance of interstitial infiltrates on light microscopy. The following patterns are recognised: no significant immune deposits in the interstitium and along the tubular basement membrane (equates to the majority of human cases); the linear fixation of IgG along that membrane (antitubular basement membrane disease which is rare in man); granular deposits of immunoglobulins and complement fractions along the tubular membrane and in the interstitium (also rare in man) (74).

1.3.1 Anti-tubular basement membrane disease:-

Anti-tubular basement membrane (TBM) disease with interstitial nephritis is the most widely studied model system of ATIN and represents an antigen-driven model of interstitial nephritis. Anti-TBM disease is induced by immunizing susceptible strains of rodents or guinea pigs with a renal tubular antigen preparation emulsified in complete Freund's adjuvant. This immunization process results in the activation and differentiation of antigen-specific T and B cells within the host, within 7 to 10 days after immunization, and antigen-specific IgG is detectable both within the circulation and as a linearly deposited antibody along the TBM. At these early time points, there may be a diffuse infiltrate of neutrophils within the interstitium as well. Functional studies performed at this time demonstrate a depressed single nephron GFR. By 2 to 3 weeks after immunization (in the rat), the neutrophils within the interstitium have been replaced with a largely mononuclear cell infiltrate, comprised of T cells, B cells, plasma cells, macrophages, and natural killer cells. At this time point, GFR is significantly depressed. At these later time points, irreversible injury to the tubulointerstitium results from a combination of antigen-specific and non-specific effector mechanisms (76).

The disease is not transferred by immune cells but with antibody, suggesting a clear role for antibodies against TBM in the pathogenesis (77). This was confirmed by injecting a heterologous anti-idiotypic antibody preparation which abolished the antibody production to the TBM, and led to a reduction in tubulointerstitial damage. Also, in a guinea-pig model where cobra-venom factor was used to deplete complement levels, there was inhibition of the tubulointerstitial disease, suggesting a role for complement. In other experiments, giving cyclophosphamide (which depletes lymphocytes, particularly B cells) early in the course of disease and in high daily dose can prevent tubulointerstitial nephritis.

When given to animals with prominent disease, cyclophosphamide slowed progression (78). Cyclosporin A or a stable analogue of prostaglandin E1 also reduced the anti-TBM antibody production and lessened the tubulointerstitial damage (79, 80).

Anti-TBM disease that develops in mice is different from that in guinea-pigs and rats since it has a late onset and there is a prominent cell-mediated immune response as part of the injury process. In mice, the effector cells can be CD4+ and class II MHC restricted or CD8+ and class I MHC-restricted. Effector T cells can generate injury by two general mechanisms: a delayed-type hypersensitivity reaction with release of lymphokines and other mediators, or by cell-mediated cytotoxicity against a target by releasing proteases called perforins (81).

Several target antigens of anti-TBM disease have now been identified. The first was a glycoprotein called 3M-1, of 48KDa, secreted by proximal tubular cells and attached to the outer surface of the TBM (82). Two other glycoproteins of 54 and 58KDa, reacting with anti-TBM antibodies, have also been isolated (83). Normal individuals who express the 3M-1 antigen are immunologically tolerant to this tubular molecule. However, this tolerance can be impaired by treating normal surveillance systems with immunosuppressive drugs, by introducing a foreign antigen which cross-reacts with a self-structure or by creating a neo-antigen. The formation of a hapten-carrier conjugate may be responsible for the production of an antibody to TBM in some cases of human ATIN related to drugs, especially methicillin (84), cephalothin, phenytoin (85), and possibly allopurinol (86).

Evidence for an antibody to TBM is lacking in the great majority of human cases of acute tubulointerstitial nephritis. Moreover, no correlation has been found between titres of circulating antibodies against this membrane and disease activity (87).

1.3.2 Immune complex-mediated tubulointerstitial disease:

Granular deposits of immunoglobulin and complement along the TBM and in the interstitium characterize this group of tubulointerstitial diseases (17). Several experimental models have been developed, triggered by either heterologous or autologous antigen. None results in ATIN but all produce interstitial infiltrates (17).

Tubulo-interstitial lesions with IgG and C3 deposits along the TBM can be produced in rabbits using different patterns of immunization (9, 88). Granular deposits along the TBM together with tubulointerstitial lesions are also found in late phases of autologous Heymann nephritis in the rat (9, 88).

A similar model of tubulointerstitial disease has been developed in rats by immunization with Tamm-Horsfall protein (89). The rats developed autoantibodies to the protein; the immune deposits appear to be a consequence of in-situ immune-complex formation (17).

True ATIN can induced in mice by challenging the appropriate hapten-carrier antigen with specific antibody (90). In this model, humoral reactions of immune-complex formation appear to play a major part in inducing the renal lesions, and these reactions may be relevant to some types of drug-induced ATIN in man (17).

1.3.3 Cell-mediated tubulointerstitial disease:

This type of ATIN is characterized by the presence of mononuclear-cell infiltrates in the absence of significant deposits (either linear or granular) along the TBM. Such a pattern is the most common form of human tubulointerstitial nephritis. Two main types of injury are recognized: a delayed-type hypersensitivity reaction, involving mainly macrophages and CD4+ T lymphocytes; and direct T-cell cytotoxicity involving mainly CD8+ T lymphocytes (17).

With regard to autologous or homologous antigens, several models of tubulo-interstitial nephritis have been raised in Lewis rats by immunization with Lewis or Brown-Norway renal basement membranes (91, 92). In Bannister's model, Lewis rats were immunised with renal basement membranes prepared from a number of rat strains except Lewis and Wistar-Furth rats, which are TBM antigen-negative (92). Nodular tubulo-interstitial nephritis with granuloma-like lesions was found in the cortex and the outer medulla. The rats developed renal insufficiency. The disease was easily transferred to normal rats by cells from lymph nodes of the immunized donors (17).

A human counterpart of cell-mediated acute tubulointerstitial nephritis is hard to recognize precisely. However, the morphology of the lesions (granulomas and /or predominance of CD4+T cells and macrophages in the interstitium) indicates that cell-mediated reactions of delayed-type hypersensitivity may occur in cases of drug-induced ATIN (93, 94).

The association of ATIN with uveitis (TINU syndrome), where epithelioid and giant–cell granulomas also occur, could fit into this group of cell-mediated ATIN (95).

In ATIN associated with non-steroidal anti-inflammatory drugs and in the rare cases related to cimetidine, as well as those cases related to cytomegalovirus infection, a predominance of CD8+ T lymphocytes is sometimes found in the interstitium and might indicate the possibility of cell-mediated cytotoxicity (96, 97). However, in most cases, CD4+ T lymphocytes were also found and sometimes were in the majority. Therefore, because the phenotypical expression of T cells does not correlate with functional activity and because the interstitial reaction is dynamic, reliable evidence for a predominantly cytotoxic reaction in human acute tubulointerstitial nephritis is lacking (17).

1.4 The immune system

The organized cells and molecules that have a specialized function in defending the body against foreign substances is called the immune system (471). Two types of responses have been identified. Innate (natural) responses mount the same qualitative and quantitative response each time the infectious agent is encountered; innate immunity comprises phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), as well as natural killer cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines such as the interferons. On the other hand, acquired (adaptive) responses 'learn' to mount more focused and efficient responses on repeated exposure to a given infection; acquired immunity involves the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to antigen (471).

There are specialized cells (antigen-presenting cells) (the prototype being the dendritic cell), that present peptides of the protein antigen to lymphocytes and collaborate with them; in response to the antigen B cells secrete immunoglobulins to remove extracellular microorganisms. T cells help B cells to synthesize the antibody and can also kill intracellular pathogens by stimulating macrophages and by eradicating virally infected cells. Innate and acquired responses usually work together to get rid of pathogens (471).

1.4.1 The acute immune response:

Infection with a pathogen stimulates an acute inflammatory (immune) response in which cells and molecules of the immune system are recruited to the affected site (471). The complement proteins are important to this process and comprise a cascade of proteolytic substances that aids the recognition and clearance of microorganisms. The stimulation of complement produces C3b, which coats the surface of the pathogen. The

neutrophil chemoattractant and activator C5a is also generated, and together with C3a and C4a leads to the release of histamine by degranulating mast cells. Histamine causes smooth muscle contraction and increases in vascular permeability (471). Cytokines and other substances released from damaged tissues activate the expression of adhesion molecules on vascular endothelium, alerting passing leukocytes to the presence of infection. The cell-surface molecule L-selectin on neutrophils recognizes carbohydrate structures such as sialyl-Lewis on the 'activated' endothelial surface (98). These molecules support neutrophil rolling along the vessel wall and, if additional chemokine signals are detected by the neutrophil, it too becomes activated, sheds L-selectin from its surface and expresses cell-surface adhesion molecules, such as the integrins that alter their conformation to be able to recognise their counter-ligands on endothelial cells. One important ligand is ICAM-1, which increases its expression on the blood-vessel wall in response to inflammatory mediators such as bacterial lipopolysaccharide and the cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNFα). Complement components, prostaglandins, leukotrienes, and other inflammatory mediators all contribute to the recruitment of inflammatory cells, as do chemoattractant cytokines called chemokines, for example the chemokine interleukin-8 is a powerful neutrophil chemoattractant. The activated neutrophils migrate through the endothelium, moving up the chemotactic gradient to accumulate at the site of infection, where they are well placed to phagocytose any C3b-coated microbes (471).

1.4.2 Mast cells:

Mast cells express high-affinity receptors for the Fc portion of IgE, and bind IgE antibodies. When a mast cell, armed with IgE antibodies, is re-exposed to the specific allergen, a series of reactions takes place, leading eventually to the release of mediators that cause immediate hypersensitivity reactions. First, antigen (allergen) binds to the

secreted or membrane-bound IgE antibodies. Multivalent antigens bind to more than one IgE molecule and cross-link adjacent IgE antibodies and the underlying mast cell IgE Fc receptors. The cross-linking of IgE Fc receptors (Fc∑RI) activates signal transduction pathways via the cytoplasmic portion of IgE Fc receptors. These signals activate two independent processes, one leading to mast cell degranulation with discharge of preformed (primary) mediators that are stored in the granules, and the other involving synthesis and release of secondary mediators. These mediators are responsible for the initial symptoms of immediate hypersensitivity and they initiate the events that lead to the late-phase response (99). In addition to inducing mediator release and production, signals from IgE Fc receptors promote the survival of mast cells and can enhance expression of the Fc receptors, providing an amplification mechanism (100).

1.4.2.1 Primary mediators:

Primary mediators contained within mast-cell granules can be divided into three categories:

1.4.2.1.1 Biogenic amines:

The most important vasoactive amine is histamine, which causes intense smooth muscle contraction, increased vascular permeability and increased secretion by nasal, bronchial and gastric glands.

1.4.2.1.2 Enzymes:

These are contained in the granule matrix and include neutral proteases (chymase, tryptase) and acid hydrolases. The enzymes cause damage to local tissues and lead to production of kinins and activated components of complement (e.g. C3a) by acting on their precursor proteins.

Tryptase is an abundant product of human mast cells. It is a serine protease with a molecular size of 134 kD, it is composed of four monomers of 32 to 34 kD, each with one catalytic site (101). Its presence is restricted to mast cells, where tryptase exists within secretary granules in a complex with heparin proteoglycan (101). Biologic activities include lysis of fibrinogen, augmentation of histamine-mediated contractility of air way smooth muscle, and degradation of vasoactive intestinal peptides (102). It is also mitogenic for fibroblasts, smooth muscle cells, and bronchial epithelial cells (103, 104). Studies have indicated that tryptase-positive mast cells may be involved in renal interstitial injury (105).

1.4.2.1.3 Proteoglycans:

These include heparin, a well-known anticoagulant, and chondroitin sulphate. The Proteoglycans serve to package and store the other mediators in the granules.

1.4.2.2 Secondary mediators:

Secondary mediators include two classes of compounds (1) lipid mediators and (2) cytokines.

The lipid mediators are generated by reactions in the mast-cell membranes that lead to activation of phospholipase A2, an enzyme that acts on membrane phospholipids to produce arachidonic acid. This is the parent compound from which leukotrienes and prostaglandins are derived by the 5-lipoxygenase and cyclooxygenase pathways.

1.4.2.2.1.1 Leukotrienes:

Leukotrienes C4 and D4 are the most potent vasoactive and spasmogenic agents known. On a molar basis, they are several thousand times more active than histamine in increasing vascular permeability and causing bronchial smooth muscle contraction. Leukotriene B4 is highly chemotactic for neutrophils, eosinophils, and monocytes.

1.4.2.2.1.2 Prostaglandin D2:

This is the most abundant mediator derived from the cyclooxygenase pathway in mast cells. It causes intense bronchospasm as well as increased mucus secretion.

1.4.2.2.1.3 Platelet-activating factor (PAF):

PAF is produced by some mast cells. It causes platelet aggregation, release of histamine, bronchospasm, increased vascular permeability, and vasodilation. It has important pro-inflammatory actions. PAF is chemotactic for neutrophils and eosinophils. At high concentrations, it activates the newly recruited inflammatory cells, causing them to aggregate and degranulate. Because of its ability to recruit and activate inflammatory cells, it is considered important in the initiation of the late –phase response. Although the production of PAF is also triggered by the activation of phospholipase A2, it is not a product of arachidonic metabolism.

1.4.2.2.2 Cytokines:

Mast cells secrete many cytokines, which contribute to the late-phase reaction of immediate hypersensitivity because of their ability to recruit and activate inflammatory cells. The cytokines include TNF, IL-1, IL-3, IL-4, IL-5, IL-6, and GM-CSF, as well as chemokines, such as macrophage inflammatory protein (MIP)-1α and MIP-1β (106). Mast cell-derived TNF and chemokines are important mediators of the inflammatory response seen at the site of allergic inflammation.

1.4.3 Macrophages:

Macrophages have critical roles in host response to injury and the mechanisms by which it is repaired (107-109). They infiltrate damaged tissues where they adapt to the local microenvironment by developing properties that either cause further injury (such as

might be advantageous in defence against infection), or alternatively evolve into cells that promote resolution of inflammation and facilitate tissue repair once the original cause of injury has been eliminated (107).

1.4.3.1 Classical activation of macrophages:

Macrophages have a range of receptors on the cell surface that enable them to recognise infectious organisms including receptors with pathogen-associated molecular patterns (PAMPs), which include Toll-like receptors (TLR), mannose receptor and scavenger receptor families. TLRs identify a range of microbial products including lipopolysaccharide (LPS) that binds to TLR4, bacterial unmethylated CpG DNA that binds to TLR9 and viral double-stranded (ds) RNA that binds to TLR3 (110). Classical activation comprises activation of macrophages with two signals. The first is the involvement of TLR and the second is yielded by the cytokine IFN-γ that is secreted by T-helper lymphocytes or natural killer cells. This dual activation leads to macrophage to produce nitric oxide, reactive oxygen species and other proinflammatory cytokines particularly TNF-α and IL-12, also the expression of MHC class Π and costimulatory molecules which promote antigen presentation. These responses are designed to intensify microbial killing and stimulate adaptive immunity (111). TLRs also identify endogenous ligands secreted by damaged tissues such as heat-shock protein 60 and 70 which are ligands for TLR4 (113) and ds DNA which interacts with TLR9 (114).

Classically activated macrophages have been found at sites of cell immune-mediated inflammation, including glomerulonephritis (115), especially during the initial induction of the inflammatory response.

1.4.3.2 Alternative activation of macrophages:

The alternatively activated macrophage developed after the exposure to IL-4 or IL-13 (117, 118). These cells show enhanced expression of mannose receptor and MHC class II, increased endocytosis, decreased nitric oxide production (due to both decreased expression of iNOS and increased production of arginase) and enhanced expression of IL-1 decoy receptor and IL-1ra (119). Thus these cells have a reduction in the killing of intracellular organisms but the enhanced matrix production suggest a role in tissue repair (120).

1.4.3.3 Type II-activated macrophage:

Recently Mosser (121) has recognized the "type II-activated macrophage". They explained that macrophages exposure to activating stimuli such as LPS or CD40L in the presence of IgG immune complexes resulted in enhanced IL-10 expression and reduced IL-12 expression but with preservation of other proinflammatory cytokines such as TNF-α and IL-6 (122, 123). These cells in vivo favour the development of Th2 type immune responses with increased T cell IL-4 production and IgG class switching by B cells (124).

Following adhesion to activated endothelium, macrophages transmigrate to the focus of injury in response to a chemotactic gradient. Renal injury, whether toxic, ischaemic, or immunologic, can lead to chemokine production by endothelium, mesangial cells and tubular cells. The most extensively studied chemokine/receptor partners for macrophage chemotoaxis are MCP-1 and its receptor CCR2, and RANTES, macrophage inflammatory protein-1α and 1β (MIP-1α and MIP-1β) and their receptors CCR1,3, and 5 (125).

1.4.4 Eosinophils:

Eosinophils are often present in increased number during ATIN and may be detected in the urine. Eosinophils are a type of granulocyte derived from bone marrow, distinguished by their morphologic features, particularly their specific granules, and associations with specific diseases. Specific granules contain lysosomal hydrolases and most of the cationic proteins unique to eosinophils (126). The core of the granule is composed of major basic protein, and the non core matrix contains eosinophil cationic protein, eosinophil derived neurotoxin, and eosinophil peroxidase (127, 128). Major basic protein, so named because it is one of the most abundant cationic (basic) eosinophil granule proteins, is a 14,000-dalton protein rich in arginine residues (129). It has no recognized enzymatic activity, but is toxic to helminthic parasites, tumor cells, and host cells (130, 131). Eosinophil cationic protein is a markedly cationic polypeptide of 18,000 to 21,000 daltons (132-134) with bactericidal (135) and helminthotoxic (136) activities. Like major basic protein, it is toxic to host cells. Eosinophil-derived neurotoxin, a protein of about 18,600 daltons that shares some sequence similarity with eosinophil cationic protein (137, 138), was named because it induced cerebrocerebellar dysfunction when injected intracerebrally into rabbits (131, 134). Eosinophil cationic protein and eosinophil-derived neurotoxin have partial sequence identity with pancreatic ribonuclease and have ribonuclease catalytic activity; eosinophil-derived neurotoxin is about 100 times more potent as a ribonuclease than eosinophil cationic protein (132, 134). Eosinophil peroxidase, an enzyme distinct from the myeloperoxidase of neutrophils and monocytes, consists of two polypeptides of about 15,000 and 55,000 daltons (139, 140). In the presence of hydrogen peroxide and halide ions, eosinophil peroxidase is toxic to helmenthic and protozoan parasites, bacteria, tumor cells, and host cells (131, 141, 142). Another

distinctive protein of human eosinophils is the protein that forms Charcot-Leyden crystals, the bipyramidal crystals that are often found in sputum, faeces, and tissues as a hallmark of eosinophil-related disease. This 17,000-dalton protein, which crystallizes in vivo and vitro (143, 144), is a hydrophobic protein with lysophospholipase activity (145). It composes about 5 percent of the eosinophil's total protein, is found in primary granules (146), and also is associated with cell membranes of eosinophils.

The effector functions of eosinophils involve acute cellular responses, such as degranulation, oxidative-burst activity, and eicosanoid release. The ability of eosinophils to be involved in other types of cellular responses suggests that eosinophils can collaborate with lymphocytes and other immunologic and mesenchymal cells in various ways that are pertinent to health and disease. For example, mature eosinophils retain the ability to synthesize proteins, including CD4 and HLA-DR (147), and so can form new proteins relevant to immunologic functions. Eosinophils have been shown to function as antigen presenting cells after in vitro induction of HLA-DR, so they could interact with CD4+ lymphocytes to elicit antigen-specific lymphocyte responses. In tissues, eosinophils might also initiate antigen-specific lymphocyte responses for antigens specific to certain mucosal sites. Other direct cell-cell interactions between eosinophils and other types of cells are feasible on the basis of their expression of CD4 and the capacity of eosinophils to respond to lymphocyte products (148). Thus, specific cytokines and lymphokines may promote other activities of mature eosinophils in addition to effector functions. Finally, cytokines elaborated by eosinophils could affect other cells nearby. Transforming growth factor-α, synthesized by eosinophils (148, 149), may stimulate endothelial cells and fibroblasts. Because of their distribution in tissues of the respiratory, gastrointestinal, and lower

genitourinary tracts, resident eosinophils may be active cellular participants in mucosal immune responses at these sites.

1.4.4.1 Chemokines and eosinophil recruitment:

Chemokines that are important for eosinophil recruitment are expressed at sites of allergic inflammation following allergen challenge. The chemokine eotaxin has particular specificity for eosinophils. Studies with eotaxin-deficient mice have shown that eotaxin is important in the early recruitment of eosinophils after allergen challenge (150). At late time points following allergen challenge, eotaxin is not involved. In fact studies with neutralizing antibodies demonstrate that chemokines such as RANTES, MCP-5, and MIP 1α are also important in eosinophil tissue recruitment (151). In man there are also differences in the timing of expression of different CC chemokines at sites of allergic inflammation. In a skin model of the late-phase reaction to allergen, MCP-3 mRNA expression peaks at 6 h post-allergen challenge coinciding with the peak eosinophil recruitment, whereas RANTES mRNA peaks later at 24 h, suggesting that in this model MCP-3 is more important than RANTES in eosinophil recruitment (152).

Chemokines bind to 7 trans-membrane-spanning chemokine receptors expressed by eosinophils. Eosinophils express CCR3 receptors which bind eotaxin as well as RANTES and MCP-3, making the CCR3 receptor an attractive therapeutic target. Studies with neutralizing antibodies to CCR3 have demonstrated that it is possible to inhibit binding of eotaxin as well as RANTES, MCP-2, MCP-3, and MCP-4 to human eosinophils. Reports with CCR3 deficient mice suggest that CCR3 mediates approximately 50% of eosinophil tissue recruitment (153).

1.4.5 Basic concepts of adaptive immune response:

The shape of antigen and the peptide-MHC complex are the basis of detection by the antibodies and the combining site on the T cell receptor respectively (154). Secreted antibodies or membrane-expressed antibodies acting as B cell receptors, usually recognize discontinuous epitopes, composed of amino acids that are brought together when the protein folds into its native structure (155). When an epitope on an antigen fits well with a particular combining site on the B cell, the resultant population of antibodies against this epitope tends to dominate the antibody response. In contrast, the epitopes recognized by α/β T cell receptors (where α and β are the two chains that make up the receptor) are linear peptides derived from intracellular breakdown of the antigen. These peptides are transported to the cell surface within the peptide-binding groove of the MHC molecule (471).

Antibodies and T cell receptors can differentiate between closely related antigens but they sometimes recognise apparently unrelated antigens. This may occur if the two antigens share an identical epitope, or if two different epitopes have similar shapes and charges. Sometimes this leads to molecular mimicry, whereby epitopes on microbial agents stimulate the production of antibodies (or the proliferation of T cells) which react with self antigens. Molecular mimicry may be a cause of autoimmune disease (155, 156). An example of this is post-streptococcal rheumatic fever, which is caused by antibodies induced by an epitope on streptococcal M protein that also recognise a similar epitope on cardiac myosin.

Some antigens (the T cell-independent antigens) can stimulate B cells without help from T cells (157). T-independent antigens include the polysaccharides or polymerized flagellin of bacteria. These have many repeating epitopes that bind to the B cell receptors

and along with activation signals which a variety of cell types can provide, they activate B cells without help from CD4 T cells. T cell-independent antigens do not induce the formation of germinal centres, memory B cells or the somatic hypermutation which results in the production of high-affinity antibodies. The extent of class switching from IgM to other classes of antibodies is also limited. For these reasons, T cell-independent antigens generally give rise to low-affinity IgM antibodies (471).

Most antigens can only stimulate B cells with help from CD4 T cells and are therefore referred to as T cell-dependent antigens. After a B-cell receptor binds such an antigen, it is internalized and processed by the B cell into short peptides, which are carried to the cell surface by MHC class Π molecules. Neighbouring CD4 T cells that are able to recognize these peptide-MHC complexes (since they have been exposed to antigen-presenting dendritic cells) become activated and express costimulatory molecules such as CD40 ligand on their surface. CD40 ligand on the activated T cell binds to its receptor (CD40) on the B cell, and this induces the B cell to begin the processes of somatic hypermutation and immunoglobulin class switching. The cytokines IL-2, IL-4, and IL-5 that are released from the T cells also provide help. Dendritic cells and macrophages, by presenting peptide-MHC class Π complexes, can also activate naive helper CD4 T cells, so that they express costimulatory molecules and release immunostimulatory cytokines. Once the immune system is stimulated by an immunogenic epitope, additional epitopes on the antigen may be drawn into the response. This effect is referred to as epitope spreading (160). It may involve other antigens (intermolecular spreading). Its clinical relevance is that in some autoimmune diseases, a complex of several molecules, may provoke a broad spectrum of autoantibodies. Other events that lead to autoantibody formation include revealing of cryptic (hidden) epitopes which can occur after a change in the processing of

antigen as may happen when proinflammatory cytokines stimulate antigen-presenting cells (161). Further, B cells may generate peptides which are not produced by dendritic cells or macrophages, as seen with the model antigen hen-egg lysozyme (162).

The continual mutation of microorganisms causes a phenomenon called antigenic drift. The mutants may not be recognised by the memory component of the immune system. In addition, the exchange of genetic material between related organismscan lead to antigenic shift (163). Very few of the memory cells which were generated during exposure to the native organism may be able to recognize the new variant. The influenza pandemics which have killed large numbers of people when the virus has swept relatively unchallenged across the globe has resulted from antigenic shift. (471)

1.4.6:- T lymphocytes:

Stem cells migrate from the bone marrow to the thymus, where they develop into T cells (164). This process continues throughout life, although the thymus does degenerate a little in older people. Most T cells in the thymus have α/β T cell receptors and undergo a series of selection procedures (165). Unlike the antibody molecule, which acts as the antigen receptor on B cells and recognizes antigen in its native (natural) state, the α/ β T cell receptor recognizes short peptides. These are generated by intracellular processing of protein antigens. Peptides are presented to the T cell receptor by MHC molecules on the surface of an antigen presenting cell. The T cell receptor recognizes an individual's own MHC molecules (self) together with peptides derived from foreign antigens. MHC molecules are highly polymorphic and the desirable immature T cells are those which can recognize self MHC molecules but are not autoreactive. This is achieved by thymic education, a process which involves both positive and negative selection (166-169). Cells are positively selected if they express a T cell receptor that can recognize the MHC

complexes on a body's own epithelial cells in the thymic cortex. Positive selection stops spontaneous apoptosis (death of the cell). More than 95% of T cells are not selected at this stage and therefore die in the thymus. In contrast, negative selection involves the induction of apoptosis in any T cell expressing a T cell receptor with a high affinity for the complex of a self peptide plus a self MHC molecule on dendritic cells and macrophages in the thymic medulla. During thymic education, some molecules on the surface of T cells increase expression and others reduce their expression. The molecules have been characterised using monoclonal antibodies. This led to a nomenclature in which a given molecule was assigned a "cluster of differentiation", or CD number, for example CD1, CD2, and CD3. The CD4 and CD8 molecules are of particular note with regard to T cell development; together with the CD3 group of molecules, they form part of the T cell-receptor complex. CD4 binds to an invariant part of the MHC class Π molecule, whereas CD8 binds to an invariant part of the MHC class I molecule. CD4 T cells usually act as helper T cells and recognize antigens presented by MHC class Π molecules, while CD8 T cells are usually cytotoxic and recognize antigen presented by MHC class I molecules. Early in T cell development in the thymus, immature T cells express both CD4 and CD8 (170). If they have an appropriate T cell receptor, these double-positive immature T cells have the potential to recognize an antigen-derived peptide presented by either MHC class I or MHC class Π molecules. As T cells mature in the thymus, the expression of one of these molecules is lost, resulting in a single–positive CD4 or CD8 T cell.

A minority of T cells in the thymus use γ and δ chain genes to produce a T cell receptor. These γ/ δ T cells rapidly leave the thymus, and some may develop outside the thymus, possibly in the gut (171). They are thought to contribute to mucosal defences. The antigens they recognise include both proteinaceous and nonproteinaceous antigens

from mycobacteria and other infectious organisms. In addition, they have an important immunoregulatory role because they influence antibody production and immunoglobulin class switching by B cells and may modify T cell responses (172).

1.4.6.1-Effector functions of T cells:

CD4 T cells are mainly cytokine-secreting helper cells, whereas CD8 T cells are mainly cytotoxic killer cells.

CD4 T cells can be divided into different subsets (173); type 1 (Th 1) helper T cells secrete interleukin-2 and interferon-γ. Type 2 (Th 2) helper T cells secrete interleukin-4, 5, 6, and 10. Cytokines influence the type of immune response generated against particular infectious agents (163, 174). For example, the release of interleukin-12 by antigen-presenting cells stimulates the production of interferon-γ (immune interferon) by Th 1 cells. This cytokine efficiently activates macrophages, enabling them to kill intracellular organisms. In general, the production of cytokine by Th 1 cells facilitates cell-mediated immunity, including the activation of macrophages and T cell-mediated cytotoxicity; while Th 2 cells help B cells produce antibodies (471).

Elimination of virally infected cells is carried out by CD8 cytotoxic T cells. The infected cell displays peptides derived from the intracellular viral protein within its MHC class I molecules and is recognised by the cytotoxic T cell. Cytotoxic T cells bind to this viral peptide-MHC complex and then kill the infected cell. They can insert perforins into the target-cell membrane, which produce pores through which proteolytic enzymes called granzymes are passed from cytotoxic T cells into the target cell. At least one of these proteolytic enzymes activates the caspase enzymes which induce apoptosis in the target cell. Cytotoxic T cells also can bind the Fas molecule on the target cell using their Fas ligand, and this also activates caspases within the target cell and induces apoptosis (175).

Any released virus is immediately susceptible to the effect of antibodies. In addition to directly killing infected cells, CD8 T cells also produce a number of cytokines, including TNF-α and lymphotoxin. Interferon-γ, another product of CD8 cells, reinforces antiviral defences by making adjacent cells resistant to infection (176).

1.4.7-Control of the immune response:

A successful immune response will get rid of the inciting antigen and,then return itself to a resting level. In addition to cleansing itself of antigen, the immune system uses several other mechanisms to down-regulate its activity. IgG itself can switch off the response to its corresponding antigen, a type of negative feedback loop. This suppression of IgG production occurs when the FcγR and the B-cell receptor on the same cell are linked by immune complexes containing the relevant antigen and transmitinhibitory signals into the nucleus of B cell (179). Cytokines participate at another level of regulatory control, for example, the secretion of interferon-γ by Th 1 cells inhibits Th 2 cells and the secretion of interleukin -10 by Th 2 cells reciprocally inhibits Th 1 cells (173). Immunoregulation involves many other interactions of the immune system with both the endocrine and nervous systems, with cross-talk between these systems involving hormones, cytokines, and neurotransmitters.

The following sections on Th 2 cells and interleukin-4, as well as the large section on chemokines are presented because of the current perceived role of these cells and mediators in interstitial nephritis.

1.4.8- Th 2 helper cell subset:

The theory that T helper cells, in humans and rodents, can be divided into functionally specific types (named Th 1and Th 2) has changed the understanding of

adaptive immune reactions (Holdsworth SR, 1999). The immune responses associated with Th 1 and Th 2 cells are accompanied by specific forms of cytokine secretion and these cytokine profiles have, in turn, helped to describe the various immune effects of these cytokines (Holdsworth SR, 1999).

In 1986, Mosmann et al (180) demonstrated that functionally distinct subsets of CD4+ T cells could be defined by their pattern of cytokine production. Th 2 cells, defined by their propensity to secrete interleukin-4 (IL-4), IL-5, and IL-10, are important in allergy, mast cell/ IgE-mediated immediate type hypersensitivity responses, and helminth infections, in which protective responses are mediated by eosinophils. In addition, cytokines produced by Th 2 cells act as regulators of the immune response. In addition, cytokines IL-13, and particularly IL-10 regulate Th 1 responses, suppress delayed hypersensitivity responses, and have inhibitory effects on macrophages, especially in the context of the activation by Th 1 cytokines such as IFN-γ (181-183). Th 2 responses are associated with high levels of antibody production promoted by cytokines such as IL-4 that stimulate B-cell growth. The profile of immunoglobulin isotypes is heavily influenced by the Th 1/Th 2 balance of the immune response. This has been studied extensively, particularly in the mouse (184-186), in which the levels of IgG1 (which has a weak affinity for Fcγ receptors) and IgG2a (which is strongly complement fixing and a high affinity for Fcγ receptors) have been related to the Th 1/Th 2 predominance of immune responses. A Th 2, IL-4 dominant response promotes a higher ratio of IgE and IgG1 to IgG2a, whereas Th 1 response patterns (IL-12 and IFN-γ driven) have higher IgG2a to IgG1 and IgE ratios.

The cytokine profile of antigen-stimulated CD4+ T cells and the pattern of T-cell immune responses are determined by a number of factors, including the type, dose, and route of antigen presentation, the epitope T cell receptor binding affinity, the nature and

degree of co-stimulatory signals, and the genetic background of the animals. One of the most important and widely studied factors is the cytokine milieu at the time of antigen presentation (173, 187, 188). IL-12 is crucial for the development of Th 1 responses (189), whereas IL-4 is required for the generation of Th 2 cells (188). The presence of IL-12, which is not produced by T cells but by antigen-presenting cells such as macrophages and dendritic cells, polarizes uncommitted T cells toward a Th1 profile (189). In the absence of IL-12 during the initiation of the immune response, T cells may lose future responsiveness to IL-12.

Although the concept of Th 1/Th 2 immune responses provides a useful framework, it is perhaps overly simplistic to consider that each immune response to an antigen will be strictly either Th 1 or Th 2, with one type of response being protective and the other harmful. The complexity of infectious and inflammatory responses implies that some cytokines (or a single cytokine) within a Th 1or Th 2 grouping may have overlapping or, at times, opposing functions. At a cellular level, there is some heterogeneity in the pattern of cytokine secretion of Th 1 and Th 2 cells (195). It has been suggested that the Th 1/Th 2 paradigm could be modified to include the concept that although an overall immune response to a specific antigen may be predominantly Th 1 or Th 2, antigen-specific T cells produce a spectrum of cytokines, with pure Th 1 and Th 2 cytokine profiles being at the extremes of a spectrum (196).

However, another review of the functional diversity of T helper lymphocytes refers to features that confirm the presence and relevance of the Th 1/Th 2 model in immune responses (187). Two factors are cited: first, the clear pattern of antigen-specific Th 1 orTh 2 cell (and associated cytokines) predominance in a number of diseases in both mice and

humans, and second, the tendency for T helper cell populations to become increasingly polarized and irreversibly committed with chronic immune stimulation.

1.4.9 Interleukin-4 (IL-4):

Interleukin-4 is a 20 kDa immuno-regulatory cytokine that is secreted by T cells, mast cells, basophils, and a subset of natural killer cells (197). It is a key modulator of allergic reactions, so it is discussed here. It is produced by Th 2 CD4+ T cells and facilitates the development of a Th 2 phenotype. In vitro, it inhibits interferon (IFN)-γ production by activated T cells (198). IL-4 inhibits many functions of activated macrophages, including the secretion of reactive oxygen intermediates (199) and nitric oxide (200), and the expression of tissue factor (201) and macrophage colony stimulating factor (202). It suppresses macrophage TNF-α and IL-1β (203) production and up-regulates expression of IL-1 antagonist (204). It stimulates macrophage 15-lipoxygenase activity, which may reduce synthesis of the pro-inflammatory leukotriene B4 (205). IL-4 also decreases monocyte expression of all three classes of Fc receptor for IgG (206). IL-4 induces IgE synthesis and promotes immediate-type hypersensitivity reactions. It also induces differentiation of naïve T cells to Th 2 lymphocytes (207, 208) that secrete additional macrophage-inhibiting cytokines such as IL-10 and IL-13 (183, 209). IL-4 is involved in the alternative activation of macrophages (see section 1.4.3.2).

1.5 Chemokines and their receptors:

Chemokines have already been introduced and a more detailed resume of their functions is presented here.

Chemokines are a family of small proteins defined by four conserved cysteine residues. These proteins trigger G protein-coupled receptors and stimulate cells to migrate through a concentration gradient, so that leukocyte recruitment is promoted. Some chemokines are homeostatic and are constitutively produced and secreted. These homeostatic proteins help to direct the trafficking of lymphocytes to lymphoid tissues and are involved in immune surveillance. Other chemokines are considered inflammatory and are only produced by cells during infection or a proinflammatory stimulus. Inflammatory chemokines recruit leukocytes to the injured or infected site. In addition, inflammatory chemokines activate the cells to mount an immune response and initiate wound healing (221).

Chemokines are proteins with a molecular weight of 8-10 Kd with 20% to 70% similarity in amino acid sequences. They have been subdivided into families on the basis of the relative location of their cysteine residues (210, 211). There are four known families of chemokines (Table 2; the common names of chemokines will be used throughout the thesis). The α- and β-chemokines, which contain four cysteines, are the largest families. In the α-chemokines, the first two cysteine residues are separated by one amino acid (cysteine-x amino acid-cysteine, or CXC), whereas in β-chemokines, the first two cysteine residues are adjacent to each other (cysteine-cysteine, or CC). Two chemokines that do not fit into this classification, lymphotactin (212) has only two cysteines, while fractalkine (213) is a membrane-bound glycoprotein in which the first two cysteine residues are separated by three amino acids (CXXXC) and the chemokine domain sits on a mucin-like stalk.

The α-chemokines can be further subdivided into those that contain the sequence glutamic acid–leucine-arginine (‘ELR’) near the N terminal (preceding the CXC sequence) and

those which do not (214). The α-chemokines containing the ELR sequence are chemotactic for neutrophils, whereas those not containing the sequence act on lymphocytes. For example, IP-10 and MIG (monokine induced by interferon-γ) attract activated T cells (215), and stromal–cell-derived factor 1 acts on resting lymphocytes (216). The β-chemokines, in general, attract monocytes, eosinophils, basophils, and lymphocytes with variable selectivity, but do not act on neutrophils. Structurally, the β-chemokines can be subdivided into two families: the monocyte-chemoattractant-protein-eotaxin family, containing the five monocyte chemoattractant proteins and eotaxin, which are approximately 65% identical to each other; and all other β-chemokines (217). As with the CXC family, the N-terminal amino acids preceding the CC residues of β-chemokines are essential for the biological activity and leukocyte selectivity of these chemokines (218, 219). Many chemokines undergo N-terminal proteolytic processing after secretion, which alters their activity (220). This may reflect a general mechanism which allows local factors to regulate and amplify chemokine activity (Luster AD, 1998) (229).

Table 2:- Chemokines and Chemokine Receptors(Adapted from(221).

Ligand systematic names	Ligand common names	Receptors	Function
CCL1	I-309	CCR8	I (inflammatory)
CCL2	MCP-1	CCR2	I
CCL3	MIP-1α	CCR1, 5	I
CCL4	MIP-1β	CCR5	I
CCL5	RANTES	CCR1, 3, 5	I
CCL7	MCP-3	CCR1,2	I
CCL8	MCP-2	CCR1,2,5	I
CCL11	Eotaxin	CCR3	I
CCL13	MCP-4	CCR1,2,3	I
CCL14	HCC-1	CCR1	I
CCL15	HCC-2	CCR1	I
CCL16	HCC-4	CCR1,8	I
CCL17	TARC	CCR4	I
CCL18	DC-CK1		
CCL19	ELC	CCR7	H (homeostatic)
CCL20	LARC	CCR6	
CCL21	SLC/6CKine	CCR7	H
CCL22	MDC	CCR4	I
CCL23	MPIF-1	CCR1	
CCL24	Eotaxin-2	CCR3	I
CCL25	TECK	CCR9	I
CCL26	Eotaxin-3	CCR3	I
CCL27	CTAK/Eskine	CCR10	
CXCL1	Groα	CXCR1,2	I
CXCL2	Groβ		I
CXCL3	Groγ		I
CXCL4	PF4	CXCR1,2	I
CXCL5	ENA-78	CXCR2	I
CXCL6	GCP-2	CXCR1,2	I
CXCL7	NAP-2	CXCR2	I
CXCL8	IL-8	CXCR1,2	I
CXCL9	Mig	CXCR3	I
CXCL10	IP-10	CXCR3	I
CXCL11	I-TAC	CXCR3	
CXCL12	SDF-1	CXCR4	H
CXCL13	BCA-1	CXCR5	
CXCL14	Bolekine		
CXCL15	Lungkine		H
XCL			
XCL1	Lymphotactin	XCR1	I
XCl2	SCM-1β		I
CX3CL			
CX3CL1	Fractalkine	CX3CR1	H,I

1.5.1 Eotaxin:

Human eotaxin has a molecular weight of 8.4 KDa, 74 amino acids residue poly peptide that is produced by number of normal cells and cell lines (222). Eotaxin-1, a highly potent eosinophil chemoattractant, was originally purified and separated as a CC-chemokine from the bronchoalveolar lavage fluid of allergen-challenged guinea pigs (223). Guinea pig and human eotaxin-1 genes were cloned subsequently (222, 223). Eotaxin-1 and the closely related MCP are clustered together on human chromosome 17q11.Two further genes encoding for CC chemokines with eosinophil-selective activity, called eotaxin-2 and eotaxin-3 (224, 225) have been identified on chromosome 7, although they are only 30% identical in sequence to eotaxin-1. The eotaxins signal exclusively via CCR3, a receptor highly expressed on eosinophils and also on other cells involved in allergic reactions, including basophils, mast calls and a subpopulation of Th 2 cells (224, 226, 227). Th 2 cells regulate eosinophil recruitment suggesting that Th 2 derived cytokines may regulate eotaxin gene expression (228-230). Both cytokines and glucocorticoids can modulate *in vitro* expression of eotaxin-1 and eotaxin-2 mRNA and protein in human lung epithelial and dermal fibroblast cell lines; TNF-α and IL-1β induce eotaxin-1 and eotaxin-2 expression, as do the Th 2 cytokines IL-4 and IL-13 (231, 232). Furthermore, TNF-α in combination with either IL-4 or IL-13 has a synergistic effect on expression. The glucocorticoid dexamethasone decreases cytokine induced eotaxin-1 and eotaxin-2 expression, an effect not altered by pre-treatment with the protein synthesis inhibitor cyclohexamide (233). These studies suggest a mechanism linking inflammatory cytokine secretion to eosinophil recruitment and *in vivo* evidence supports this concept. If IL-4 and IL-13 are administered intra-nasally to naïve mice, airway epithelial cell eotaxin-1

expression is induced, as well as lung eosinophilia (234). IL-4 knockout mice in an allergic dermatitis model had reduced eosinophilic infiltration and an associated reduction in eotaxin-1 mRNA (235). In humans, eotaxin-1 has been detected at elevated levels in the bronchial epithelium of patients with asthma, as well as in lesions of skin biopsies of allergic dermatitis sufferers (236, 237).

Evidence has described differential expression patterns of eotaxin-1 and eotaxin-2, for example lung fibroblasts produce eotaxin-1 protein in response to in vitro Th 2 cytokine stimulation however, no eotaxin-2 is detected in such circumstances, even at mRNA level (238). Furthermore, in human atopic subjects undergoing allergen induced late-phase allergic skin reactions, results suggested that eotaxin-1 has a role in the early (6h) recruitment of eosinophils, whilst eotaxin-2 is more involved with later (24 h) eosinophil infiltration (239). Hence the implication is that the eotaxins all have different expression profiles with regard to cell specificity, kinetics and eosinophil effector function. Indeed Berkman and co-workers (240) have provided evidence that at the mRNA level, eotaxin-1 and eotaxin-2 may be co-expressed in human lungs of non-challenged asthmatic patients, whilst eotaxin-3 is expressed following allergen challenge. However, little additional data on eotaxin-3 expression have been reported. Eotaxin-3 was described first by Shinkai et al, who stimulated vascular endothelial cells with IL-4 before subjecting the cDNA to differential display analysis (225). The noval CC chemokine detected was named eotaxin-3 as it demonstrated in vitro chemotaxis of eosinophils, it acted on cell lines transfected with CCR3 and furthermore when the protein was injected in vivo into primates, local tissue eosinophilia was noted at injection sites. Th 2 derived cytokines IL-4 and IL-13 were shown to regulate eotaxin-3 expression in endothelial cell lines, conversely IL-1β and TNF-α had no effect (225). Eotaxin-3 was cloned also by Kitaura and co-

workers (241), who reported constitutive eotaxin-3 mRNA expression in human heart and ovary tissue. Eotaxin-3 was also found to be 10-fold less potent as a CCR3 ligand than eotaxin-1. It was reported that the three eotaxins show different activity profiles with respect to CCR3 binding and the release of toxic reactive oxygen species from human eosinophils (242).

1.5.2 RANTES:

The C-C chemokine RANTES (regulated upon activation of normal T cell expressed and secreted), is a 68-amino acid protein, that can be expressed by stimulated fibroblasts (243), mesangial cells (244), and tubular epithelial cells (245).

RANTES is inducible in T cell lines and circulating T cells by exposure to mitogens and antigens and is also produced by monocytes/macrophages. RANTES mediates monocyte and activated memory CD4+ and CD8+ T cell chemotaxis (246-248). RANTES can activate T cells via activation of phospholipase D (249). It induces calcium influx and up-regulates IL-2 receptors on the surface of these cells suggesting a role in T cell proliferation (250). RANTES has been implicated in the development of inflammatory infiltrates in kidney allografts (251), coronary artery allografts (252) and also in vasculitic glomerulonephritis (253). In vivo, blockade of RANTES activity using the antagonist Met-RANTES produced significant reduction in proteinuria and leukocyte infiltration in a murine model of glomerulonephritis (254), while intra-dermal injection of RANTES into rat skin induced accumulation of basophils and also the generation of histamine at this site (255).

1.5.3 Chemokine Receptors:

Chemokines trigger cell migration and stimulation by binding to specific G-protein-coupled cell-surface receptors on target cells (256, 257). Sixteen human chemokine receptors had been identified (at the time of writing): five human CXC chemokine receptors (CXCR1 through CXCR5), ten human CC chemokine receptors (CCR1 through CCR10), and one human CXXXC chemokine receptor (CX3CR1). Chemokine receptors are expressed on different types of leukocytes. Some receptors are restricted to certain cells (e.g, CXCR1 is mainly expressed by neutrophils), whereas others are more widely expressed (e.g, CCR2 on monocytes, T cells, natural killer cells, dendritic cells, and basophils). In addition, chemokine receptors are constitutively expressed on some cells, whereas they are inducible on others. CCR1 and CCR2 are constitutively expressed on monocytes but are expressed on lymphocytes after stimulation by interleukin-2 (258). In addition, some constitutive chemokine receptors can be down-regulated; CCR2 is down-regulated by lipopolysaccharide, making the cells unresponsive to monocyte chemoattractant protein 1(which activates only this receptor), but it remains responsive to macrophage inflammatory protein 1α (which activates CCR1 and CCR5) (259). In contrast, the expression of other chemokine receptors is restricted to cell states of activation and differentiation. For example, CXCR3 is expressed on activated Th 1 cells, whereas CCR3, in addition to being expressed on eosinophils and basophils, is preferentially expressed on activated Th 2 cells (se above for definitions of Th 1 and Th 2 cells) (227). In this way, transient up-regulation of chemokine receptors on leukocytes allows for the selective amplification of either a cell-mediated Th 1-type immune response or an allergic Th 2-type response.

Some chemokine receptors are expressed on non-hematopoietic cells, suggesting that the chemokines have roles in addition to leukocyte chemotaxis. Most chemokine receptors bind more than one chemokine but CC receptors bind only CC chemokines and CXC receptors bind only CXC chemokines. This probably is due to structural differences between CC and CXC chemokines (260). Chemokine receptors, like other members of the family of G-protein-coupled receptors are functionally linked to phospholipases through G proteins (256, 257, 261). Many chemokine-induced signalling events are inhibited by Bordetella pertussis toxin, suggesting that chemokine receptors are linked to G proteins of the Gi class. Receptor activation leads to a cascade of cellular activation, including the generation of inositol triphosphate, the release of intracellular calcium and the activation of protein kinase C (260). Chemokine-receptor signalling also activates small guanosine triphosphate-binding proteins of the Ras and Rho families (262). Rho proteins are involved in cell motility through regulation of actin-dependent processes such as membrane ruffling, pseudopod formation, and assembly of focal adhesion complexes. Thus, chemokine receptors activate multiple intracellular signalling pathways that regulate the intracellular machinery necessary to move the cell in its chosen direction.

Chemokines also interact with two types of non-signalling molecules. The first one is the erythrocyte chemokine receptor, called DARC (Duffy antigen receptor for chemokines) (263). This receptor, known since the 1950s as the determinant of the Duffy blood group, is expressed on erythrocytes and endothelial cells. Although DARC is structurally related to chemokine receptors, it is distinctive in that both CXC and CC chemokines bind to it and chemokine binding does not induce calcium flux. This receptor may bind and clear chemokines from the circulation. The second type is a group of heparan sulfate proteoglycans. Chemokines are basic proteins and they bind avidly to negatively

charged heparin and heparan sulfate (264, 265). Heparan sulfate proteoglycans capture chemokines in the extracellular matrix and on the surface of endothelial cells, a process that may help to establish a local concentration gradient from the point where chemokine secretion begins (266).

1.5.3.1 CCR3:

CCR3 is the specific receptor for the CC-chemokines, eotaxin -1, 2, 3, and they interact with high affinity. RANTES and MCP-2, MCP-3, and MCP-4 also have CCR3 as a receptor but, unlike eotaxin, they also recognise additional receptors on monocytes, granulocytes, T cells and NK cells. CCR3 is expressed on eosinophils (from which eotaxin mediates histamine release) and basophils but not on neutrophils, monocytes or freshly isolated peripheral blood lymphocytes (267). CCR3 is also expressed by Th 2 cells but not Th 1 cells (227). About 1% of peripheral blood T cells have been found to express CCR3, but following expansion with PHA and IL-2 *in vitro*, lines derived from sorted CCR3+ cells have an enriched CCR3+ population (19%) which produces the Th 2-associated cytokines, IL-4 and IL-5. The production of these cytokines correlates with CCR3 expression. In such cell lines, expanded from CCR3+ and CCR3- T cells, both CD4+ and CD8+ cells produce IL-4, but to a greater extent in the CCR3+ lines.

CCR3 expression has been shown on lymphocytes co-localising with eosinophils at sites of allergic inflammation (267). Thirty-two T cell clones with four different antigenic specificities were analysed for the expression of CCR3. Thirteen out of 24 expressed high levels of CCR3, with nine of these producing IL-4 and/ or IL-5. This is another indication that CCR3 expression predominates in Th 2, rather than Th 1, cells. In nasal polyps, it was seen that CCR3+ T cells were always found in association with eosinophils, and areas of CD3+ cells without eosinophils were always CCR3-. This observation is substantiated by

the fact that there are no CCR3+ cells in rheumatoid synovium, which is characterised by wide spread T cell infiltrates but no eosinophilia. It is likely that CCR3+ T cells play an important role at sites of allergic inflammation where eosinophils are present. They may up-regulate adhesion molecules required for eosinophil diapedesis, or they may prime and prolong eosinophil survival. CCR3 expression may be induced in increasing numbers of T cells following encounter with allergen and may have potential as a prognostic marker for allergic inflammation. In atopic asthma, approximately 81% of the cells expressing CCR3 mRNA were found to be eosinophils and 89% of the eosinophils were positive (236). The other cell types were macrophages and mast cells. Sallusto et al. suggested that CCR3 ligands may attract eosinophils, basophils and also Th 2 cells, which, when activated by antigen, could provide a source of IL-4 and IL-5 required for the survival of the allergic effector cells (227).

1.5.3.2 CCR5:

The chemokine receptor CCR5 is one member of a family of structurally and functionally related seven-transmembrane-spanning, G-protein-coupled receptors. CCR5 binds to three of the CC-chemokines, namely macrophage inflammatory protein-1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β), and RANTES (regulated upon activation normal T cell expressed and secreted), but it does not bind monocyte chemoattractant protein-1 (MCP-1) (268, 269). Elevated expression of RANTES has been demonstrated in renal (251, 270, 271) and cardiac allograft (272) rejections, and the chemokines MIP-1α and MIP-1β were shown to be elevated in liver allograft rejection (273, 274) and in the early phase of cardiac rejection (272). Local expression of these chemokines may be responsible for the interstitial and vascular mononuclear cell infiltrates of T cells and macrophages that characterize renal allograft rejection (274). The

identification of CCR5 as the major co-receptor for macrophage-tropic HIV-1 strains has led to a large increase in knowledge about the physiological and pathophysiological role of CCR5 (275). Approximately 20 to 30% of peripheral T cells and 10% of monocytes are CCR5 positive (276). By in situ hybridization, CCR5 expression was detected in infiltrating cells in allograft rejection but not in intrinsic renal cells of normal kidney (277). In a study on cryosections, Rottman et al, described CCR5 by immunohistochemistry. In one case of interstitial nephritis, CCR5 was found on interstitial infiltrating cells, endothelium and vascular smooth muscle cells (278).

1.6 Adhesion molecules

Leukocyte adhesion and transmigration are regulated largely by the binding of complementary adhesion molecules on the leukocyte and endothelial surfaces, and chemical mediators (chemoattractants and certain cytokines) affect these processes by modulating the surface expression or avidity of such adhesion molecules. The adhesion receptors involved belong to four molecular families (the selectins, the immunoglobulin superfamily, the integrins and mucin-like glycoproteins).

Vascular cell adhesion molecule (VCAM-1) is important for the adhesion and recruitment of lymphocytes, eosinophils and monocytes (most of the ATIN cell infiltrates), and it will be discussed in more detail below.

1.6.1 VCAM-1:

VCAM-1 is an important adhesion and recruitment molecule for eosinophils that is expressed by endothelial cells.

Human VCAM-1 is a transmembrane glycoprotein characterized by the presence of seven C2-type immunoglobulin domains (279-283). Approximately 80 kDa in predicted molecular weight, human VCAM-1 contains a 674 amino acid residue extracellular segment, a 22 amino acid residue transmembrane domain, and a 19 amino acid residue cytoplasmic tail. There are multiple-N-linked glycosylation sites, and each C2 domain is associated with a pair of cysteines that form disulfide linkages, stabilizing the overall domain (284). Although VCAM-1 with seven domains is considered the predominant form (279-281), alternatively spliced forms occur. In rabbits, an eight domain variant has been reported (279), and in humans, a six domain form (the 4^{th} domain being absent) occurs (281, 282). In the mouse, the situation is more complex. Soluble forms of VCAM-1 have been identified in tissue culture supernatants and in blood (283, 285). Blood levels are elevated in diseases as diverse as acute myelomonocytic leukaemia (285), bronchial asthma (286), acute phase multiple sclerosis (287) and sepsis (288).

The ligands (or co-receptors) for VCAM-1 have been identified and are the α4β1 and α4β7 integrins (289-292). Integrins are non-covalently linked heterodimers composed of one large α subunit (120-180 kDa) and one small β subunit (90-120 kDa). The principle ligand or co-receptor for VCAM-1 is α4β1/VLA-4 (291, 293). Normally, VCAM-1 has a low to nominal expression on un-stimulated endothelium (294, 295) but it is inducible by a number of cytokines (IL-1, TNF-α, IL-4 and IL-13). When induced, VCAM-1 plays a significant role in migration for leukocytes that express VLA-4 (e. g, lymphocytes, monocytes, eosinophils, basophils). If an antigenic challenge is "allergic" in nature and involves IgE antibody, mast cells will release IL-4 (296). Although both TNF-α and IL-4 induce endothelial VCAM-1 expression, IL-4, unlike TNF-α, does not up-regulate E-selectin or ICAM-1(297, 298). This would remove adhesion molecule support for almost

all neutrophil and monocyte extravasation, and result in a predominantly eosinophilic infiltration. VCAM-1 also plays an important role in lymphocyte homing and migration.

1.7-Pathology of Acute tubulo-interstitial nephritis:

In the light of the sections on the immune system, chemokine recruitment of immune cells and adhesion receptors, the current knowledge of the pathology of ATIN is now reviewed.

Although the tubulo-interstitium can only respond in a limited manner to a variety of insults, the pattern of response can help define the acuity of the process, the long term prognosis and in some patients the pathogenesis and/or aetiology of the disorder. The initial division of the histologic pattern of ATIN is dependent on the presence or absence of significance inflammatory infiltrate in the interstitium. Where the infiltrate is predominant, the characteristics of the cellular components of the infiltrate may further assist in differentiating the types of ATIN with differing pathogenesis.

In acute or active forms of tubulointerstitial nephritis the interstitium is oedematous and there is a cellular infiltrate that may contain lymphocytes, plasma cells, or polymorphonuclear leukocytes, including eosinophils. If there is prominent neutrophilic polymorphonuclear leukocytes, people think it is more likely to be associated with ascending infection. Invasion of the tubules by lymphocytes may be seen to resemble the tubulitis of allograft rejection. In more chronic forms, interstitial fibrosis and tubular atrophy is the most prominent feature that may be accompanied by an infiltrate comprised only of small lymphocytes.

1.7.1-Mechanism of insult in ATIN:

The mechanisms by which various aetiologic agents can mediate renal tubulo-interstitial injury can be direct through cytotoxic effects or indirect by the induction of systemic inflammatory or immunologic reactions. Direct cytotoxic mechanisms are dose and exposure duration dependent, such as that which is seen in analgesic and lead nephropathy; such processes are often chronic and do not induce ATIN.

Indirect reactions are often idiosyncratic as occurs in the ATIN associated with non-steroidal anti- inflammatory agents (NSAIDs). The nature of the injury may be determined to some extent by factors unrelated to the agent such as pre-existing renal disease, or extra renal factors that can affect renal dosage such as abnormal liver function. In addition, the differences in susceptibility of different nephron segments can modify the renal response to these agents, such as with heavy metal exposure.

As discussed in section 1.3, studies in experimental models as well as observation in human disease provide compelling evidence for immune mechanisms of tubulo-interstitial disease (9, 299).

1.7.2-Detailed pathological features of ATIN:

Grossly, the kidneys are enlarged, and the degree of enlargement is proportionate to the extent of involvement. Infectious processes with suppuration produce abscesses and, if there is associated obstruction, pyonephrosis. Extension of the infectious process through the renal capsules results in perinephric abscess. Drug reactions and ATIN due to immunologic injury result in large, pale, and swollen kidneys. The external surface is smooth (1).

The most striking histological finding is the presence of numerous cells, mainly mononuclear, in the renal interstitium. Tubular changes, characterized by areas of

epithelial injury and interstitial oedema, or fibrosis and oedema, are usually seen.The absence of glomerular lesions and significant glomerular deposits by immunofluorescence is required for differential diagnosis from primary glomerular disease, lupus glomerulonephritis, essential mixed cryoglobulinaemia, and vasculitis, all conditions in which prominent interstitial cell infiltrates may be present.

1.7.2.1-Microscopic findings:

Under light microscopy, the interstitial infiltrates of mononuclear cells and associated areas of oedema are multifocal and vary in intensity. Most of the mononuclear cells are lymphocytes but plasma cells can be seen occasionally. There are also numerous monocyte/macrophages. Polymorphonuclear cells make up a very limited number of the cell infiltrate as compared to lymphocytes and when present in higher number may indicate acute pyelonephritis. Eosinophils constitute only a small proportion of the interstitial cells, even in drug-induced ATIN, where their presence is probably indicative of an allergic response (17) (there is no specific agreement about whether this should be called acute eosinophilic tubulointerstitial nephritis).

Epithelioid and non-caseating giant-cell granulomas are demonstrated in some cases, especially in those in which ATIN is related to drugs (93, 94). Interstitial granulomas were found in 27% (300) and 45% (301) of patients with drug-induced ATIN. Patients with interstitial granulomas were found to have an oliguric presentation and permanent renal damage more often than those without (300). Granulomas are not a specific finding of drug-induced ATIN as they can be also found in ATIN related to infections or in cases of unknown aetiology. Most of the interstitial lymphocytes are of T lineage, with CD4+ cells and CD8+ T cells occuring in roughly equal proportions (17).

Expression of HLA class Π antigen on T lymphocytes has been observed (97, 302) while CD25 expression (interleukin-2 receptor) was rarely found (97).

Many interstitial cell infiltrates have been shown to express LFA-1 and VLA-4 integrin molecules by immunohistochemical studies (303).

Tubular injury includes tubulitis (T-lymphocytes infiltrate between tubular cells), breaks of the TBM, and necrosis of tubular cells and loss of tubules, depending on the aetiological agent. According to Ivanyi, B, et al. (304), tubulitis more often involves the distal nephron. Aberrant expression of HLA-class Π molecules by tubular cells was found in ATIN (97, 305), but no significant correlation between HLA-DR in tubular epithelium and intensity or the phenotype of interstitial infiltrates has been determined (305).

In ATIN, the glomeruli are often spared. Arterial and arteriolar changes are usually absent. When present in older persons, they are unrelated to the primary tubulo-interstitial process and reflect aging-associated-hypertension, or both (1).

1.7.2.2-Immunohistological findings

Immunohistochemical techniques are often used to delineate pathogenic mechanisms (and see section 1.3). Linear deposits of antibody and complement along the TBM may suggest antibody directed against or cross-reactive with TBM antigens (eg., in some patients with Goodpasture's syndrome and in renal allografts); granular deposits of antibody and complement in the TBM or interstitium (or both of these) suggest an immune complex pathogenesis; and tubulo-interstitial nephritis with a T cell and other mononuclear cell infiltrate without deposits of antibody and complement associated with the TBM, suggests a cell- mediated reaction associated with delayed-type hypersensitivity (e.g., drug reactions, tubulointerstitial nephritis with uveitis) or cytotoxic T cell injury (e,g., first-set allograft rejection).

1.8 Types of acute tubulo-interstitial nephritis

Acute tubulo-interstitial nephritis related to drugs, TINU syndrome and idiopathic ATIN are now reviewed. ATIN associated with systemic diseases is not a focus for this thesis and will not be considered further.

1.8.1 Drug-induced acute tubulo-interstitial nephritis:

In the early reports of ATIN due to methicillin, onset of renal dysfunction usually appeared after 10 to 20 days of drug therapy. It is now appreciated that the start of ATIN can manifest other types of time kinetics. For example, it can happen quickly within 2 to 3 days after re-challenge with a drug to which an individual has been previously sensitized. It can also happen de novo in response to a previously tolerated medication by the individual (76). With the enormous increase in the use of different chemotherapeutic agents over the past five decades, drug-induced acute tubulointerstitial nephritis has come to dominate this area of medicine. Although, we must not forget that overall, drug-induced ATIN is relatively rare (17).

The exact incidence of drug-induced ATIN is uncertain, as many patients with reversible acute renal failure usually do not undergo renal biopsy. Richet et al found that drug-induced ATIN comprised 0.8% of all cases with acute renal failure (260). Furthermore, ATIN was found in quarter of renal biopsies done in patients with drug-related acute renal failure.

There are different drugs including β-lactam antibiotics, non-steroidal anti-inflammatory drugs, diuretics, anticonvulsants, sulphonamides, rifampicin, phenindione, cimetidine, omeprazole and an even more heterogeneous group of other drugs perhaps

cause allergic ATIN (12, 306-308) (table 3). The majority of the reports are single case descriptions and most of these patients had received multiple drugs (17) and the evidence is very weak.

1.8.1.1-Mechanism of drug allergy:

Allergic drug reactions continue to be a serious problem as they are unpredictable and diverse in nature. There are also problems of diagnosis and, other than withdrawal of the drug, treatment is limited. A major difficulty in predicting these reactions is that the immunological processes underlying drug allergy are poorly understood. Broadly anaphylactic reactions, for example to betalactam antibiotics, are associated with specific IgE antibody (309), whereas skin reactions and other inflammatory responses, for example to sulphonamides, phenytoin, carbamazepine and penicillins, are linked to the presence of sensitized T cells (310). The primary effector cells of IgE-mediated reactions are mast cells that are activated by antigen within seconds to release histamine and other chemical mediators; this is often followed by eosinophil recruitment to tissue sites. In the case of cell-mediated reactions, T cells are presumed to release pro-inflammatory cytokines upon activation by the drug. Hence, the T cell is central to understanding drug allergy: it is important in the induction phase of the IgE antibody response as a source of interleukin 4 (IL-4) and other cytokines, and in both the induction and effector stages of T-cell mediated reactions.

1.8.1.2 T-cell recognition of drugs:

Drug-specific T cell clones can be grown and isolated from the peripheral blood of patients allergic to many drugs including penicillins, sulphonamides and lidocaine (311). The majority of drug specific T cell clones express the α β type of T cell receptor, are of

CD4+ or CD8+ phenotype, and are MHC class I or Π restricted (310), although some T cell clones that recognize lidocaine express the γ δ receptor type (311).

Patterns of cytokine production by drug-specific T-cell clones are variable. Thus, benzylpenicillin-specific clones produce predominantly a Th 1-like pattern with high IL-2 and interferon γ (IFN-γ) production, whereas sulphamethoxazole and lidocaine-specific clones show a mixed Th 0 or Th 2 phenotype (310, 312, 313).

Some benzylpenicillin-specific human T cell clones proliferate in response to synthetic MHC class Π-binding peptides that have the appropriate HLA-DR anchor residues and pericilloylated lysine in selected positions along a polyalanyl back bone. These responses are highly dependent on the precise position of the hapten but less so on the carrier sequence (313).

1.8.1.3 Drug metabolism and antigen generation:

An important factor in immune responses to drugs is whether the drug is chemically reactive and capable of covalent conjugation to carrier proteins. If not, it may require oxidative metabolism to a reactive intermediate. Examples of reactive drugs that spontaneously generate haptens are the penicillins that form amide linkages with the side–chain of lysine residues, and sulphydryl drugs such as the anti-arthritic agent D-penicillamine that conjugate by disulphide bonding to cysteine residues. Other drugs that cause allergic reactions, such as sulphonamides, carbamazepine and phenytoin, are not reactive: they are either metabolized to reactive species or may associate noncovalently with immune recognition molecules.

T cell clones derived from patients with allergic reactions to drugs such as sulphametoxazole and propyphenazone showed significantly increased proliferation in response to the eliciting drug in the presence of cytochrome P450-active liver microsomes

(314). Although, the liver is the major site for the drug-metabolizing cytochrome P450 enzymes, blood monocytes express high levels of the cytochrome P450 enzyme CYP1B1, and could be responsible for drug oxidation in the blood, skin and other peripheral tissues.

Table 3 Drug associated with acute tubulointerstitial nephritis (adapted from (17)).

Antibiotics	**Analgesics and salicylates**	**Others**
β-lactam antibiotics Amoxicillin Ampicillin Carbenicillin Cloxacillin Methicillin Nafcillin Oxacillin Penicillin G Piperacillin Cefaclor Cefotaxime Cefotetan Cephalexin Cephalothin Cephradine **Other antibiotics** Erythromycin Ethambutol Gentamicin Isionazide Lincomycin Polymyxin sulphate Quinolones: Piromidic acid Ciprofloxacin Norfloxacin Rifampicin Spiramycin Sulphonamides Cotrimoxazole Tetracyclines Minocycline Vancomycin	**Analgesics and salicylates** Aminopyrine Antrafenin Aspirin Floctafenin 5-Aminosalicylic acid Glafenin Paracetamol (acetaminophen) Salicylazosulfapyridine Sulfinpyrazone **Non-steroidal anti-inflammatory drugs** Alclofenac Clometacin Diclofenac Diflunisal Fenclofenac Fenoprofen Flurbiprofen Ibuprofen Indomethacin Ketoprofen Mefenamic acid Naproxen Niflumic acid Phenazone Phenylbutazone Piroxicam Pirprofen Sulindac Tolmetin Zomepirac	**Diuretics** Chlorthalidone Frusemide Indapamide Thiazides Tienilic acid Triamterene **Anticonvulsive agents** Carbamazepine Diazepam Diphenylhydantoin Phenobarbitone Phenytoin Valproic acid **Others** Acyclovir Ajmaline Allopurinol α-Methyldopa Amphetamine Azathioprine Bethanidine Captopril Cimetidine Clofibrate Contrast agents D-Penicillamine Foscarnet Gold bismuth salts Herbal medicines Interferon-α Interleukin-2 Phenindione Ranitidine Warfarin sodium

1.8.1.4-Clinical and pathological features of drug-associated acute tubulo-interstitial nephritis:

The reaction of acute tubulo-interstitial nephritis (ATIN) occurs in only a small number of patients taking any drug and appears never to be dose-related, except in some cases involving penicillin G and allopurinol (12, 21).

It is suggested from the literature that there may be a maculopapular rash, fever is usual and arthralgia common; sometimes the liver is involved and acute renal failure may develop, with dialysis being necessary in about a third of the patients. Microscopic haematuria is reported to be present in the majority of patients, macroscopic haematuria common, and red cell casts may be observed in the urine (315). Nephrotic range proteinuria is almost exclusively found in ATIN when related to non-steroidal anti-inflammatory drugs. The renal failure is often non-oliguric Eosinophilia is variable; it is said to be commoner in methicillin-related cases, but occurs overall in only 50% of the patients; eosinophiluria may be present (23, 316), but the absence of eosinophilia or eosinophiluria does not appear to be helpful in excluding the possibility of it being diagnosed. Hyperchloraemic acidosis and impaired urinary concentration have been reported and may persist for months after withdrawal of the drug. The value of symptoms and signs of systemic allergy in predicting ATIN were evaluated in a collaborative study: the positive predictive value for fever, arthralgia, blood eosinophilia and hepatocellular damage was low (only 0.60) because these symptoms were also found in 24% of patients with drug- induced acute tubular necrosis (317). Because of this overlap, it appears that the diagnosis of ATIN can be established only by renal biopsy.

Many of the laboratory tests used for diagnosing a hypersensitivity reaction lack both sensitivity and specificity (21). Circulating antibodies to penicillin, rifampicin, or

glafenin and their derivatives have been found in some cases of ATIN attributed to these drugs. Circulating antibodies against the tubular basement membrane have been described in some case reports of ATIN after the use of methicillin, cephalothin and diphenylhydantoin (17).

Most patients recover fully, provided the drug responsible is removed. The recovery of renal function depends on how long the renal failure had continued before discovery of ATIN (11). The benefits of treatment with steroids are highly controversial. The evidence to support the use of steroids in drug-induced ATIN comes from anecdotal reports (318) as well as from small, uncontrolled, non-randomized studies (11, 308, 319). In one study of fourteen patients, those treated with prednisolone (n=8) had an earlier and more complete return to base line serum creatinine than those left untreated (n=6) (308). The risks of this therapy must be weighed against its benefits in any given patient.

Neilson et al(9) believe that a limited course of high dose prednisolone is advisable for biopsy-proven ATIN, if renal failure has persisted for more than one week after the removal of any inciting factor and that steroids should be discontinued if no response is obtained after 3 to 4 weeks of treatment. However, if steroids are to be of benefit, it would seem to be more logical to begin treatment immediately the diagnosis is established providing there are no contraindications and to reduce the dose in accordance with the response, whether this an improvement in renal function or a continuation of the symptoms.

1.8.1.4.1-Antibiotics

1.8.1.4.1.1-β-Lactam

Many cases of methicillin and penicillin-induced ATIN were reported during the 1970s. The incidence of renal dysfunction ranged between 12% and 20% of patients treated for staphylococcal infections, or receiving prophylactic treatment before cardiac surgery (25). Methicillin is now no longer used; and other anti-staphylococcal antibiotics such as flucloxacillin are preferred.

The sex incidence of penicillin-induced ATIN is about 2-3 males: 1 female (12). Signs and symptoms of ATIN are reported to appear between 2 and 60 days after the start of treatment (320). Macroscopic haematuria, skin rash, blood eosinophilia and eosinophiluria are present in one-third of the patients. Half of the adults with ATIN have increased blood urea. Recovery occurs in 90% of cases. Interestingly sodium wasting, hyperkalaemia and distal tubular acidosis are prominent features in some patients (320). Allergic reactions are much less frequent with current penicillin derivatives used in practice than in methicillin-related ATIN, including ampicillin (94, 301, 318), amoxicillin (301), penicillin G (90, 318, 321) and piperacillin (322).

Re-challenge with the drug or with a chemically related one can quickly lead to recurrence of the renal and extra-renal symptoms and all β-lactam antibiotics are best avoided in patients who have developed penicillin-related ATIN during treatment with a penicillin compound or a cephalosporin (25). The frequency of cross-allergenicity between the two groups of drugs is not reported with any certainity, but probably is around 5%-10%. However, cephalosporins are rarely responsible for ATIN (21).

1.8.1.4.1.2-Sulphonamides

Many cases of ATIN following the use of normal or high doses of co-trimoxazole (trimethoprim and sulphamethoxazole) have been reported (21, 94, 301, 318). Signs of hypersensitivity are often absent, severe interstitial infiltrates with eosinophils and granulomas are frequently found in renal biopsies, and full renal recovery does not seem to occur in the majority of affected individuals.

1.8.1.4.1.3-Other antibiotics associated with ATIN

Since the first report of rifampicin-associated ATIN in 1971 (323), it has always been found in association with tuberculosis. The treatment regimen has often been intermittent and only a small number of cases have resulted from continuous daily treatment. A few hours or days after taking the drug, the patients developed chills, myalgia, fever, lumbar pain, nausea or vomiting and dark urine. Skin rash, eosinophilia, thrombocytopenia, haemolysis and even hepatitis are possible but inconsistent features. Most patients with rifampicin-induced ATIN have abrupt oliguria and require dialysis (301, 324) but tubular dysfunction with or without progressive renal failure has also been described (325).

Renal biopsies show a typical ATIN with focal tubular necrosis or atrophy in 50% of cases. In the remainder, marked injury of proximal tubules and a little interstitial infiltration is observed. Interstitial granulomas are sometimes present (326). High titres of anti-rifampicin antibodies have occasionally been found during the acute phase. These observations argue for an immune-complex pathogenesis and suggest, at least in some cases, that rifampicin has a direct toxic effect (13).

Most patients make a full recovery, but a few suffer permanent interstitial fibrosis (12). There is no evidence that steroids hasten recovery and renal failure may progress despite continuous prednisolone therapy.

In a small number of cases other antibiotics have been implicated in the development of ATIN for example, vancomycin, tetracycline, erythromycin, nitrofurontoin, and also quinolone derivatives, such as piromidic acid, norfloxacin, levofloxacin and ciprofloxacin.

1.8.1.4.2-Non-steroidal anti-inflammatory drugs:

The first case reports of ATIN from these drugs were made in 1979 (327). Almost all the various NSAIDs in clinical use, which vary in their chemical structure, have been implicated. There may be no symptoms or signs of hypersensitivity but the renal insufficiency has a progressive onset, discovered several months or years after the start of treatment, because the affected patients usually remain polyuric rather than becoming oliguric. More than 80% of ATIN cases related to NSAIDs develop a nephrotic syndrome compared to less than 1% when it is related to betalactams (21).

Withdrawal of the offending drug usually leads to resolution and there is no evidence that steroids hasten or improve the result (12).

1.8.1.4.3-Other groups of drugs associated with ATIN

1.8.1.4.3.1-Analgesics and salicylates

Biopsy-proven ATIN has been found in a few patients receiving therapeutic doses of paracetamol (301, 318). Salicylate derivatives may lead to ATIN including 5-aminosalicylic acid, used as primary treatment and maintenance therapy in inflammatory bowel disease (21, 328).

1.8.1.4.3.2-Diuretics

Thiazides and frusemides have been most commonly implicated in diuretic – induced ATIN: both are of course both chemically related to sulphonamides. Acute renal failure develops several weeks after the start of treatment with signs and symptoms of systemic allergy. On biopsy, there are interstitial infiltrates and epithelioid granulomas; immunofluorescence is negative. Withdrawal of the drug, with or without steroid treatment, leads to rapid recovery of renal function in all cases (12).

Bendrofluazide (318), hydrochlorothiazide alone (93) and tierilic acid (301, 324) have also been thought responsible.

1.8.1.4.3.3-Miscellaneous

The potential for drugs to cause ATIN is large. However, some additional relevant examples may also include the following.

Acute tubulo-interstitial nephritis due to allopurinol sensitivity has been described sometimes with granuloma formation, mostly in patients with pre-existing renal impairment and relative over-dosing or on treatment with thiazides, which lead to increased blood concentrations (86, 329, 330). A return to base line renal function occurs after allopurinol is stopped.

Cimetidine has been implicated as a cause of ATIN in several patients. Several weeks after the start of treatment, fever, myalgia and non-oliguric renal insufficiency develop (21, 96, 262). Eosinophils are found in serum, urine and within the biopsies of renal interstitium. Most patients regained normal renal function after withdrawal of the drug, but residual renal damage is found in some cases (321, 331, 332). Ranitidine is responsible for some cases of acute tubulo-interstitial nephritis (333, 334).

Omeprazole is a commonly used proton pump inhibitor that has been implicated in many cases of ATIN (335, 336). Hypercalcaemia has been reported with omeprazole-induced ATIN and attributed to granuloma-derived PTH-related peptide but steroid therapy has returned the serum calcium and creatinine to normal levels (337).

There are many other drugs which have been suspected of causing ATIN, for example, captopril, diphenylhydantoin, valproate, warfarin, phenobarbital, streptokinase, acyclovir, interferon-α, Chinese herbal medicine, recombinant interleukin 2, clozapine and rofecoxib.

1.8.2-TINU syndrome (tubulo-interstitial nephritis and uveitis syndrome):

Idiopathic tubulointerstitial nephrits and uveitis syndrome (TINU Syndrome) is a relatively uncommon syndrome (1). When the causal agent for ATIN cannot be identified, it is termed idiopathic acute tubulointerstitial nephritis (I ATIN). It may appear alone or associated with uveitis (338), but the concomitant development of these disorders raises the concept of a renal-ocular syndrome. In 1975, Dorbin et al (339) described a new syndrome consisting of acute renal failure secondary to ATIN associated with lymph node and bone marrow granulomas and anterior uveitis. Moreover, sporadic cases of ATIN have been described with granulomas in bone marrow without uveitis (340), with hypocomplementemia, eosinophilia and tubular deposits of IgE and complement C_3 (341, 342) and with deafness (343). The great majority of the cases have been adolescent females. The uveitis normally follows the onset of renal problems, and often appears as they are resolving. The condition is normally preceded by symptoms such as asthenia, myalgias, loss of weight, vomiting, fever, anorexia, and abdominal pain (338, 344), headache or nausea (345). An increase in the ESR, anaemia (95) and

hypergammaglobinemia (346) are often found. The kidneys are affected by ATIN with a predominant mononuclear infiltrate in the majority of cases (344, 346). The clinical form of renal disorder at onset is acute renal insufficiency with preserved diuresis (345, 347) and less commonly, polyuria or Fanconi's syndrome (345).

Although the aetiology is still unknown, the immunochemical findings point to an autoimmune cause, with involvement of cell mediated immunity (348). These findings consist of an increase in the levels of immunoglobulins and circulating immunocomplexes (338), decrease in T cells (347), absence of specific immunofluorescence, and presence of helper T lymphocytes in the renal interstitium. The suppression of the peripheral immune reactivity in contrast with the increase in immune reactivity in inflammed sites makes this condition similar to sarcoidosis.

Systemic corticosteroids usually resolve the uveitis (344). Azathioprine has not proved effective in the prevention of exacerbations of uveitis (95), in contrast to the favourable response to cyclosporine A. In almost all cases, the nephropathy responds to steroid treatment (345). Some cases have resolved spontaneously (344).

The evolution is generally benign, with complete resolution of symptoms in almost 100% of reported cases. The uveitis follows its course independently from the nephropathy and sometimes tends to relapse (344).

1.8.3.-Epstein-Barr virus (EBV) and acute Tubulo-interstitial nephritis:

1.8.3.1-History:

The Epstein-Barr virus (EBV) was found 45 years ago in the cultured cells f

using electron microscope by Epstein, Achong, and Barr (349).

In 1968, EBV was discovered as the cause of infectious mononucleosis (350) and its DNA was detected in tissues from patients with nasopharyngeal carcinoma in 1970 (351). In 1980, EBV was associated with non-Hodgkin's lymphoma and oral hairy leukoplakia in patients with the acquired immunodeficiency syndrome (AIDS) (352, 353). Following these discoveries EBV DNA has been found in tissues from other cancers, including T cell lymphomas and Hodgkin's disease (354, 355). EBV infects 90 per cent of humans and persisting for the lifetime of the person.

EBV has been linked to ATIN (see section 1.8.3.2.5) and one aim of the present thesis was to investigate this association further.

1.8.3.2-Virus features:

1.8.3.2.1-Replication:

EBV is a ubiquitous human herpes virus. Two EBV types circulate in most human populations. The two types differ in only a few genes, but there are significant and consistent genetic differences between the two alleles characteristic of the few genes that are type specific. Some of the differences are important in biological activity. The two types were originally referred to as types A and B; however, a change to types 1 and 2 was suggested to make the nomenclature similar to that for herpes simplex virus types. EBV is the prototype lymphocryptovirus.

The viral genome is within a nucleocapsid surrounded by the viral envelope. In order to enters the B cell, the viral gp 350 (major envelope glycoprotein) binds to the CD21 (viral receptor and the C3d complement receptor), on the surface of the B cell. The major-histocompatibility-complex (MHC) class Π molecule serves as a cofactor for the infection of B cells (231). The viral genome composed of linear DNA molecule that contains 100 viral proteins (356). These proteins control the appearance of viral genes, replicate viral DNA, build-up the virion basic elements, and adjust the human immune response. Epithelial cell infection by EBV in vitro leads to active replication, with lysis of the cell and creation of the virus (357). On the otherhand, B cells infection by EBV in vitro leads to a latent infection, with immortalization of the cells. After infecting B cells, the linear EBV genome becomes circular and forms an episome. Viral clonning is automatically stimulated in very small percentage of infected B cells. Human Infection with EBV usually happens by contact with saliva. The virus replicates in the oropharynx cells, and all seropositive individuals engergetically spill virus in the saliva (358, 359).

1.8.3.2.2-Latent infection:

Persistance of the EBV in human beinging is localised to memory B cells (360). Spilling of EBV from the mouth is stopped in patients treated with acyclovir, while the number of EBV-infected B cells in the blood stays unchanged (358). Furthermore, as EBV can be cleared in bone marrow-transplant recipients who have received therapy that kills their hematopoietic cells, but not their oropharyngeal cells (361), supports the suggestion that B cells are the site of EBV persistence. In normal adults, around 50 B cells per million in the blood are infected with EBV, and the number of latently infected cells endures constant over years (360, 362). Only 10 viral genes are appeared in latently infected B cells despite the 100 genes appeared during replication in vitro (356). Two types of non-

translated RNA, six nuclear proteins, and two membrane proteins are appeared in these latently infected B cells.

The EBV nuclear antigen (EBNA) 1 protein attached to viral DNA to keep the EBV genome in the B cell as a circular DNA episome (363). EBNA-2 stimulates the appearance of EBV latent membrane protein (LMP) 1, LMP-2, and cellular proteins that augment the development and alteration of B cells. The EBNA-3 proteins also control the appearance of cellular genes (364), whereas EBNA leader protein help the EBNA-2 to stimulate LMP-1.

LMP-1 can work as an oncogene (365), and induction of this protein in transgenic mice leads to B cell lymphomas (366). LMP-1 mediated signalling resembles stimulated CD40 molecule on the B cell surface (367). LMP-1 attachs to receptors of the tumour necrosis factor in vitro (368) and, in EBV-positive lymphomas, in vivo (369). Ultimately nuclear factor-κ B (NF-κ B) transcription factor is stimulated in vitro and in vivo, as well as stimulation of c-jun, induction of cellular adhesion molecules, cytokine production, and B-cell growth.

In the presence EBV LMP-2, reactivation of EBV from latently infected cells stopped by blocking tyrosine kinase phosphorylation (370). The non-converted types of EBV-encoded RNA (EBER) have no proteins, but they are crucial for oncogenesis and resistance to apoptosis (programmed cell death) (371). Dieases associated with EBV show viral gene expression as one of three patterns of latency (372); only EBNA-1 and EBER are expressed in type one; EBNA-1, LMP-1, LMP-2, and EBER are expressed in type two; and all the latency genes are expressed in type three. A fourth pattern of latency is seen in B cells from the peripheral blood of healthy persons infected with EBV in the past, in

which only EBER and LMP-2, and sometimes EBNA-1 RNA have been detected (373) (Table 4).

Table 4- Expression of EBV latent genes (373)

Pattern of latency	EBNA-1	EBNA-2	EBNA-3	LMP-1	LMP-2	EBER
Type 1	+	-	-	-	-	+
Type 2	+	-	-	+	+	+
Type 3	+	+	+	+	+	+
Other	±	-	-	-	+	+

1.8.3.2.3-LMP-1:

The first ORF (open reading frame) of EBV encodes a well-characterized transforming protein, LMP-1. LMP-1 is a 63 KDa integral membrane phosphor-protein with at least three domains: (a) a 20-amino acid hydrophilic amino-terminus lacking the characteristics of signal peptides; (b) six markedly hydrophobic 20 amino acid, alpha helical, transmembrane segments, separated by five reverse turns, each eight to ten amino acids in length; and (c) a 200-amino acid carboxy-terminus, rich in acidic residues.

Immunofluorescence microscopy shows that about half of the LMP-1 molecules are in plasma membranes (374). LMP-1 is phosphorylated on serine and threonine residues at a ratio of 6:1 and is not phosphorylated on tyrosines (375). Half or more of LMP-1 is associated with the cytoplasmic cytoskeleton as defined by resistance to extraction with buffers supplemented with non-ionic detergents (375). The cytoskeletal form is

phosphorylated, whereas the soluble LMP-1 is not phosphorylated (375). The half-life of the soluble form is less than 2 hours, whereas the half life of the cytoskeletal form of LMP-1 is of the order of 3 to 15 hours. LMP-1 is cleaved near the beginning of the carboxy-terminal cytoplasmic domain, resulting in a soluble c-terminal domain of about 25 Kd (375). The principal serine and threonine phosphorylation sites are near each other in the 25-kd cleavage product (375). LMP-1 forms discrete patches in the plasma membrane that are often further organized into a single cap-like structure (376). Unlike growth factor receptors, which form patches and caps in response to ligand binding, LMP-1 constitutively forms patches in LCL (lymphoblastoid cell lines) plasma membranes in the absence of exogenous growth factors (376). LMP-1 induces many of the changes usually associated with EBV infection of primary B lymphocytes or with antigen activation of B-lymphocytes, including cell clumping; increased villous projections; increased Vimentin expression; increased cell surface expression of CD23, CD39, CD40, CD44 and class II major histocompatibility complex (MHC); increased IL-10 expression; decreased expression of CD10; and increased exhibition of the cell adhesion molecules LFA-1, ICAM-1, and LFA-3. LMP-1 protect B-lymphocytes from apoptosis (377). These actions mediated in part through the induction of bcl-2 by LMP-1 and probably also through induction of A20 (377). LMP-1 not only increases plasma membrane expression of adhesion molecules, but also functionally activates adhesion and induces higher levels of LFA-1 mRNA (364). Each of these effects in BL (Burkitt's lymphoma) cells is dependent on the particular cell background. Some BL cell types already express high levels of adhesion molecules. In such cells LMP-1 has no effect on adhesion molecule expression. The effects of LMP-1 on epithelial cell growth were first demonstrated by expressing LMP-1 in the skin of transgenic mice, in which LMP-1 induces epidermal hyperplasia and

alters keratin gene expression (378). Similar effects were observed when LMP-1 was expressed in immortalized human keratinocytes grown in monolayer cultures, where LMP-1 alters keratinocyte morphology and cytokeratin expression (379), or stratified air-liquid interface raft cultures of immortalized epithelial cell lines, where LMP-1 inhibits cell differentiation (380). EBV recombinants with mutations in LMP-1 that result in expression of proteins which are deleted for the amino-terminal cytoplasmic domain and the first trans-membrane domain or larger deletions of the amino-terminus result in a non-transforming phenotype (381). Surprisingly, this effect is not due to specific interactions mediated by the amino-terminal cytoplasmic domain (382). Deletion of any part of the amino-terminal cytoplasmic domain result in no more than a 90% effect on transforming efficiency and the transformed cells grow well (383). In contrast, deletion of all of the carboxy-terminal cytoplasmic domain results in a complete loss of the ability to transform primary B lymphocytes, and deletion of all but the first 44 amino acids of the carboxy-terminus results in the inability to transform primary B lymphocytes without fibroblast feeder layers (384). The LMP-1 interactive protein also interacts with the CD40 cytoplasmic domain and with the lymphotoxin beta cytoplasmic domain, probably explaining the similarity between LMP-1 and CD40 cross-linking effects on B lymphocyte growth. LMP-1 also induces higher level expression and associates with TRAF 1, another tumour necrosis factor receptor associated protein. LMP-1 and EBNA-1 are the only latent infection-associated genes that are also transcribed in lytic EBV infection.

1.8.3.2.4-EBERs:

The EBV-encoded, small non-polyadenylated RNAs are by far the most abundant EBV RNAs in latently infected cells. Estimates of abundance place the EBERs at 10^7 copies per cell (385). Most of the EBERs localize to the cell nucleus, where they are

complexed with cellular La protein (386). La protein complexes are recognized by specific antisera from patients with systemic lupus erythematosus. The EBER RNAs have stable secondary structures so that in purified RNA or RNA-La protein complexes the RNAs are extensively intramolecularly base paired (387). La protein is associated with the 3' terminus of the EBER RNAs (387).

EBER1 and EBER2, adenovirus VA, and U6 cell RNA, may have similar primary sequences, secondary structures, and association with La protein in order to accomplish similar functions. The EBERs could be expected to be expressed as the earliest or one of the earliest EBV RNAs.

1.8.3.3-Immune response to EBV:

EBV Infection in human leads in both natural and adaptive immunity to the virus. Antibodies directed against viral structural proteins and the EBNAs are important for the diagnosis of infection. Natural killer cells and CD4+ and CD8+ cytotoxic T cells regulate growth of EBV-infected B cells during initial infection (388). In glandular fever, 40 per cent of CD8+ T cells are directed to one replicative EBV protein sequence, whereas 2 per cent are directed to one latent EBV protein sequence (389). Post acute infection, HLA-restricted cytotoxic T cells are crucial in regulating EBV, and CD8+ T cells are directed to the same percentages of replicative and latent antigens (390). EBV exhibits a cytokine and a cytokine receptor that essential to change the body's response to allow infection's persistence.

The EBV BCRF1 protein resembles IL-10 (inhibitory cytokine) in 70% of its amino acid sequences (391). The BCRF1 protein inhibits IFN-γ synthesis by human peripheral-blood mononuclear cells as IL-10 in vitro (392).

The EBV BARF1 protein works as a soluble receptor for CSF-1. Since CSF-1 increases the induction of IFN-α by monocytes, BARF 1 protein can act as a decoy receptor to stop the action of the cytokine (393). Since IFN-γ and IFN-α abolish the proliferation of EBV-infected cells in vitro, the BCRF1and BARF1 proteins enhance the virus to evade the host's immune system during acute infection or reactivation.

EBNA-1 can stop its breakdown by proteosomes in the cell (394). Viral proteins are normally degradated by proteosomes to peptides for presentation to cytotoxic T cells, so the ability of EBNA-1 to inhibit its breakdown leads the protein to avoid triggering the stimulation of cytotoxic T cells. The EBV has two proteins that abolish apoptosis. The EBV BHFR1 protein is similar to the human bcl-2 protein, which also blocks apoptosis (395), whereas EBV LMP-1 controls the appearance of cellular proteins that stop apoptosis, including bcl-2 and A20 (366).

1.8.3.4-Diseases associated with EBV:

1.8.3.4.1-Infectious mononucleosis:

EBV infections in childhood are asymptomatic, while infections of adulthood leads to infectious mononucleosis (396). Half of patients with infectious mononucleosis manifest fever, lymphadenopathy, and pharyngitis; splenomegaly, palatal petechiae, and hepatomegaly are each present in more than 10 per cent of patients. Most patients with infectious mononucleosis have an increased number of peripheral mononuclear cells, heterophile antibodies, high serum aminotransferase levels, and atypical lymphocytes. The atypical lymphocytes are primary T cells, many of which are responding to the EBV-infected B cells. Most of the symptoms of infectious mononucleosis are attributed to the growth and stimulation of T cells in response to infection. Few per cent of the peripheral B

cells may be infected with EBV and activation of B cells by EBV, lead to production of polyclonal antibodies, causes elevated titers of heterphile antibodies and occasionally causes an increase in cold agglutinins, cryoglobulins, antinuclear antibodies, or rheumatoid factor.

1.8.3.4.2-Chronic active EBV infection:

Chronic active EBV infection is uncommon disease, explained by the presence of a severe illness of

started with abnormal EBV antibody titers; histological evidence of organ disease, such as pneumonitis, hepatitis, bone marrow hypoplasia, or uveitis; and demonstration of EBV antigens or EBV DNA in tissue (397). There are association with elevated titres of virus-specific antibody.

1.8.3.4.3-Other diseases:

Apart from being the causative agent of infectious mononucleosis, EBV has been linked to B cell lymphoproliferative disorders in immunocompromised hosts (non-Hodgkin's lymphomas, Burkitt's lymphoma), Hodgkin's disease, lymphocytic interstitial pneumonitia in children, nasopharyngeal carcinoma and oral hairy leukoplakia in patients with acquired immune deficiency syndrome (AIDS)(396).

1.8.3.5-EBV and acute tubulo-interstitial nephritis:

Most of the reported articles about the association between ATIN and EBV come from the association with the infectious mononucleosis.

Renal involvement with EBV infection occurs in ~16% of the cases that come to medical attention (398). Infectious mononucleosis not infrequently has manifestations of mild renal involvement. Haematuria or proteinuria have occurred in 2% and 18% of patients studied

respectively (399). Such changes are self-limited and are rarely associated with diminished renal function (398).

The spectrum of renal involvement with EBV is broad. Mild nephropathy may result in only microscopic haematuria or asymptomatic proteinuria. Rhabdomyolysis resulting in acute renal failure has been described in association with EBV infection (399). Acute tubulointerstitial nephritis, sometimes accompanied by mesangial hypercellularity or foci of tubular necrosis, is the most common pathological finding in EBV-associated acute renal failure (399). The abnormalities usually occur acutely at the onset of illness or during the initial week of infection and may resolve spontaneously after 7-10 days. The pathogenesis of EBV-associated tubulointerstitial nephritis remains unclear. Many reports have described a predominance of cytotoxic/suppressor T cells in cases of EBV-associated acute renal failure and tubulointerstitial nephritis (400).

Therapy for ATIN is mainly supportive, but pharmacological treatment with immunosuppressive drugs may be appropriate, particularly in cases associated with autoimmune disease or glomerulonephrits (76). Controlled, prospective clinical trails of steroid therapy for EBV-associated tubulointerstitial nephritis are lacking, but several small studies supporting its use were recently summarized in a review by Michel and Kelly (286). Several patients with tubulointerstitial nephritis had diuresis or a fall in the creatinine level within 72 hours of initiation of steroid therapy, whereas a few untreated patients recovered more slowly or not at all.

Aims of the study:

Acute tubulointerstitial nephritis (ATIN) is an important cause of renal morbidity. This study examines the clinical and histological features of ATIN, any correlation between these two aspects, and their relationship to renal outcome.

Specifically, the first hypothesis to be addressed was that the clinical features at presentation would dictate the renal outcome. To analyse clinical features, a database was set up and a retrospective analysis of patients' records identified the presenting features, aetiological factors, clinical outcome, including renal outcome. The study included all cases of biopsy-proven ATIN between 1984 and 2002, being identified from a complete database of all renal biopsies performed at the Queen Elizabeth Hospital during this time period.

The second hypothesis was that leukocyte recruitment to the tubulointerstitium would reflect the aetio-pathogenic process inducing the disease or disease outcome. Histologically, ATIN was characterised by a mononuclear cell infiltrate with interstitial oedema. The pattern of infiltrating cells was compared in renal biopsies from drug-induced ATIN, idiopathic ATIN (where no antecedent aetiological factor could be identified) and ATIN associated with TINU syndrome. Immunohistochemistry was used to define different cell subtypes (eosinophil protein markers, CD3, CD4, CD8 and CD68). This provided quantitative parameters that allowed accurate analysis of patterns of infiltrating cells in each ATIN subgroup, allowing assessment of the possible roles of these infiltrating cells in the outcome of ATIN subgroups and whether there was any difference in the infiltrating cells between ATIN subgroups.

The third hypothesis was that the recruitment of leukocyte subsets in ATIN was related to the chemokines expressed within renal tissue and so the infiltrating leukocytes

would mirror this by expression of specific chemokines receptors. In the event, only a limited analysis of all the possible chemokines and chemokine receptors that might be expressed could be undertaken. The chemokine receptor CCR3 was analysed in detail between different ATIN subgroups and colocalised with cell type of expression. The presence of interleukin-4 (IL-4) and eotaxin were also assessed by immunohistochemistry.

Finally, the fourth hypothesis was that Epstein-Barr virus (EBV) infection of proximal tubular cells might induce ATIN. Previous studies of patients with idiopathic chronic tubulointerstitial nephritis had suggested that this virus might play a pathological role. The role of EBV in ATIN was examined using immunohistological analysis for EBV associated proteins, including latent membrane protein-1 (LMP-1), and by RNA/RNA in situ hybridization for EBV-encoded, small non-polyadenlyated RNAs (EBERs).

Chapter 2:

Materials and methods

2.1-Analysis of immunohistochemistry staining of the renal tissues

2.1.1-Patients:

In a retrospective study, the reports of all acute renal failure (which is defined as a deterioration in renal function occurring over hours or days) biopsies (n=976) done at Queen Elizabeth University Hospital from 1984 to 2002 were reviewed and the diagnosis classified. The indication for biopsy was symptomatic or asymptomatic renal insufficiency, proteinuria and haematuria with signs of recent onset (preserved kidney size).

The records of all patients with ATIN (n=78) were reviewed with regard to a past history of renal disease, causative agent, the clinical data, and the clinical course, including therapeutic measures.

The causes of ATIN were defined according to the most probable temporal association.

Renal biopsies were taken with informed consent from the patients. The control samples were taken from the unaffected pole of nephrectomy specimens, removed for renal cell carcinomas.

ATIN was defined as a condition in which there is an inflammatory infiltrate in the interstitial tissues of the kidney, particularly the cortex, with interstitial oedema and acute damage to tubules. The inflammatory cells are mainly lymphocytes, macrophages, plasma cells and eosinophils, in various proportions. There may be granulomas but the term ATIN was not used if there was tuberculosis, nor if there was predominantly chronic renal damage, as there usually is in sarcoidosis. This definition excludes:

1- Conditions in which there are collections of neutrophil polymorphs, particularly ascending infection of the kidney, also called pyelonephritis.

2- Conditions in which the interstitial inflammation is part of more widespread disease in the kidney, such as in lupus nephritis and renal vasculitis (5, 401).

3- Conditions in which there is known or suspected direct bacterial infection of the kidney, such as leptospirosis.

2.1.2-Statistical methods:

Patients with ATIN were divided into three distinct groups for comparison: Drug-induced ATIN, Idiopathic ATIN, and TINU syndrome. The groups were compared for clinical and histological characteristics. Statistical significance was determined by the $\chi 2$ distribution tests. A two- sided P value of < 0.05 was considered statistically significant. Tarone-Ware test was used to measure the P value as it takes into account the number of cases at risk at each time point.

The outcome of the patients with ATIN was measured by dividing the patients into two distinct groups for comparison: those with reversible and those with irreversible renal insufficiency. Renal insufficiency was defined as fully reversible if creatinine level at 3 months was less than 150 μmol/l, and irreversible if it was higher than that level. The influence of some selected variables on discrimination between the two groups was investigated (clinically relevant parameters were selected). A computer system with the statistical package for the social sciences (SPSS version 10) was used for analysis.

2.2-Immunohistochemistry: Antigen retrieval and indirect immunostaining:

The tissue specimens were obtained by percutaneous biopsy, fixed in formalin, embedded in paraffin, and cut in 4 µm thick sections. Immunohistochemistry was performed using a streptavidin/horseradish peroxidase based method and visualised using diaminobenzidine and Mayer's haematoxylin. This entailed three basic steps: dewaxing and hydration of tissue sections, antigen retrieval and immunostaining.

Sections were first placed in xylene for 5 minutes. This was repeated with a fresh bath of xylene before placing it in ethanol 99% for 5 minutes, followed by another ethanol 99% bath for 5 minutes. Sections were rinsed under water and transferred to a Tris-Buffer Saline (TBS) bath (Appendix A), then they were labelled and a well created by a PAP pen. Endogenous peroxidase activity was blocked for 30 minutes with 30% hydrogen peroxide in methanol in a ratio of 1:100 at room temperature and then washed for 10 minutes in TBS pH 7.6 (Appendix A).

In order to retrieve antigen masked by the waxing process, sections were incubated in boiling 10 mM citric acid, pH 6.0, simmered for 30 minutes in a microwave oven. Following this they were washed in running water, before being rinsed by TBS pH 7.6. This was used in the staining for LMP-1.In the staining for CD3, CD8 and CD4 we used TRIS/EDTA solution, PH 8.0 (Appendix B), for antigen retrieval instead of citric acid. For the staining of CD68, mast cell tryptase and Eosinophil Major Basic Protein (E-MBP) the antigen was retrieved by trypsinisation. This involved preheating 200 ml of phosphate buffer saline [PBS] pH 7.4 (Appendix C) to 37ºC (approximately 45 seconds in a microwave), adding 0.2g trypsin and the sections to the PBS, and incubating at 37ºC for 15 minutes. The sections then washed well in running water.

Primary antibodies and appropriate isotype controls were added at the required concentrations (which gives a good staining at a lower dilution and avoids non-specific staining) and incubated for one hour in a humidified chamber at room temperature, then washed in TBS. Biotinylated goat anti-mouse secondary antibody (Dako Ltd, Cambridge, UK) was added at the appropriate concentration to all sections and incubated for 45 minutes and washed in TBS.

In order to amplify the strength of detection system, a streptavidin ABC/HRP complex (Dako Ltd, Cambridge, UK) was added for 45 minutes and washed with TBS. Diaminobenzidine (DAB) (Appendix D) and hydrogen peroxide (Vector Laboratories Ltd, Peterborough, UK) were added for 2 minutes and slides were developed and washed with water. Sections were then counterstained using Mayer's haematoxylin for 1 minute and washed for a few minutes under running water to develop, then dehydrated in alcohol, cleared in xylene and mounted.

Primary antibodies against the Latent Membrane Protein-1 [LMP-1]; CD3; CD8; CD68; Mast Cell Tryptase (Dako, Ltd, Cambridge, UK), CD4 (Novocastra laboratories Ltd, Newcastle, UK) and Eosinophil Major Basic Protein [E-MBP] (Biogenesis Ltd, UK) were optimised to work on paraffin-embedded sections. But after lengthy optimisations, others such as those for chemokines, chemokines receptors and eosinophil peroxidase proved to be more difficult, so frozen sections were chosen.

Wegener's Granulomatosis nasal tissues were used as a control for CD3, CD4, CD8, CD68 and mast cell tryptase, while allergic nasal polyps used as a control for eosinophil major basic protein (EMBP) and Hodgkin's disease lymph node was used as a control for the LMP-1 staining.

The primary antibodies were tested several times and by different methods (two or three stage indirect immunohistochemistry methods) on control tissues to achieve optimal dilutions and optimal staining intensity with minimal background, before they were applied to renal tissues. For each primary antibody there are two control tissues (positive and negative) and normal kidney tissue.

Table 5- Flow Chart of ABcomplex method

Avidin-biotin-enzyme complex (ABC) method:

1-Place slides in metal (plastic) racks and place them in xylene 1. (dewax) for 5 minutes.
2-Drain slides and Place them in xylene 2 for 5 minutes.
3-Drain slides and Place them in IMS 1 (rehydrate) for 5 minutes.
4-Drain slides and Place them in IMS 2 for 5 minutes.
5-Wash them in running water for 3-5 minutes.
6-Dry around each section and circle them with PAP Pen (wax Pen).
7-Block endogenous peroxidase using 30% hydrogen Peroxide in methanol for 30 minutes at room temperature (1ml H_2O_2 in 100 ml methanol).
8-Wash them in running water for 5 minutes.
9-Pretreat with heat if required i.e Microwave.
a-Place in plastic slide holder and immerse into a beaker containing 3.15 gram citric acid in 1.5 litre of distilled water and its PH adjusted to 6.0 using 10 NNaOH solution.
b- Put the buffer into the microwave and on full power to the boil. When boiling remove the beaker and add the sections, Put the beaker back into the microwave, set the timer to 30 minutes and heat until boiling again then turn the microwave down to (level 6) and leave until time finished.
c- after microwave wash in running water for 10 minutes.
10- Rinse in Tris buffer (TBS) PH 7.6
11- Incubate with optimally diluted primary antibody in appropriate 10% normal Swan serum for 60 minutes.
12- Wash in Tris buffer (TBS) PH 7.6 for 10 minutes (on stirrer).
13- Add secondary antibody (Duet kit solution C diluted 1:100) for 45 minutes.
N.B at this point make –up tertiary reagent (Duet kit Solution A+B diluted 1:1:100) and leave to stand.
14- Wash in Tris buffer (TBS) PH 7.6 for 10 minutes (on stirrer).
15- Add tertiary reagent for 45 minutes.
16- Wash in Tris buffer (TBS) PH 7.6 for 10 minutes.
17-Visualise with DAB solution for 2 minutes.
18- Wash in running water for 5 minutes.
19-Counterstain with Mayer's Haematoxylin for 1minute.
20- Wash under cold water for 2 minutes.
21- Wash under hot water for 1 minute.
22- Rinse again in cold water.
23- Drain and place into IMS 2 for 5 minutes.
24- Drain and place into IMS 1 for 5 minutes.
25- Drain and place into xylene 2 for 5 minutes.
26- Drain and place into xylene 1 for 5 minutes.
27- Keeping the rack in xylene, take slides one at a time out of the rack, wipe dry, and mount using one small drop of Piccolyte and an appropriately sized coverslip.
Press out air bubbles using finger nail.

2.3-Immunohistochemistry of frozen renal tissue:

Tissue was immediately snap frozen and cut into 6µm cryostat sections, mounted onto glass slides and allowed to dry at room temperature for at least 1 hour.

Slides were fixed in acetone for 15 minutes at room temperature, allowed to dry for 40 minutes, then wrapped in foil and stored at –70ºC until use.

Immunohistochemistry was performed using a streptavidin/horseradish peroxidase based method and visualised using diaminobenzidine and Mayer's haematoxylin.

Sections were taken out the freezer and left at room temperature to warm-up, then encircled by PAP-Pen. Endogenous peroxidase activity was blocked with 30% hydrogen peroxide (H_2O_2) in methanol solution for 30 minutes, and washed for 10 minutes in TBS pH 7.6 (Appendix A).

Primary antibodies and appropriate isotype controls were added at the optimised concentrations and incubated for 1hour at room temperature (except RANTES antibody was incubated overnight), then washed in TBS pH 7.6 (Appendix A). Biotinylated goat anti-mouse secondary antibody (Dako Ltd., Cambridge, UK) was added at appropriate concentration to all sections and incubated for 45 minutes and washed in TBS pH 7.6.

The protocol was the same as described in section 2.2 from this step onwards. In the case of the CCR5 antibody staining, a 2-step indirect immunoperoxidase method was used (with rabbit anti-mouse peroxidase labelled as secondary antibody) because it gave little background staining.

The primary antibodies against the eosinophil peroxidase (Serotec, Oxford, UK), human eotaxin and RANTES (R&D, UK) were used.

The expression of some chemokines receptors that are upregulated on activated Th cells including the chemokine receptors CCR3 (Leukosite, Cambridge, UK) on Th2 cells and

CCR5 (R&D, UK) on Th1 cells were also studied.These and other antibodies used are listed in table 6.

Appropriate IgG1, IgG2a and IgG2b isotype controls were included (Dako Ltd, UK). Two slides of nasal polyps were used as control tissues (positive and negative) for each of the above mentioned primary antibodies.

2.4- Analysis of immunohistochemistry:

Positive staining was measured using Aequitas IA image analysis software (Dynamic Data Links, Cambridge, UK).

The cells were counted using X40 power for the whole specimen, and the surface area of each slide was measured using Aequitas IA data analysis software, and the computer image was calibrated to give the area in μm^2. All parameters, including the threshold detection levels, were held constant throughout. Quantitative analysis was performed for CD3+ T cells, CD4+ T cells, CD8+ T cells, CD68+ macrophages and eosinophil major basic protein (E-MBP). Data from the image analysis and quantitative analysis were analysed using non-parametric tests. The comparisons were between the patients groups, therefore unpaired tests were carried out.

For the ATIN frozen sections, which were stained for chemokines and chemokine receptors, semiquantitative analysis was carried out as only seven frozen cases were available, most of the stain were not sharply localized and for that reason it could not counted. Semiquantitative analysis comprised three categories according to the extent of immunostaining in the tissues as follows: (+) under 1/3 of the tissue stained, (++) for 1/3-2/3 of the tissue stained and (+++) over 2/3 of the tissue stained

Table 6- Antibodies used for immunohistochemistry

Antibody	Company	Type & Number of control tissues	Optimal concentration	Type&Number of tissue
LMP-1	Dako	Lymph node (12)	1 in 3200,MM	Paraffin section (80)
E-MBP	Dako	Nasal polyps (6)	1 in20, MM	Paraffin section (34)
E-Peroxidase	Serotec	Nasal polyps (2)	1 in 200, MM	Frozen sections (7)
Eotaxin	R&D	Nasal polyps (2)	1 in 50, MM	Frozen sections (7)
CCR3	Leukosite	Nasal polyps (2)	1 in 100, MM	Frozen sections (7)
CCR5	R&D	Nasal polyps (2)	1 in 100, MM	Frozen sections (7)
RANTES	R&D	Nasal Polyps (2)	1in 20, GP	Frozen sections (7)
IL-4	Peprotech	Nasal polyps (2)	1 in 400, MM	Frozen sections (7)
VCAM-1	Dako	Nasal polyps (2)	1 in 50, MM	Frozen sections (7)
HLA-Class2	Dako	Nasal polyps (2)	1 in 50, MM	Frozen sections (4)
CD25	Dako	Nasal polyps (2)	1 in 50, MM	Frozen sections (4)
CD3	Dako	Nasal WG (4)	1in 200, MM	Paraffin sections (34)
CD8	Dako	Nasal WG (4)	1 in 200, MM	Paraffin sections (34)
CD4	Novocastra	Nasal WG (2)	1 in 10, MM	Paraffin sections (14)
CD68	Dako	Nasal WG (2)	1 in 200, MM	Paraffin sections (27)
Mast Cell tryptase	Dako	Nasal WG (2)	1 in 200, MM	Paraffin sections (23)

Where MM= mouse monoclonal, GP= Goat polyclonal, WG= Wegener's Granulomatosis.

2.5-Immunohistochemical double-staining method:

The original method described a peroxidase-labelled system in conjunction with an alkaline phosphatase-labelled system, the two enzymes being separately developed at the end of the two simultaneous reactions (402). So, the tissue was snap frozen and cut into 6 µm cryostat sections. The double immunohistochemistry staining was performed using a streptavidin/horseradish peroxidase and alkaline phosphatase based methods and visualised using diaminobenzidine for the first method and fast blue for the later one. Briefly, endogenous peroxidase activity was blocked with a solution of methanol, 30% hydrogen peroxide (H_2O_2) for 30 minutes at room temperature, and then washed for 5 minutes in Tris-buffered saline (TBS) pH 7.6 (Appendix A). The first primary antibody and appropriate isotype controls were added at the required concentrations and incubated for 30 minutes at room temperature then washed in TBS for 10 minutes. HRP polymer (Dako Ltd, Cambridge, UK) was added to all sections and incubated for 30 minutes and then washed in TBS. Diaminobenzidine (DAB) (Appendix D) was added for 10 minutes and slides were developed and washed with distilled water.

The second primary antibody and appropriate isotype controls were added at the required concentrations and incubated for 30 minutes at room temperature then washed in TBS for 10 minutes. Alkaline phosphatase polymer (Dako Ltd, Cambridge, UK) was added to all sections and incubated for 30 minutes at room temperature then washed in TBS for 10 minutes. Fast blue (Vector Laboratories Ltd, Peterborough, UK) was added for 20 minutes and slides were developed and washed with water. Sections were then counterstained using Mayer's haematoxylin for 1 minute and washed for a few minutes under running water to develop.

Antibodies against Eotaxin (R&D, UK) and CCR3 (Leukosite, Cambridge, UK) were used as first primary antibodies, while Eosinophil peroxidase (Serotec, Oxford, UK) was used as a second primary antibody. Appropriate IgG1 and IgG2a isotype controls were included (Dako Ltd, UK).

Table 7- Antibodies used in the double-staining method.

Name of antibody or isotype	Type of antibody	Concentration
Eotaxin	Mouse monoclonal	1/50
CCR3	Mouse monoclonal	1/100
Eosinophil peroxidase	Mouse monoclonal	1/50
IgG1	MM, isotype to Eotaxin, Eosinophil peroxidase.	1/50
IgG2a	MM, isotype to CCR3	1/50

2.6- In situ Hybridisation Using Digoxigenin-Labelled Probes.

(Non-isotopic RNA/RNA InSitu Hybridisation)

(A) Preparation of tissues and sections:-

The slides were baked overnight at 250ºC in racks, then vectabonded using DEPC water (Sigma-UK) (Appendix E) under sterile conditions. The slides were covered with foil and left to dry at 50ºC overnight. Using a sterile technique with xylene and alcohol the sections were cut at 4 µm, then the slides covered with foil and left to dry at 50ºC overnight.

(B) Material and methods:-

Tissues were drawn from the files of the Department of Pathology, University of Birmingham. All tissues were routinely processed. Blocks were embedded in paraffin wax and had been stored routinely for up to 25 years or more.

Epstein-Barr virus infected lymphoid tissues (Hodgkin's disease) were used as positive controls.

2.6.1-In Situ Hybridisation:

In collaboration with the Department of Pathology, a paraffin section EBER-ISH procedure using digoxigenin-labelled riboprobes together with a sensitive, amplified detection system was used for the identification of EBV in paraffin sections from ATIN.

Probes: Plasmids pBSJJJ1 and pBSJJJ2, containing EBER-1 and EBER-2 specific fragments, respectively, were the gift of Dr. P.Murray, Birmingham, U.K.

Their construction has been described previously (403). In short, EBER specific fragments derived from plasmids PJJJ1 and PJJJ2, were subcloned into the EcoRI/Hind3 and

BamHI/EcoRI sites of the pBluescript KS vector(GIBCO-BRL, U.K), which contains promoters for T 7 and T 3 RNA polymerases. The recombinant plasmids were purified by standard techniques, linearized with the appropriate restriction enzymes according to the manufacturers recommendations, extracted with phenol/chloroform/isoamylalcohol (25:24:1) and with chloroform, precipitated with ethanol and re-dissolved in water.

In vitro transcription of 1 ug linearized plasmid template was performed in the presence of digoxigenin-11-UTP, using either T 7 or T 3 RNA polymerase as recommended by the manufacturer (GIBCO-BRL, U.K), to produce single stranded RNA probes, complementary (antisense probe, AS) or anti-complementary (sense, S, negative control probe) to EBER-RNA transcripts. The digoxigenin-labelled probes were adjusted to between 100 and 200 bases by limited alkaline hydrolysis, and stored at 4ºC in the presence of RNAase inhibitor until use.

2.6.2-In Situ Hybridisation:

Precautions were taken to minimize contamination with RNAase up to the hybridization stage. Gloves were worn, glassware was incubated overnight at 200ºC prior to use, and all solutions and buffers were prepared using distilled water treated with 0.1%(v/v) diethylpyrocarbonate (DEPC, Sigma, U.K) (Appendix E), and to keep the work station as sterile as possible. Paraffin sections of ATIN tissues and control (Hodgkin's disease) were collected onto glass slides.

Day 1: The slides were deparaffinized twice in xylene, rehydrated in alcohols, rinsed twice in water, washed off with phosphate buffer saline PH 7.4(PBS) (Appendix C) and left in PBS for 5 minutes. The backs of the slides and around the sections were dried then sections were digested with 100 µl diluted pronase E at 0.07ng/ul per section for 5 minutes

at room temperature. In order to cover the whole section with pronase E, a parafilm was used with good effect to avoid air bubbles. Proteolysis was stopped by washing the slides in PBS and leaving in fresh PBS for 5 minutes. This was tipped off the slides which were left in fresh absolute alcohol for 5 minutes (twice). The backs of the slides and around the sections were dried and left to air dry. An appropriately sized gene frame (ABGene-U.K) was placed around each section. The amount of AS and S probe required was worked out and diluted with hybridization solution (Sigma-U.K) at a ratio of 1:1:100 (bottle 1 of AS or S: bottle 2 of AS or S: hybridization solution).

N.B: bottle 1 of AS (E1 T7), bottle 2 of AS (E2 T3), bottle 1 of S (E1 T3) and bottle 2 of S (E2 T7).

The appropriate amounts of diluted probe were put onto each section, and a siliconised cover slip pressed on to form a sealed chamber, then the slides were left to hybridise at 50^{o} C overnight.

Day 2: The cover slips and gene frames were taken off and the sections washed with 2 X SSC (standard saline citrate buffer) (Appendix F) for 5 minutes, then with 0.1 X SSC for 5 minutes (twice). This was tipped off and the slides washed with PBS for 5 minutes. The sections were circled with a PAP-pen (Bioscience, Cambridge-U.K) to create a well and 100 µl diluted goat serum (Dako-U.K) was placed on each section and left for 10 minutes, then the serum was tipped off and 50 µl diluted anti-digoxin antibody (Sigma-U.K) placed onto each section and left for 1 hour.

The slides were washed with PBS for 5 minutes, then the PBS was tipped off and 100 µl diluted secondary antibody (Biotinylated goat anti-mouse/Dako kit, Bottle C) placed onto each section and left for 30 minutes. The slides were washed with

PBS for 5 minutes, the PBS tipped off and 100 µl diluted streptavidin-biotin complex/ horseradish peroxidase (Dako kit, Bottle A and B) placed onto each section for 30 minutes, then washed with PBS for 5 minutes.

Diaminobenzidine (DAB), hydrogen peroxide (30%) and urea (Sigma, U.K) were added for 10 minutes, and then the slides were developed and washed with water for 2 minutes. Sections were counterstained using Mayer's haematoxylin for 30 seconds and washed for 2 minutes under running water to develop. The slides were dehydrated in alcohol twice for 2x5 minutes, cleared in xylene for 5minutes and mounted in DPX (Surgipath, U.K).

2.7-Measurement of the index of chronic damage in acute tubulo-interstitial nephritis:

The index of chronic damage is a simple method of measurement of chronic damage in renal biopsy specimens, which could be useful in clinical management, prognosis, comparisons between different centres and trials. The index of chronic damage ranges from 0 to 90%, increasing severity of chronic damage being associated with shortened renal survival (402).

Measurements of the index of chronic damage were made by Professor A. J. Howie. The section was selected only on the grounds that it was technically satisfactory and as completely representative as possible of the size of the specimen, including all pieces if there were more than one. Images at magnification x10 were examined with Aequitas IA image analysis software (Dynamic Data Links, Cambridge, UK). The threshold was adjusted to highlight everything in a defined area. All cortex was outlined using the freehand drawing facility. The size of this area was measured by the system in arbitrary units. Freehand drawing was then used to outline an area of chronic damage. This included glomeruli showing global sclerosis but not segmental sclerosis, areas of interstitial fibrosis, which appeared more solid and deeply stained than normal or oedematous interstitial tissues, and atrophic tubules, defined as tubules smaller than normal, with thickened basement membranes, or tubules large enough to be considered as cysts. Arteries and arterioles were not judged to have chronic damage unless they were completely occluded. The total area outlined was measured. Areas of cortex and chronic damage were summed for each specimen, and the percentage of chronically damaged cortex was calculated to the nearest integer to give the index of chronic damage (401).

The measurements of the ATIN index of chronic damage ranged between 0% and 75% (median= 0%), with 51% of the patients having a 0% index of chronic damage (40/78), and 77% of them having an index of chronic damage less than 10% (61/78).

Chapter 3:

Results: Clinical Findings

3.1 Clinical features of acute tubulointerstitial nephritis:

3.1.1 Epidemiology:

From 1984 to 2002, 6652 percutaneous renal biopsies were performed in the pathology department of the University of Birmingham/ University Hospitals Birmingham NHS Trust. 976 biopsies (14.6% of all biopsies) were in acute renal failure.

Acute tubulointerstitial nephritis was present in renal biopsies from 78 patients (1% of all biopsies and 8% of acute renal failure biopsies) (Table 8 and figure 1).

Table 8- Number of renal biopsies, acute renal failure (ARF), and acute tubulo-interstitial nephritis (ATIN).

Year	No. of biopsies	A.R.F	ATIN	Year	No. of biopsies	A.R.F	ATIN
1984	158	24	2	1994	474	54	5
1985	204	36	2	1995	450	53	3
1986	204	30	0	1996	577	76	8
1987	226	39	2	1997	521	65	5
1988	201	27	1	1998	521	56	6
1989	204	37	3	1999	464	52	6
1990	290	37	2	2000	392	80	12
1991	317	53	4	2001	321	58	4
1992	347	62	5	2002	393	69	3
1993	388	68	5	**Total**	**6652**	**976**	**78**

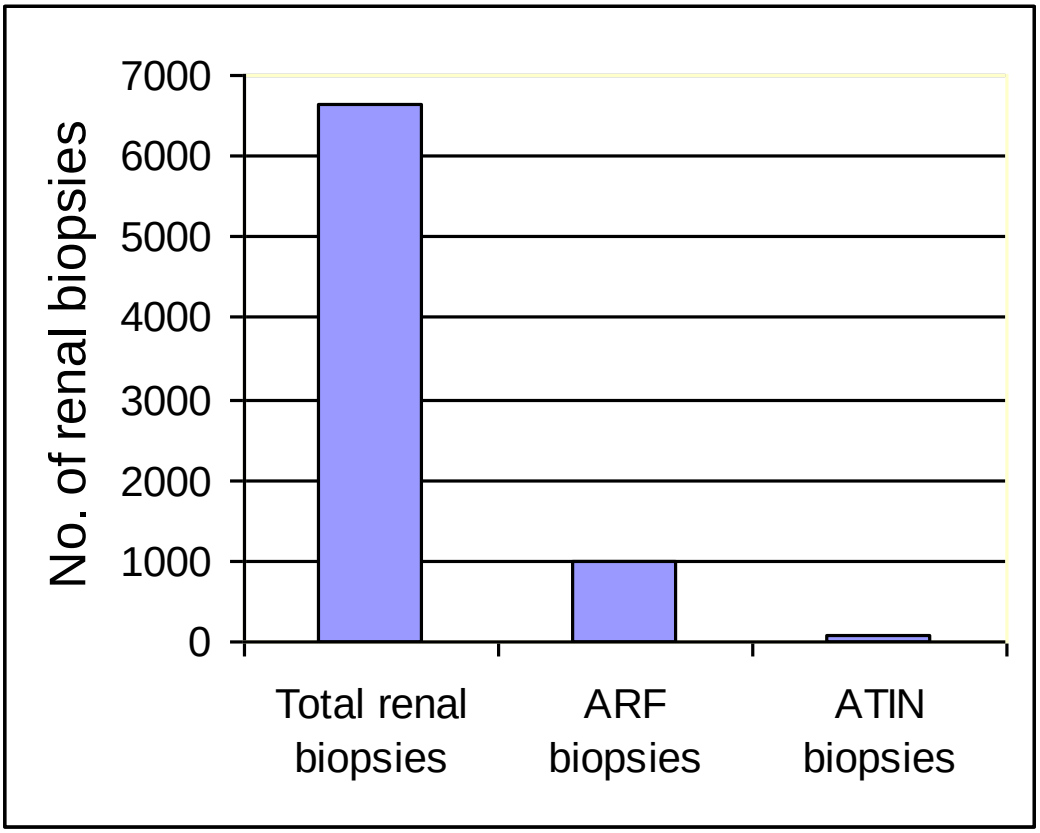


Figure 1-The total number of renal biopsies performed and the number of biopsies associated with acute renal failure (ARF) or acute tubulointerstitial nephritis (ATIN) during 1984-2002.

Two patients had a second clinical episode of ATIN, which was confirmed by renal biopsy. The known causative events were the intake of drugs (n=66, 85%), or TINU syndrome (n=7, 9%), but no cause could be verified (idiopathic) in 6% of the cases (n=5) (Figure 2).

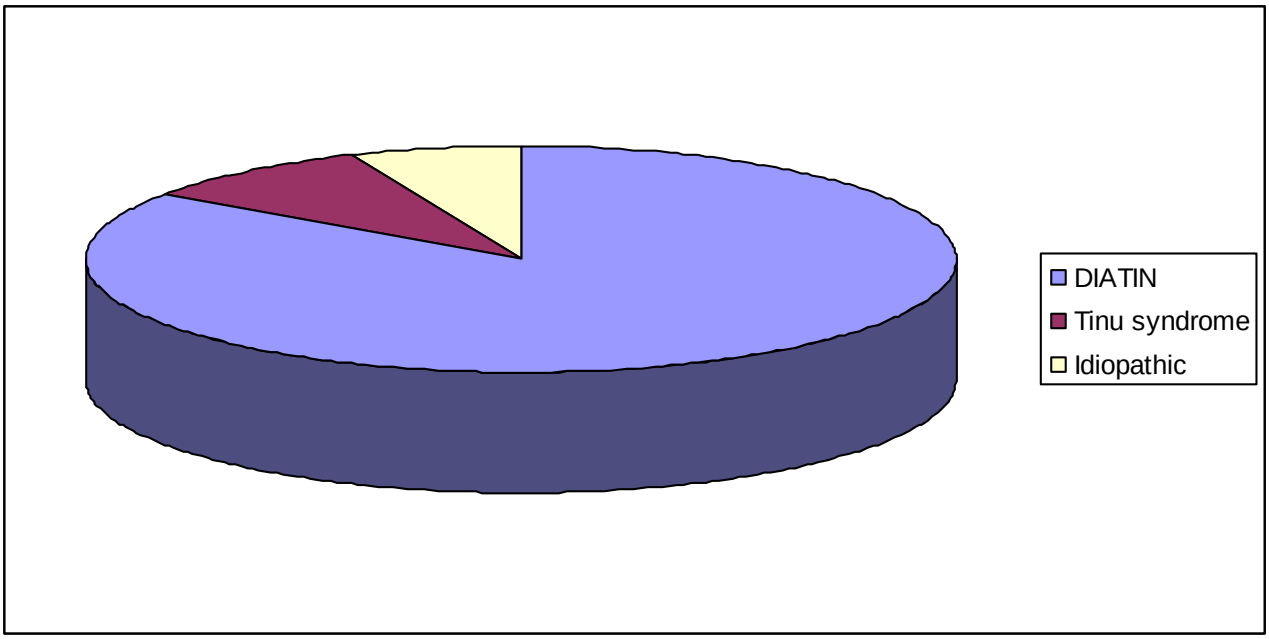


Figure 2- The causes of acute tubulointerstitial nephritis. DIATIN= drug-induced acute tubulointerstitial nephritis.

3.1.2- Causative event:

The medications suspected of having induced ATIN were antibiotics (n= 30, amoxicillin in 3, penicillin in 6, ciprofloxacin in 2, cephalosporin in 2, ampicillin in 1, cefuroxime in 1, doxycycline in 1, tetracycline in 1, oxytetracycline in 1, tazocine in 1, trimethoprim in 1, and mixed antibiotics in 10); analgesics (n= 1, acetaminophen and aspirin in 1); NSAID (n=17, ibuprofen in 3, naproxen in 3, diclofenac in 3, indomathacine in 2, mefenamic acid in 1, naproxen and indomethacine in 2, and mixed NSAID in 3); and various other drugs (n=18, omeprazole in 3, diazide in 2, chlorambucil in 1, phenytoin in 1, cisplatin in 2, moduretic in 2, warfarin in 1, bendrofluazide in 1, sulphazalasine in 1 and mixed drugs in 4). Seven cases were due to TINU syndrome (Tubulointerstitial nephritis and Uveitis) and no causes were verified in the remaining five cases (Idiopathic).

3.1.3- Patient demographics and clinical features:

The patients' mean age at presentation was 55 years (15-85), and 53% were men. Acute symptoms were noted in 50% of the cases and some patients had several symptoms or features: rash in 16%, fever in 35%, gross haematuria in 28%, and eosinophilia in 15%. The classic triad of fever, arthralgia and rash was present in only seven of seventy eight (9%) patients. The clinical features of ATIN varied widely as specific symptoms were present as in other renal diseases, while non-specific symptoms were also present reflecting systemic illness. In this study, the presence of these symptoms, signs and investigative finding among patients with ATIN are presented in table 9. It is acknowledged that the data was incomplete for many variables. All parameters were present or absent except for hypertension and immunoglobulin concentrations which were continous numeric data. What the data indicates is that there is a wide variation in findings in individuals with ATIN across the symptoms, signs and investigative findings tested. The only certainty is that the majority of patients will present with significant levels of malaise and tiredness.

Table 9- Retrospective analysis of different symptoms, signs and investigative findings in patients with ATIN.

Symptoms, signs and investigations	No. of positive/total patients
1-Gross haematuria	22/78 (28%)
2-Polyuria	11/78 (14%)
3-Nocturia	9/78 (11%)
4-Dysuria	10/77 (13%)
5-Rash	13/78 (16%)
6-Arthralgia	32/78 (40%)
7-Eye symptoms	7/78 (7%)
8-Fever	28/78 (35%)
9-Malaise	65/77 (83%)
10-Tiredness	65/77 (83%)
11-Hypertension	64/78 (81%)
12-Weight loss (> 10% loss)	16/78 (20%)
13-Short of breath.	12/78 (15%)
14-Raised immunoglobulin levels	8/57 (14%)
15-ANCA®	10/58 (17%)
16-Anti-DNA antibodies	1/46 (2%)
17-Anti-nuclear factor	21/60 (34%)
18-Proteinuria(1+ or more on dipstik)	42/78 (53%)
19-Microscopic haematuria (1+ or more on dipstick)	37/78 (47%)
20-Glycosuria	14/78 (18%)

® ANCA= antineutrophil cytoplasmic antibodies.

Other investigations were compared between the three different groups of ATIN {drug-induced ATIN, TINU syndrome, and Idiopathic ATIN} using a Kruskal-Wallis test (non-parametric test) (table 10). For each variable, the normal range is given in the table. However, the absolute value of each variable for each patient was used in the statistical analysis.

The results showed a significant difference in the lymphocyte counts between the three groups with a P value of 0.026 (Table 10 and Figure 3), where drug-induced ATIN and TINU syndrome showed a lower normal concentration of lymphocyte counts (1-1.8X 10^9 /L) while idiopathic ATIN showed an upper normal concentration of lymphocyte counts (2-2.9X10^9 /L). Further analysis using Dunn's test showed a significant difference between drug-induced ATIN and Idiopathic ATIN groups. The importance of this observation is uncertain, especially given the uncertain aetiology of the idiopathic group.

Other investigations such as C-reactive protein, bilirubin, alkaline phosphatase, aspartate transaminase, serum calcium, phosphate, potassium and sodium concentrations showed no significant difference between the three groups.

Table 10- The comparison of the investigative findings between the three subgroups of ATIN using a Kruskal-Wallis test.

Investigation	Diagnosis Groups	Number of cases	P value
1- W.B.C ($4\text{-}11\ X10^9/L$)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	64 5 7 76	P = 0.199
2-Eosinophils ($0.04\text{-}4X10^9/L$)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	50 3 7 60	P = 0.77
3-lymphocytes ($1.5\text{-}4\ X10^9/L$)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	59 5 7 71	**P = 0.026** See text for details.
4- Hb% (12-16 g/dl)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	65 5 7 77	P = 0.534
5- ESR (<20 mm/1st hour)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	49 3 7 59	P = 0.54
6-C-reactive protein (<10 mg/L)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	51 5 7 63	P = 0.119

7- Bilirubin (<17 μmol/L)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	63 5 7 75	P = 0.458
8-Alkaline Phosphatase (25-115 u/L)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	63 5 7 75	P = 0.40
9-Aspartate aminotransferas(7-40 u/l)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	62 5 7 74	P = 0.255
10-Serum calcium (2.2-2.67 mmol/L)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	61 5 7 73	P = 0.161
11- Serum phosphate (0.8- 1.5 mmol/l)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	34 2 7 43	P = 0.208
12- Serum potassium (3.5- 5.5 mmol/l)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	63 5 7 75	P = 0.369
13- Serum sodium (135- 145 mmol/l)	Drug-induced ATIN Idiopathic ATIN TINU syndrome Total	63 5 7 75	P = 0.4

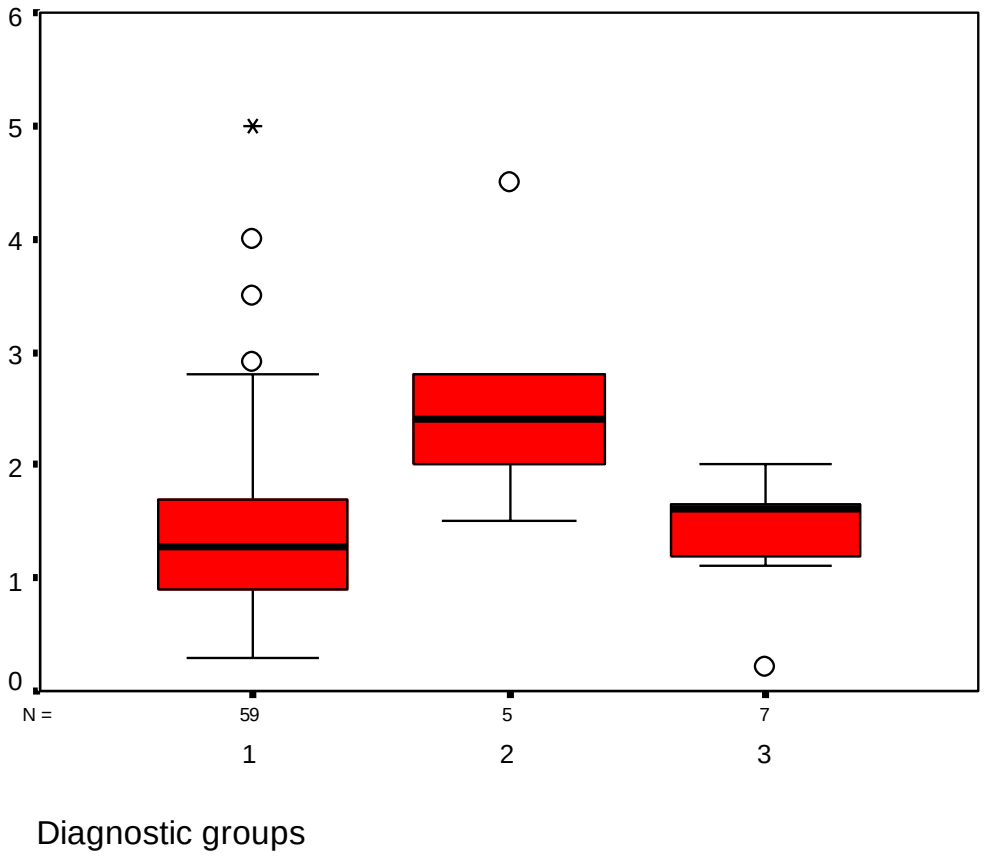


Figure 3- Comparison of lymphocyte counts($X10^9$/L) between the diagnostic groups, where 1=drug-induced ATIN, 2=Idiopathic ATIN and 3=TINU syndrome

3.1.4- Creatinine concentrations at different time points:

The creatinine concentrations at presentation, one week, one month, three months and one year were compared between these three groups (drug-induced ATIN, Idiopathic ATIN, and TINU syndrome) using a Kruskal-Wallis test (Table 11, Figures 4 and 5). This comparison showed a significant difference (P = 0.020) between these three groups at presentation, with a higher concentration of creatinine for drug-induced ATIN, moderate concentration of creatinine for TINU syndrome and a lower concentration of creatinine for

Idiopathic ATIN. Using Dunn's test, there was a significant difference between drug-induced ATIN and Idiopathic ATIN goups, while no difference between TINU syndrome and drug-induced ATIN. There was no significant difference between creatinine concentrations at one week, one month, three months and one year for these diagnostic groups.

Table 11- The Kruskal-Wallis statistical analysis of the creatinine concentrations at different time points between the three diagnostic groups where DIATIN= drug-induced ATIN.

Creatinine concentration	Diagnosis groups	No. of cases	P value
Presentation	DIATIN Idiopathic ATIN TINU syndrome Total	65 5 7 77	**0.020** see text for details
One week	DIATIN Idiopathic ATIN TINU syndrome Total	63 5 7 75	0.051
One month	DIATIN Idiopathic ATIN TINU syndrome Total	56 4 7 67	0.672
Three months	DIATIN Idiopathic ATIN TINU syndrome Total	43 4 7 54	0.678

One year	DIATIN Idiopathic ATIN TINU syndrome Total	32 1 7 40	0.958

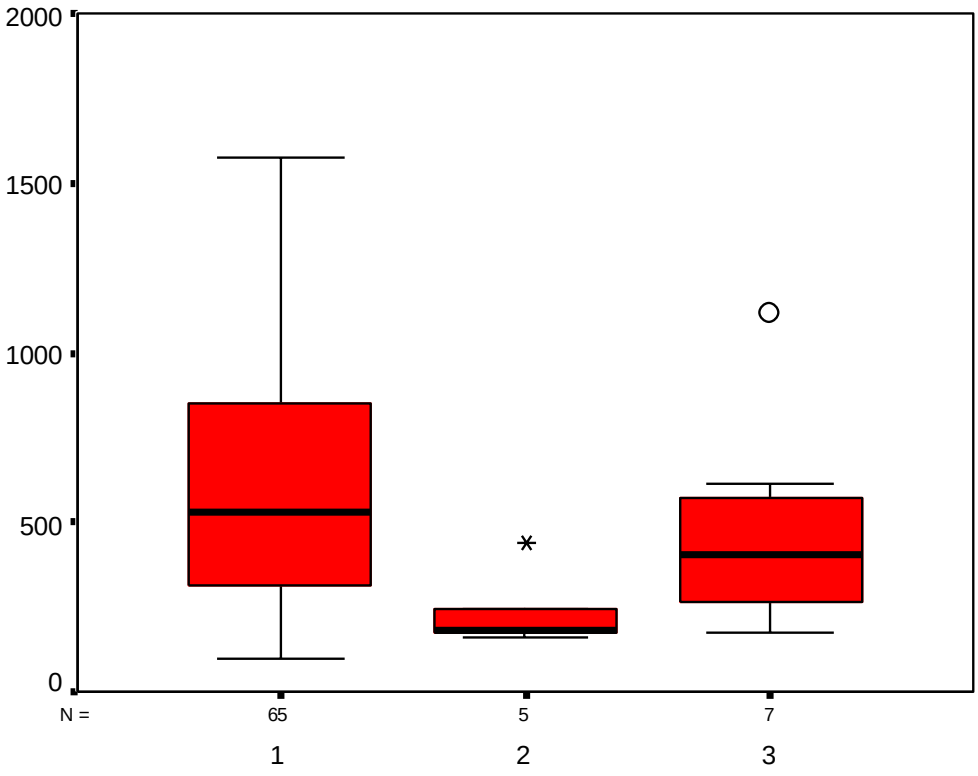


Figure 4- Creatinine concentrations (µmol/L) at presentation of the three diagnostic groups where 1= drug-induced ATIN, 2=Idiopathic ATIN, 3=TINU syndrome (Kruskal-Wallis test).

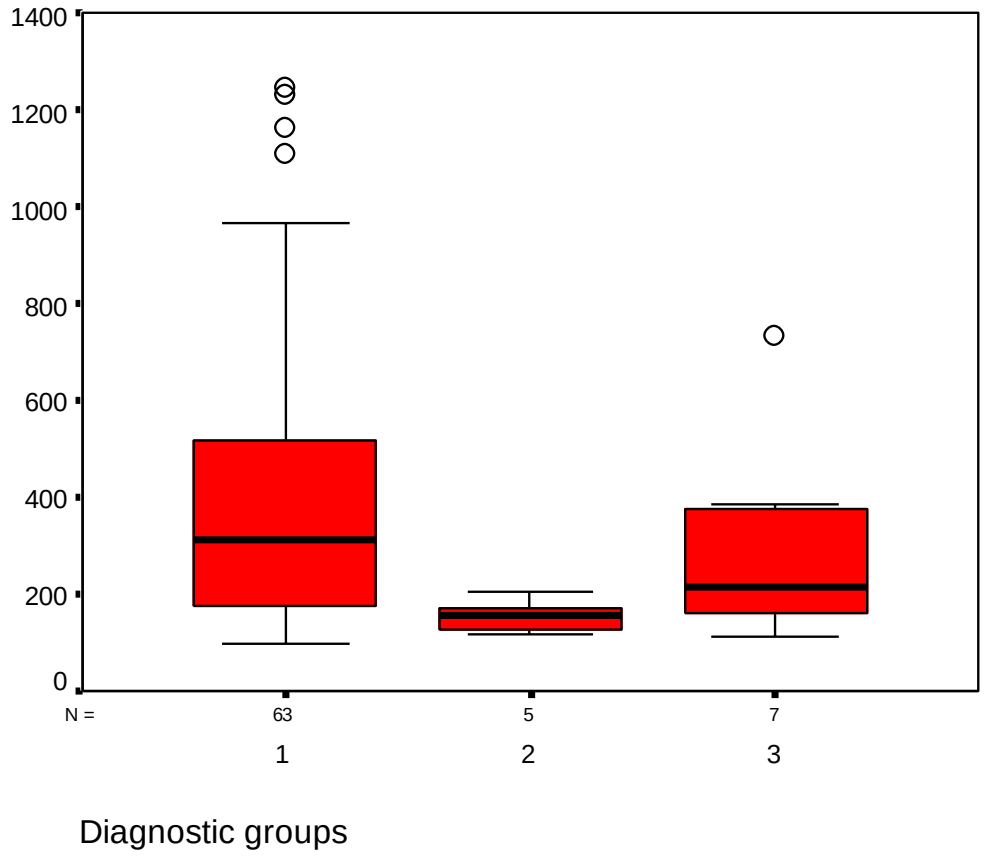


Figure 5- Creatinine concentrations (µmol/L) at one week for the three diagnostic groups where 1= drug-induced ATIN, 2=Idiopathic ATIN, 3= TINU syndrome (Kruskal-Wallis test).

3.1.5- Drug induced acute tubulo-interstitial nephritis:

Drug-induced ATIN represents the main cause of acute tubulointerstitial nephritis in this study, with 63% of the ATIN attributed to drugs (49/78). The mean age for the

patients in this group was 55 years (Median= 58 years, range 17-85) and 28 of them were males and 21 were females. Creatinine concentrations were available for 46 patients at 3 months, one patient died at one month and no data was available for the remaining two (creatinine concentration ranged from 64 to 485 μmol/l, median= 127 μmol/l) (Figure 6).

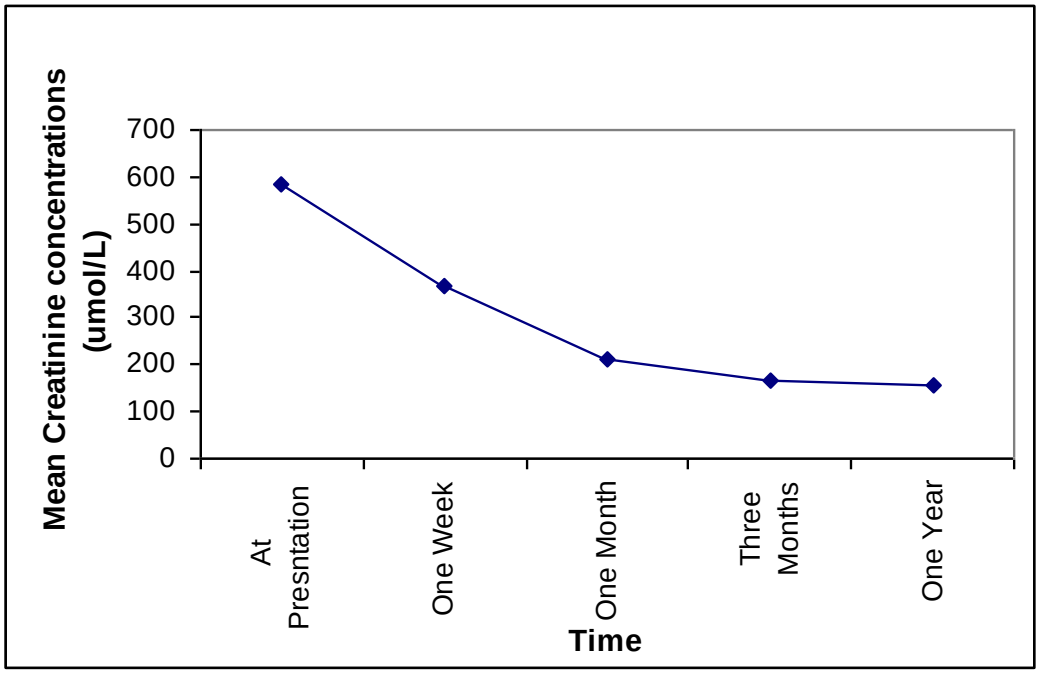


Figure 6 -The renal follow-up of Drug-induced ATIN patients.

3.1.6-Non steroidal anti-inflammatory drug-induced acute tubulo-interstitial nephritis:

In this study NSAID was responsible for 17/78 (22%) of ATIN. The names of the NSAIDs implemented are shown in Table 12.

The mean age for the patients in this group was 66 years (Median= 70 years, range 39-78 years), 9 patients were males and 8 were females. Creatinine concentrations were available on 12 patients at 3 months, 3 patients died by this time and no data was available for the remaining two (creatinine concentrations ranged from 104 μmol/l to 332 μmol/l, median= 154 μmol/l) (Figure 7).

Table 12- The Names of NSAIDs that were attributed to causing ATIN in the patient group.

Name of drug	No. of cases
Diclofenac	3 cases
Ibuprofen	3 cases
Indomethacin	2 cases
Mefenamic acid	1 case
Mixed NSAID	3 cases
Naproxen	3 cases
Naproxen+ indomethacin	2 cases
Total	17 cases

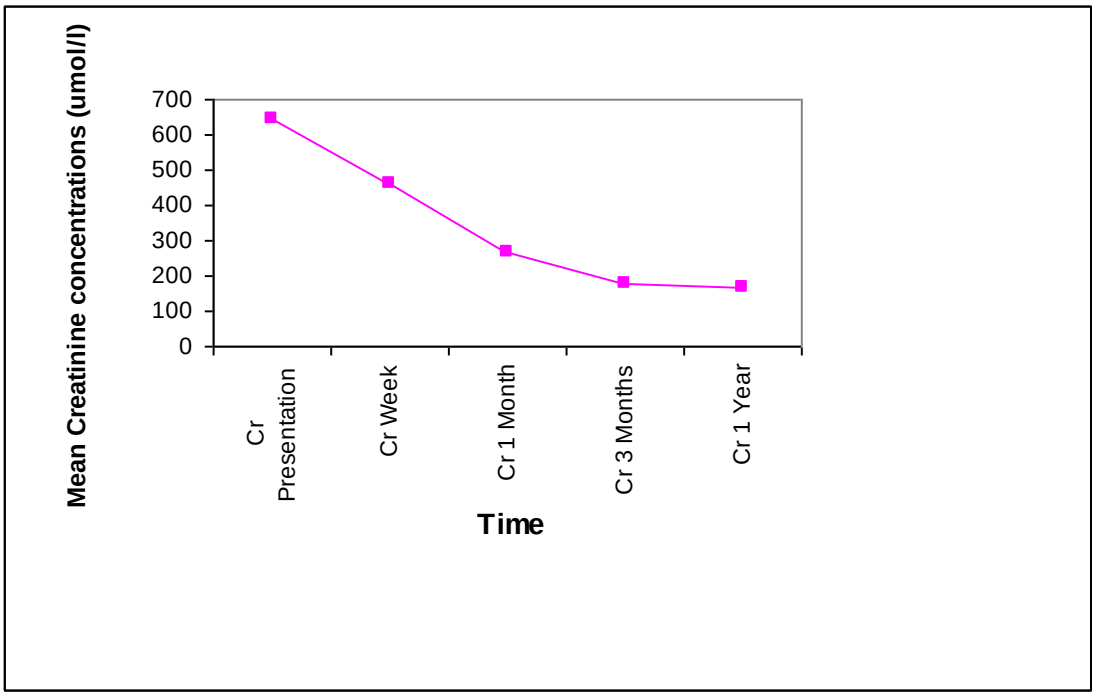


Figure 7- The renal follow-up for NSAID-induced ATIN patients

3.1.7 -TINU syndrome:

In this study there were seven cases of TINU syndrome (7/78 = 9%), which was first reported in 1975 as an association between acute tubulo-interstitial nephritis and anterior uveitis, sometimes associated with bone granulomas (339, 404).

In this study, six out of seven (85.7%) cases of Tinu syndrome occurred in women with ages ranging from 31-61 years (mean= 42years, median=38 years) (Table 13). Six of the TINU syndrome patients had a positive drug history prior the presentation with ATIN, and there was no history of drug ingestion for the seventh one. Six of the seven (86%) cases had suffered from anaemia and had an elevated ESR and in six cases the uveitis

predated the presentation with ATIN, while the seventh one was postdated by 6 months. The presence of uveitis was confirmed by a consultant ophthalmologist. All the seven patients were treated with steroids and renal outcome was good at 3 months (creatinine 91-219 μmol/l, median = 118 μmol/l) (Figure 8).

Table 13- Some parameters of Tinu syndrome patients.

Patient number	Age at presentation	Sex	Uveitis pre/post ATIN	Lymphocyte count ($1.5\text{-}4X10^9/L$)	Treatment
1	31 years	Female	Predated	1.6	Steroid
2	31 years	Female	Predated	1.6	Steroid
3	32 years	Female	Predated	2	Steroid
4	38 years	Female	Predated	1.1	Steroid
5	44 years	Female	Predated	1.7	Steroid
6	55 years	Female	Postdated	1.3	Steroid
7	61 years	Male	Predated	0.2	Steroid

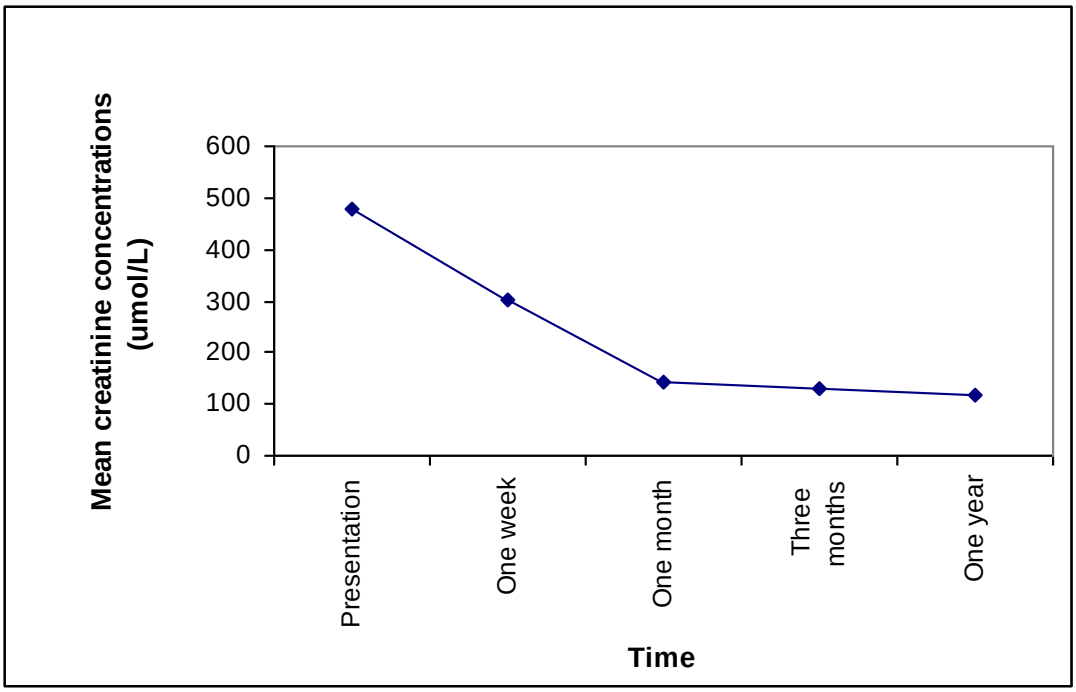


Figure 8- The renal follow-up of TINU syndrome patients.

3.1.8-Idiopathic acute tubulo-interstitial nephritis:

In this study, no cause was verified in five (6%) cases of ATIN (Idiopathic ATIN). Three out of five (60%) cases were male with ages ranging from 15- 73 years (mean= 44 years, median= 48 years) (Table 14). There was no drug history for any of the idiopathic ATIN patients prior the presentation with ATIN. All five patients were treated with steroids and renal outcome was good at 3 months (creatinine 101-144 µmol/l, median= 130 µmol/l) (Figure 9).

Table 14- Idiopathic acute tubulointerstitial nephritis parameters:

Patients number	Patients Age	Patients sex	Lymphocyte count (1.5-4X10^9/L)	Type of treatment	Creatinine at 3 months (70-<150 µmol/L)
1	15 years	Male	2.4	Steroid	132 µmol/l
2	28 years	Male	2	Steroid	101 µmol/l
3	48 years	Female	2.8	Steroid	144 µmol/l
4	55 years	Female	1.5	Steroid	130 µmol/l
5	73 years	Male	4.5	Steroid	122 µmol/l

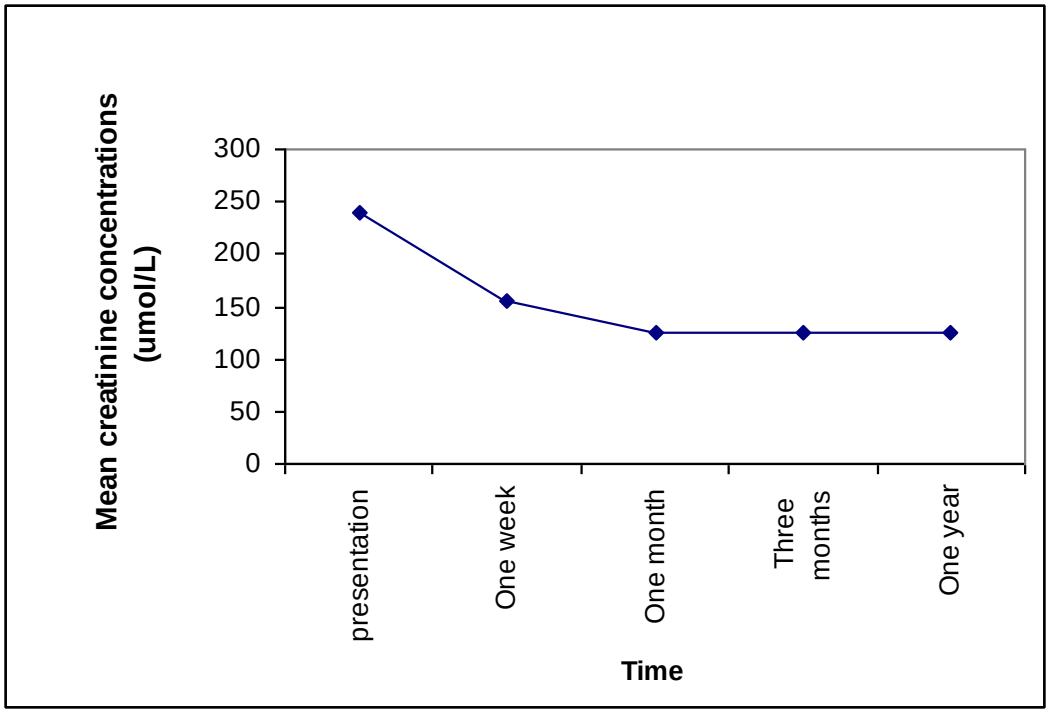


Figure 9- Follow-up of mean creatinine concentrations (µmol/L) of Idiopathic ATIN.

3.1.9-Therapy:

The treatment for ATIN primarily entails removing the causative agent and replacing renal function, if necessary. In this study, 70% of the patients had stopped the causative agents (mainly drugs) on admission and 18% had not stopped their medications and no information was available for 12%. A comparison between the patients who had stopped their medications (causative agents) on admission and those who had not among the different groups, showed no statistical significance (P value=0.10) (Table 15).

Table 15- Comparison between patients who had or had not stopped their mediciation on admission.

		Diagnosis groups			Total	Percentage
		1	2	3		
Whether Drug stopped or not?	N.A	4	4	1	9	12%
	NO	11	1	2	14	18%
	YES	51	0	4	55	70%
	Total	66	5	7	78	100%

Where the diagnosis groups 1=Drug-induced ATIN, 2=Idiopathic ATIN, 3=TINU syndrome, N.A= Not available.

3.1.10- Analysis of treatment:

For further analysis, the patients were divided into 4 groups according to the type of treatment received, 1= those who received steroids, 2= those who received antibiotics, 3= those who received dialysis + steroids and 4= those who received no treatment.

62% of our patients received steroid therapy in a dose of 45-60 mg/day for a period of 4-12 weeks, and 28% of them received steroids and dialysis together. There was no significant difference between the different types of treatments.

A comparison between the different types of treatment among different diagnostic groups show no statistical difference (P = 0.249) (Table 16).

Table 16- The different treatment and diagnostic groups. Collectively 90% of patients received corticosteroid treatment, either without (62%) or with (28%) concurrent dialysis.

		Diagnosis Groups			Total	Percentage	P value
		1	2	3			
Treatment Groups	1	36	5	7	48	62%	P = 0.249
	2	2			2	2.5%	
	3	22			22	28%	
	4	6			6	7.5%	
	Total	66	5	7	78	100%	

Where Treatment groups, 1=steroids, 2= antibiotics, 3= dialysis+steroids, 4= No treatment. Diagnosis groups, 1= DIATIN, 2=Idiopathic ATIN and 3=TINU syndrome.

3.1.11- Outcome:

Renal insufficiency was reversible (Cr < 150 μmol/l) in 47 episodes of acute tubulointerstitial nephritis (59%), irreversible (Cr >150 μmol/l) in 23 (29%) and there was no data available for five patients (6%) at 3 months after diagnosis. Of the 23 patients in whom renal function was irreversible (Cr > 150 μmol/l), seven (30 %) required dialysis at presentation. There were four patients who died within 3 months due to causes other than renal problems (5%) and another two died at five and nine months from diagnosis (Table 17).

Table 17- The details of deceased patients and causes of death**.**

Patients number	Sex	Age at diagnosis	Diagnosis	Cause of death	Time of death from diagnosis
1	Male	58 years	DIATIN	Gastrointestinal bleeding.	One week
2	Female	74 years	DIATIN	Chest infection, CVA	One week
3	Female	72 years	DIATIN	Ischaemic heart disease.	One month.
4	Male	60 years	DIATIN	Pneumonia, respiratory failure.	Three weeks
5	Male	31 years	DIATIN	Disseminated abdominal Hodgkin's disease.	Five months
6	Male	48 years	DIATIN	Terminal case of tongue carcinoma.	Nine months

DIATIN= Drug-induced acute tubulointerstitial nephritis, CVA=Cerebro-vascular accident.

An analysis of the clinical features of ATIN in terms of presence or absence of all symptoms, signs and investigative findings in relation to reversibility of renal function has been carried out. Patients, who did not have malaise and tiredness and who had fever, normal or high level of haemoglobin, lower or normal potassium level and those with low or normal phosphate tended to have reversible renal function (Table 18). Other indices such as high blood pressure, arthralgia, initial renal symptoms, and gender had no significant relationship with the outcome.

Table 18-Parameters showing a significant or near significant statistical correlation with renal outcome at 3 months (using $\chi 2$ distribution test) For 'n' values, see Table 10.

Parameters	Explanation	Statistical significance
1- Malaise and Tiredness	There is a tendency for patients who did not have malaise and tiredness to have reversible renal function (i.e Cr. < 150 µmol/L).	P = 0.072
2- Fever	Patients with fever tend to have reversible renal function.	P = 0.021
3-haemoglobin	Patients with normal or high haemoglobin concentration tend to have reversible renal function.	P = 0.018
4- serum Potassium	Patients with normal or low potassium concentrations tend to have reversible renal function. This is probably because hyperkalaemia is an indirect measure of impaired renal function.	P = 0.002
5- serum Phosphate	Patients with low or normal phosphate concentration tend to have reversible renal function.	P = 0.03

Haemoglobin (12-16 g/l)

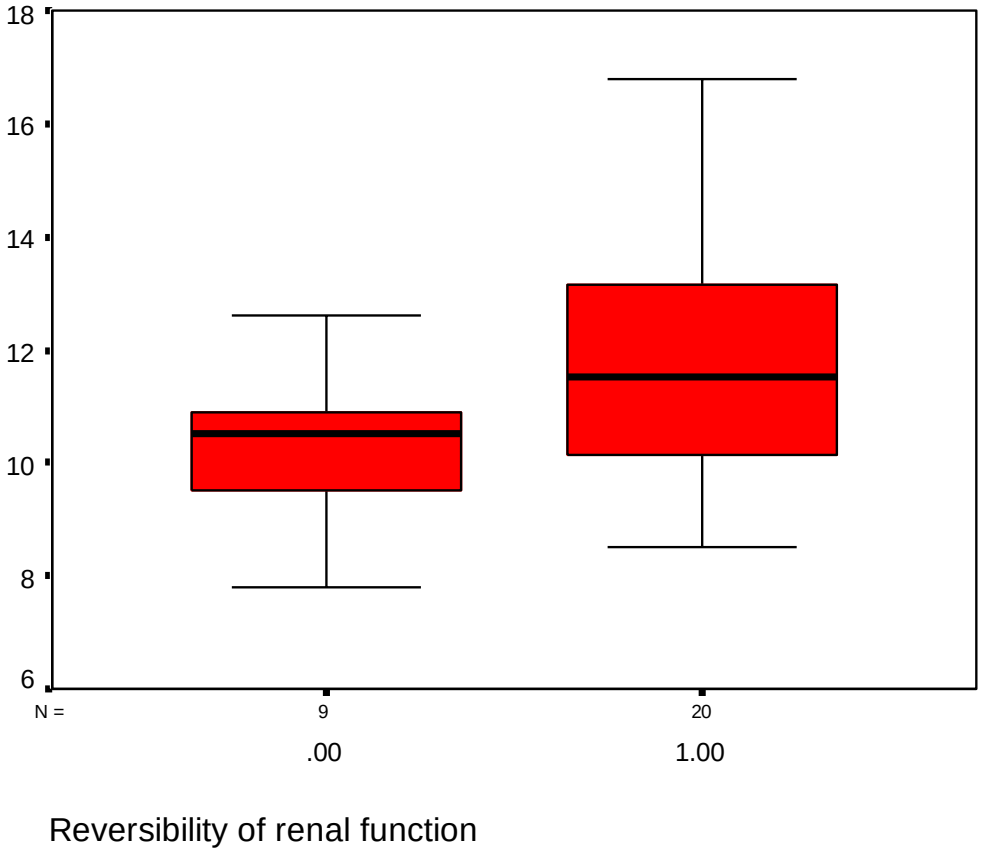


Figure 10- Comparison between the concentrations of haemoglobin at presentation and the reversibility of renal function. Where .00 =Irreversible and 1.00 = Reversible renal function (Cr < 150 µmol/L).

Serum phosphate (0.8-1.5 mmol/l)

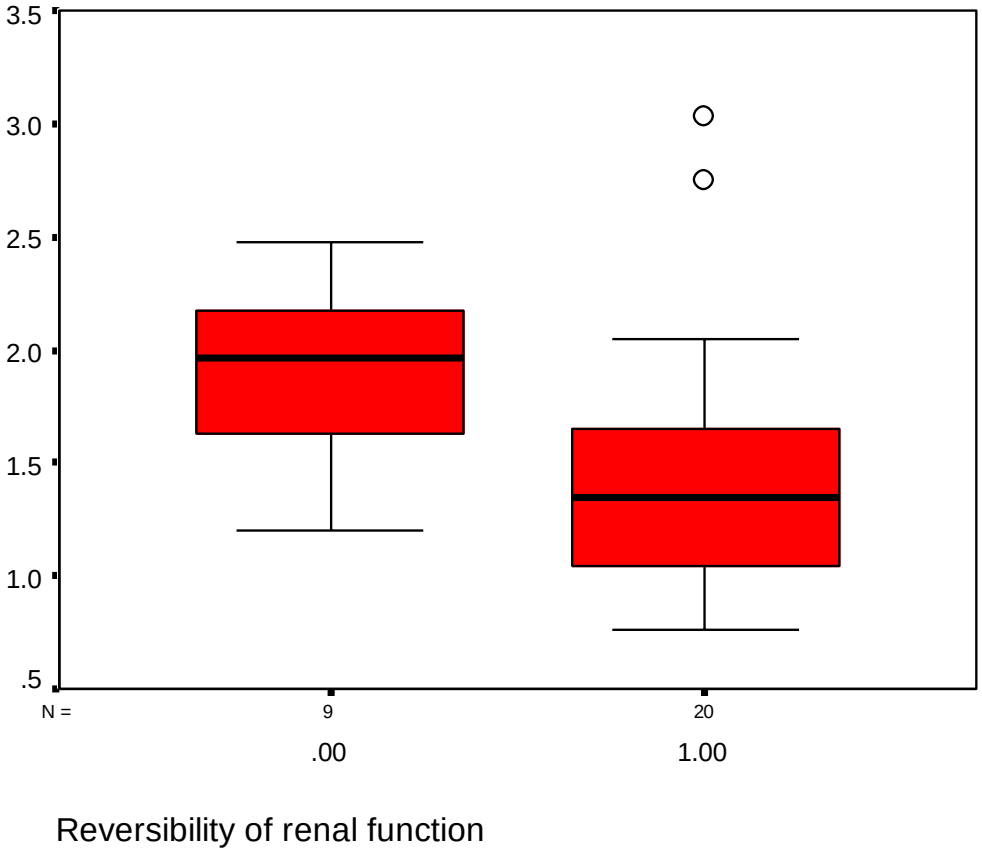


Figure 11- Comparison between the concentrations of phosphate at presentation and the reversibility of renal function. Where .00 =Irreversible and 1.00 = Reversible renal function (Cr < 150 µmol/L).

Serum potassium (3.5-5 mmol/l)

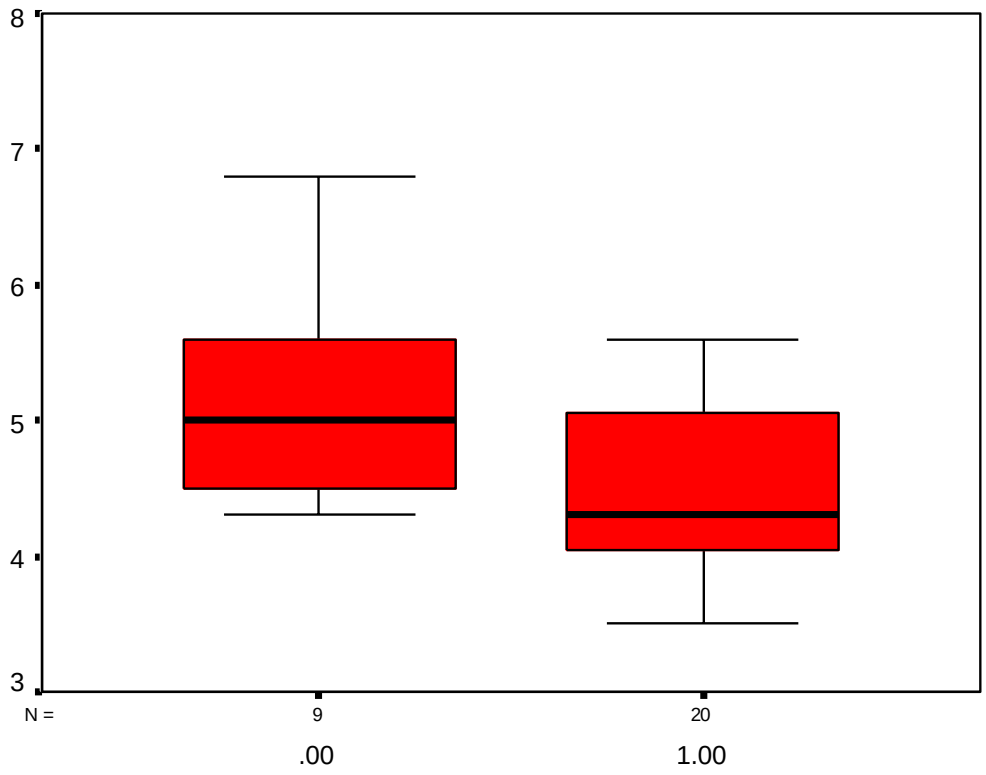


Figure 12- Comparison between the concentrations of potassium at presentation and the reversibility of renal function. Where .00 =Irreversible and 1.00 = Reversible renal function (Cr < 150 µmol/L).

The one year follow-up of renal function for the patients with ATIN (by measuring the mean creatinine concentrations at presentation, one week, one month, three months, and one year) showed a progressive decline of creatinine concentrations from presentation to reach a mean concentration of 149μmol/l at one year (Figure 13).

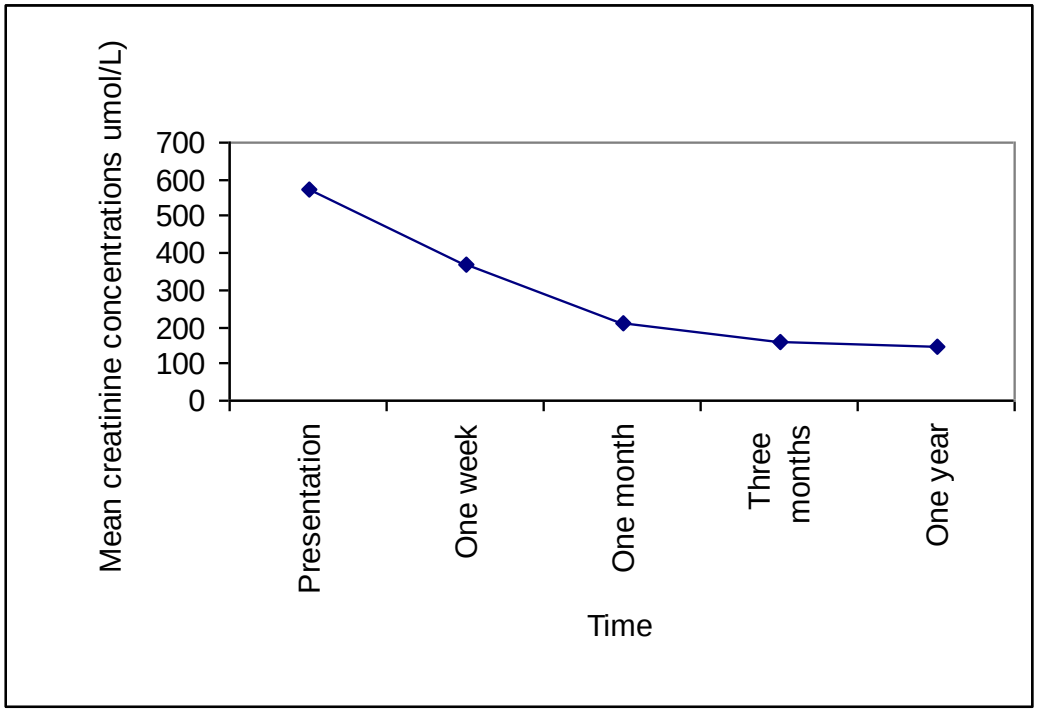


Figure 13- Follow-up of mean creatinine concentrations (μmol/L) in all patients with ATIN at presentation, one week, one month, three months and one year.

To determine the renal functional outcome, a Kaplan-Meier functional survival curve was used (Figure 14), which shows that nearly 20%, 55%, 65%, and 75% of our patients had an improvement in renal function (i.e Cr concentration < 150 μmol/l) by one week, one month, three months and one year respectively.

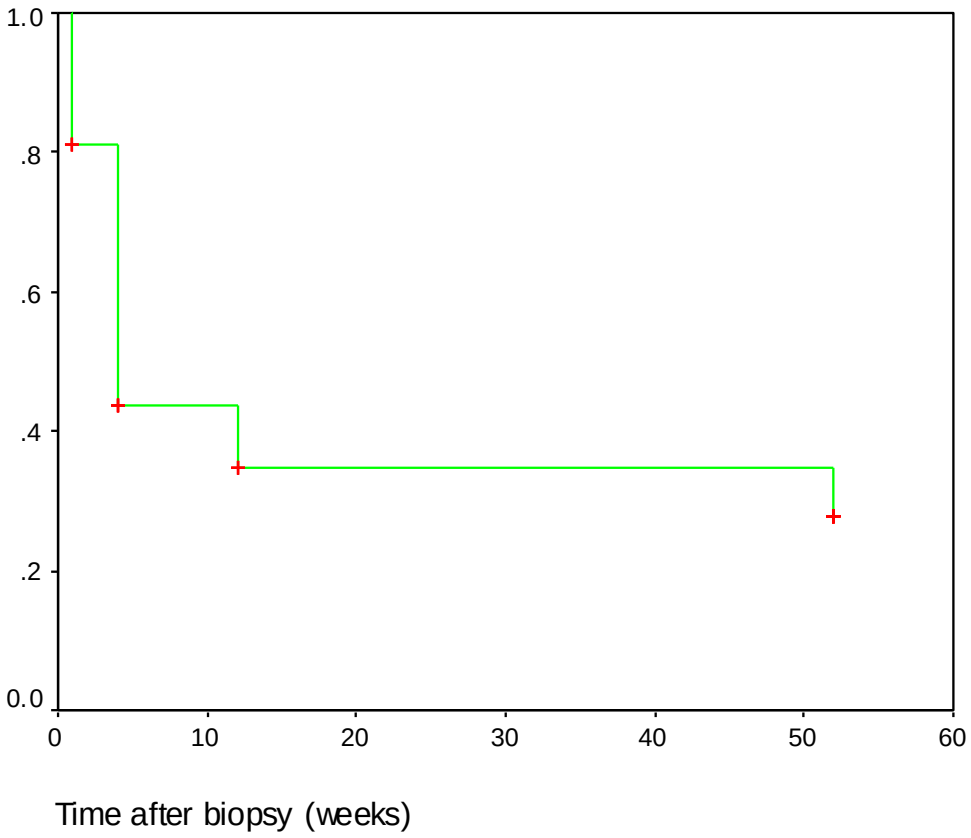


Figure 14- The improvement in renal function to a creatinine concentration < 150 μmol/l by Kaplan-Meier analysis in the entire ATIN population over one year.

Comparing the outcome for renal function between the different diagnostic groups showed a significant statistical difference (P value 0.0169) between these groups. The data was censored for deaths. For this analysis, ATIN patients who had received NSAID were analysed separately from ATIN patients due to other drugs (figure 15).

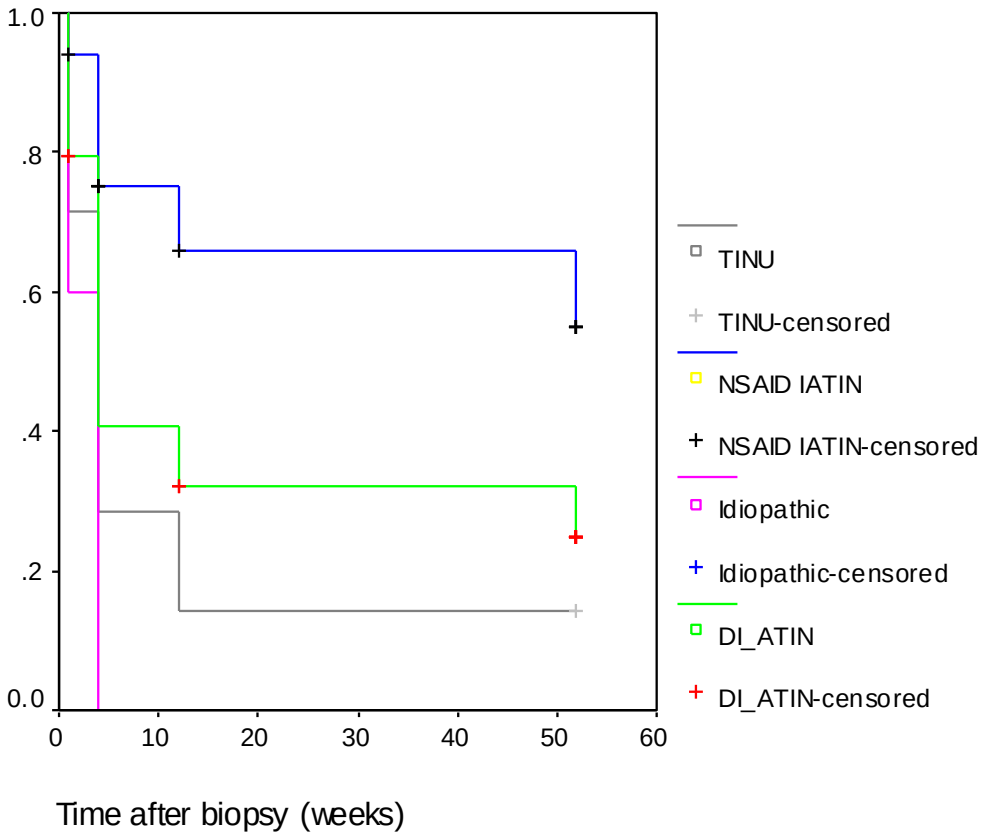


Figure 15- The improvement in renal function to a creatinine concentration < 150 µmol/l in the different diagnostic groups, where Green= DIATIN (excluding NSAID ATIN), Pink= Idiopathic ATIN, Blue= NSAID I ATIN and Grey= TINU syndrome. The data was analyzed over one year (+ = censored to follow-up period).

To analyze the statistical significance and find the responsible group for the difference in renal outcome at three months, a separate comparison between the different diagnostic groups was carried out (Table 19). So, when comparing the renal survival function between group 1 (drug-induced ATIN excluding NSAID) and group 3 (NSAID-induced ATIN) a statistical difference of (P= 0.0174) was found. The comparison between the

groups 1, 2 (Idiopathic ATIN), and 4 (TINU syndrome) showed no significance difference (P= 0.275). On the other hand, the comparison between groups 2, 3 and 4 showed a significant statistical difference (P= 0.0071).

From the above, it seems that group 3 (NSAID-induced ATIN) is the one responsible for the difference and carries a bad prognosis in comparison to other groups.

Table 19 – Comparison of renal function outcome at three months between the different groups and their statistical significance.

Diagnosis groups	Statistical significance
-Groups 1, 2, 3, and 4.	P = 0.0169
-Group 1 and 3	P = 0.0174
-Groups 1, 2, and 4	P = 0.2755
-Groups 2, 3 and 4	P = 0.0071
-Group 1 and 2	P = 0.0661
-Group 1 and 4	P = 0.4252
-Group 2 and 4	P = 0.3382
-Group 3 and 4	P = 0.0182
-Group 2 and 3	P = 0.0014

Chapter 4:

Results: Immunohistological and Other Investigations

4.1-Immunohistochemical analysis of infiltrating cells in renal tissues of ATIN:

4.1.1- Background:

Acute tubulointerstitial nephritis accounted for 8% of all acute renal failure biopsies in this study. It is characterised by a heavy infiltration of the renal interstitium with mononuclear cells. The purpose of the following experiments was to define the infiltrating cells using specific markers for lymphocytes (CD3, CD4 and CD8), macrophages (CD68) (figure 16, 17, 18 and 19 respectively) and eosinophil proteins (eosinophil major basic protein and eosinophil peroxidase) (figure 20C and 21D respectively).

4.1.2- Experimental methods and materials:

Paraffin fixed sections of renal biopsy specimens derived from histopathologically confirmed cases of ATIN were analysed for the expression of cellular antigens of T lymphocytes (CD3-figure 16D,CD4-figure 17D and CD8-figure 18D) macrophages (CD68-figure 19D), eosinophils proteins (figures 20C and 21D), mast cell tryptase (figure 22D) and Epstein-Barr virus's latent membrane protein 1(EBV's LMP-1) (figure 34B).

Nasal tissues from patients with Wegener's granulomatosis were analysed for the expression of T lymphocyte (CD3- figure 16A and B, CD4- figure 17A and B, CD8- figure 18A and B), macrophage (CD68- figure 19A and B) and mast cell tryptase (figure 22A and B) cellular antigens, as a positive and negative control for each antibody respectively, while for eosinophil major basic protein (figure 20A and B) and eosinophil peroxidase (figure 21A and B) nasal polyp tissues were used as positive and negative control respectively.

Lymph node tissues of Hodgkin's disease were used as a positive control (figure 34A) for the expression of LMP-1.

A three stage indirect immunoperoxidase method was used to stain the sections for all the above mentioned cellular antigens because preliminary studies showed it is a more sensitive method than a two stage method when working with paraffin tissues.

Frozen sections of renal tissue specimens from patients with ATIN were analysed for the expression of cellular antigens CD25 (figure 23D), HLA-class Π (figure 24D), HLA-class Π isotype (IgG1) (figure24B) and eosinophil peroxidase (figure 21D). For controls, frozen sections from nasal polyps were analysed for the expression of CD25 (figure23A) as a positive control and (figure 23B) as a negative control, HLA-class Π (figure 24A) as a positive control and (figure 24B) as a negative control and eosinophil peroxidase (figure 21A) as a positive control and (figure 21B) as a negative control. Normal kidney tissues were stained for the expression of eosinophil peroxidase and CD25 (figure 21C and 23C respectively).

4.1.3-Results of the staining:

4.1.3.1-T lymphocyte cellular antigens (CD3, CD4 and CD8)

The expression of T lymphocyte cellular antigens (CD3, CD4 and CD8) (figure 16D, 17D and figure 18D respectively) was characterized by focal and scattered infiltration throughout the renal interstitium with penetration between tubular epithelial cells by T lymphocytes, especially those expressing CD3 (figure 16D) and CD8 (figure 18D).

4.1.3.2-Macrophage marker:

The expression of CD68 by macrophages in ATIN (figure 19D) was more than in normal kidney (figure 19C). The staining pattern of macrophages in the renal biopsies comprised focal infiltration of spindle shaped cells.

4.1.3.3- Eosinophil markers:

The expression of eosinophil markers (eosinophil major basic protein and eosinophil peroxidase) (figure 20C and 21D) showed a scattered distribution of cells in the renal interstitium with some focal accumulation in some specimens. The eosinophil major basic protein expression (figure 20C) showed the presence of intact eosinophils as well as ruptured cells, reflecting the state of activity of the cell.

4.1.3.4- Mast cell marker:

The expression of mast cell tryptase in ATIN (figure 22D) showed a scattered focal infiltration in the renal interstitium, which was not different to that observed in normal tissues.

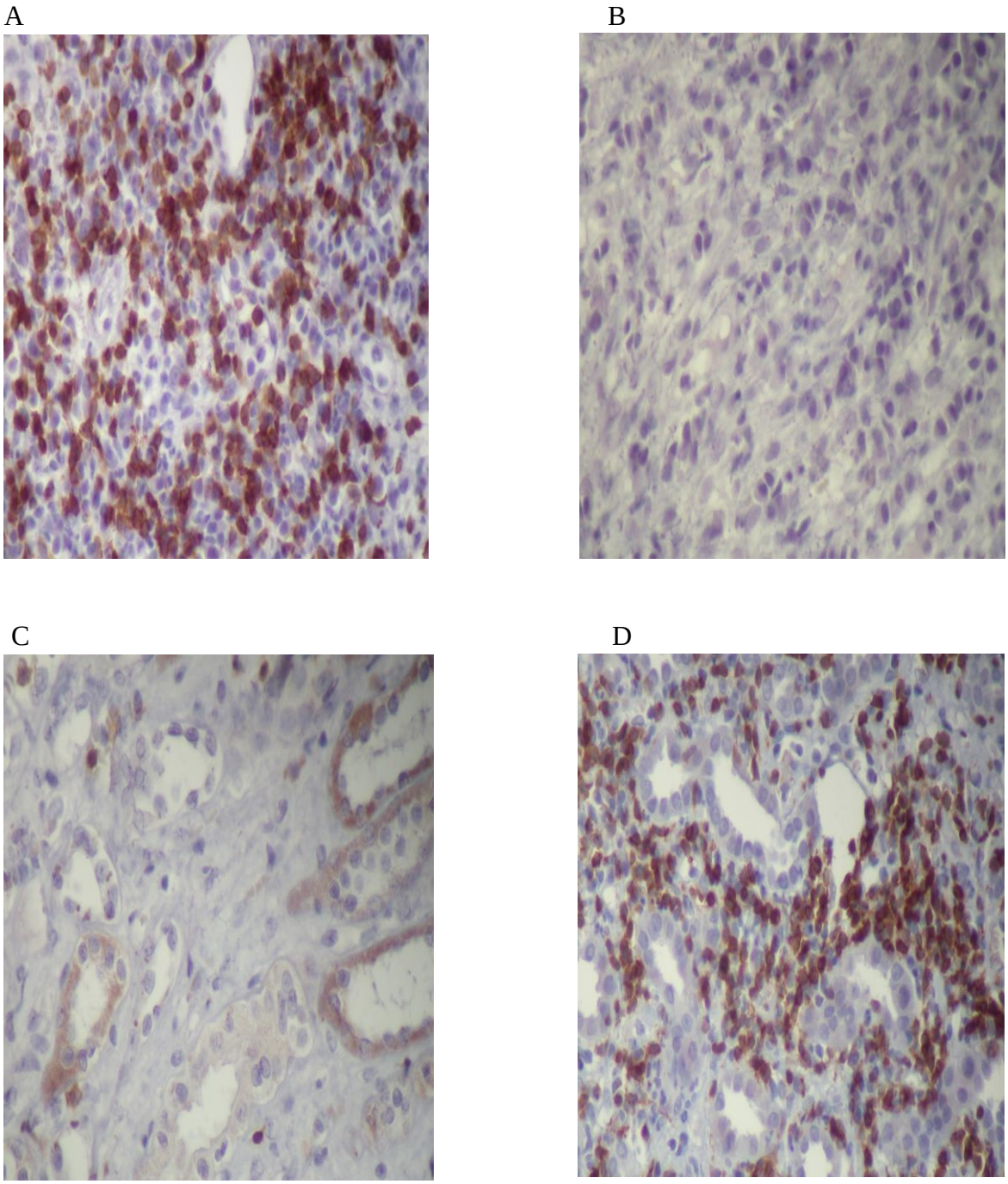
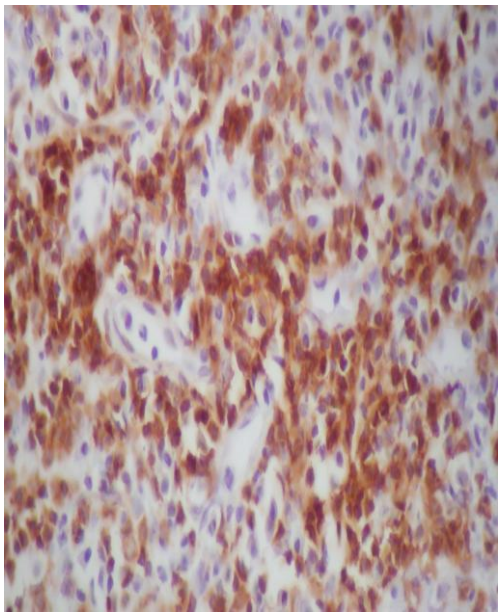


Figure 16- Immunohistochemical localization of CD3+
A: Staining for CD3 in Wegener's granulomatosis nasal tissues. (Positive control)
B: Staining for Isotype control (IgG1) in Wegener's nasal tissues. (Negative control)
C: Staining for CD3 in normal kidney tissue.
D: Staining for CD3 in acute tubulointerstitial nephritis tissue.

A



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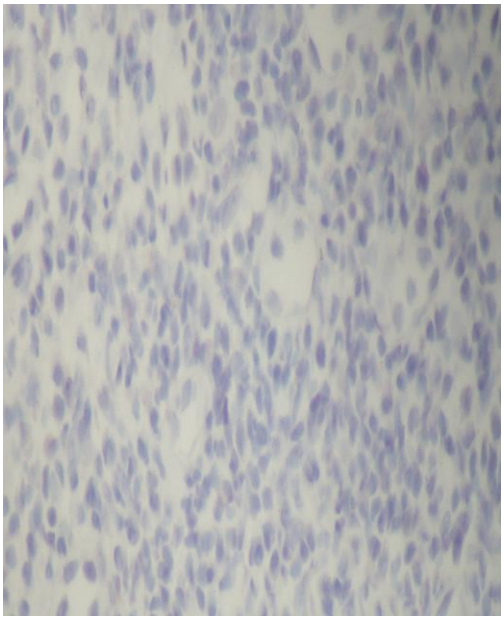

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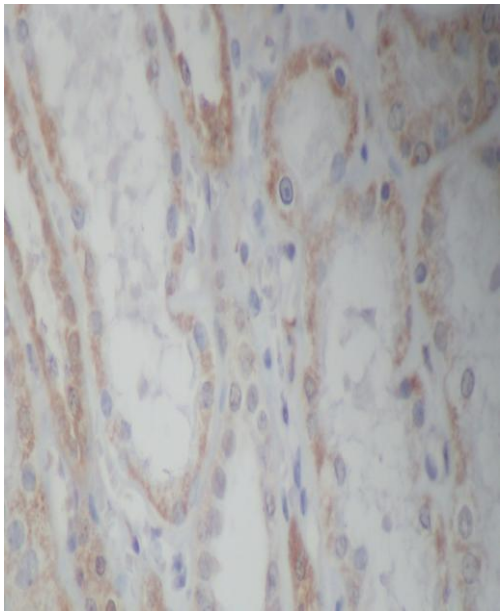

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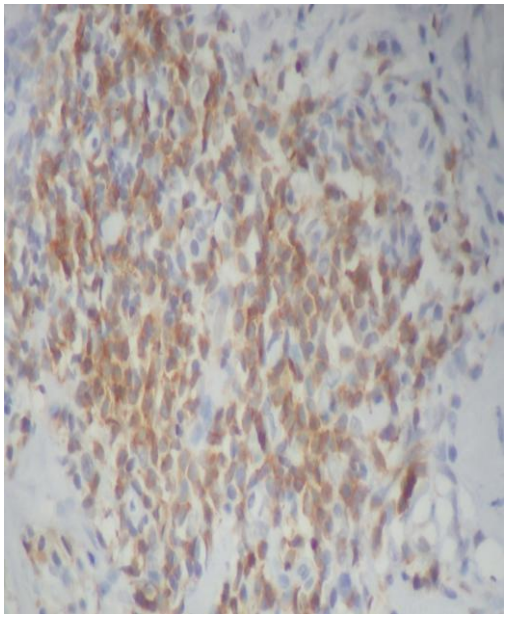
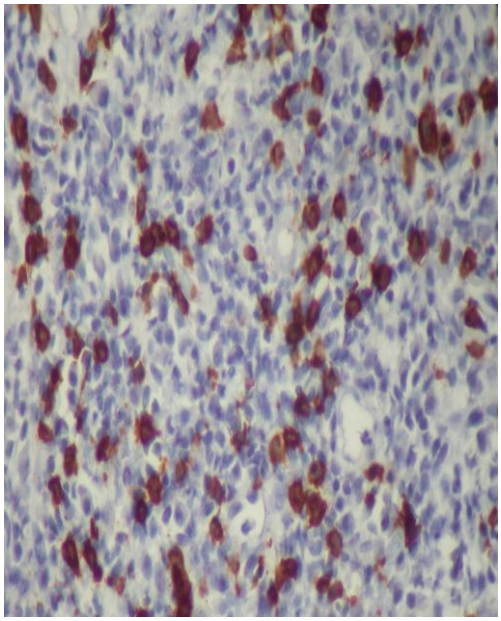


Figure 17 - Immunohistochemical localization of CD4+
A: Staining for CD4 in Wegener's granulomatosis nasal tissue. (Positive control)
B: Staining for Isotype control (IgG1) in Wegener's granulomatosis nasal tissue. (Negative control)
C: Staining for CD4 in normal kidney tissue.
D: Staining for CD4 in acute tubulointerstitial nephritis tissue.

A



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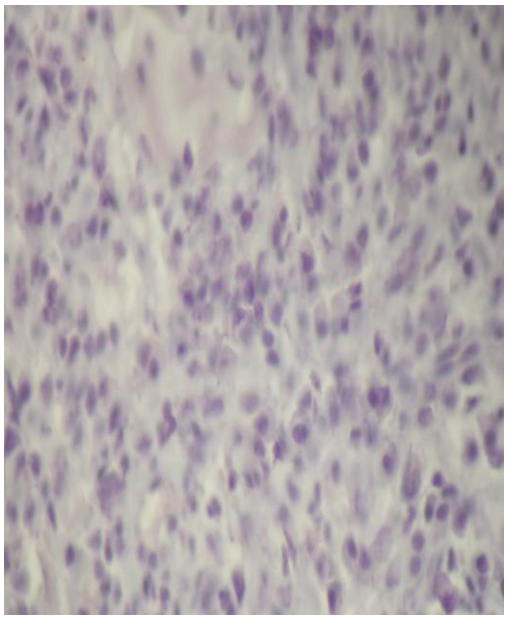

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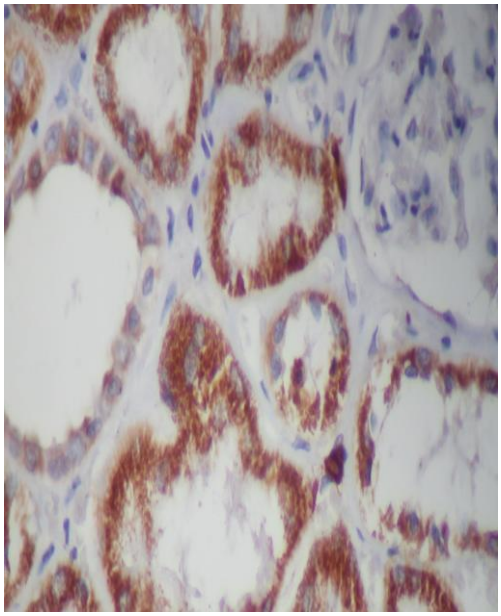

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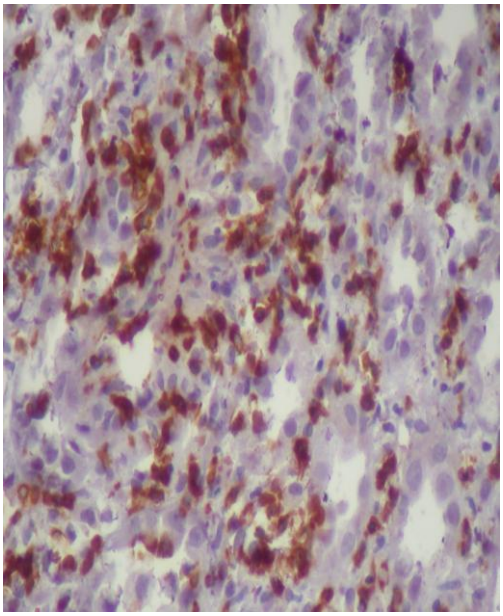
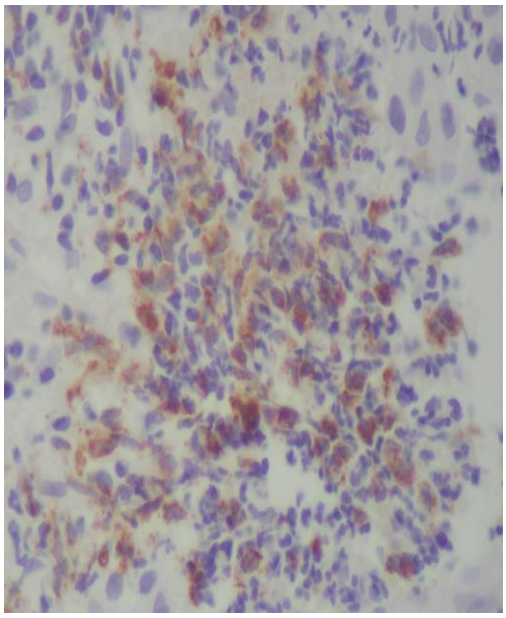


Figure 18- Immunohistochemical localization of CD8+
A: Staining for CD8 in Wegener's granulomatosis nasal tissue. (Positive control)
B: Staining for Isotype control (IgG1) in Wegener's granulomatosis nasal tissue. (Negative control)
C: Staining for CD8 in normal kidney tissue.
D: Staining for CD8 in acute tubulointerstitial nephritis tissue.

A



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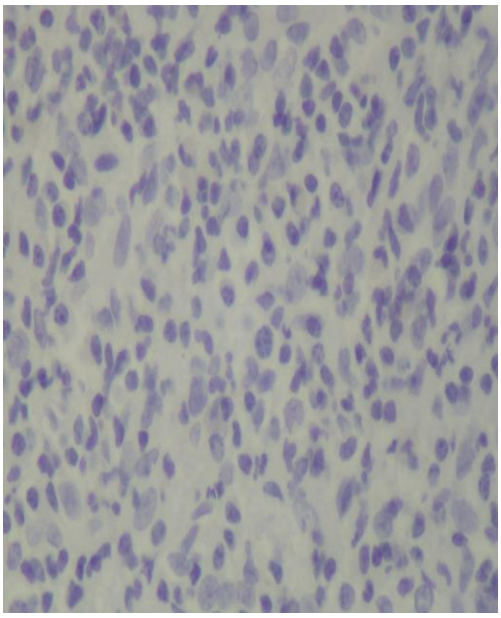

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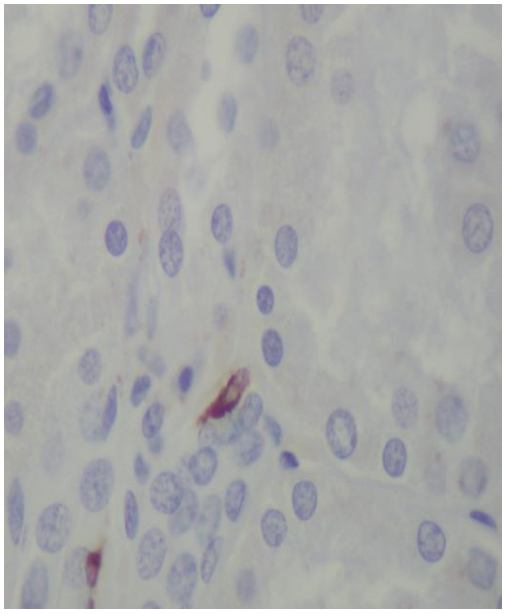

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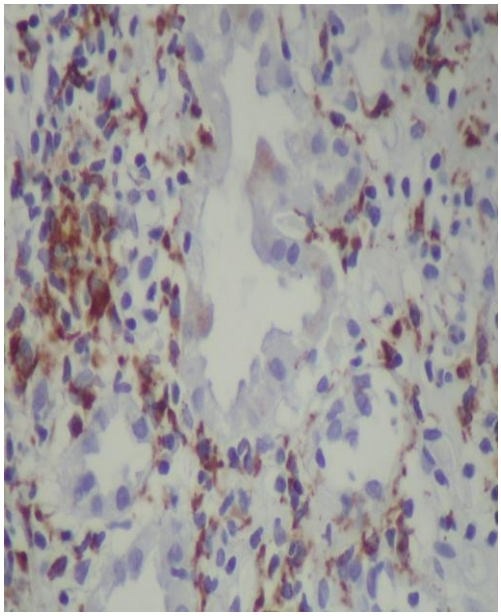
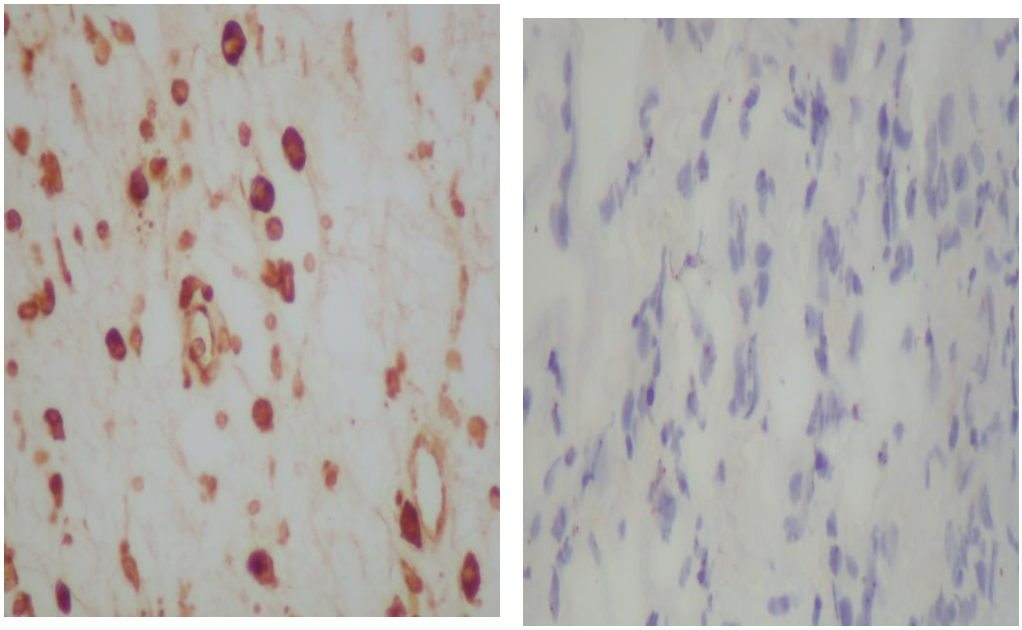


Figure 19 - Immunohistochemical localization of CD68+
A: Staining for CD68 in Wegener's granulomatosis nasal tissue. (Positive control)
B: Staining for Isotype control (IgG1) in Wegener's granulomatosis nasal tissue. (Negative control)
C: Staining for CD68 in normal kidney tissue.
D: Staining for CD68 in acute tubulointerstitial nephritis tissue.

A

B



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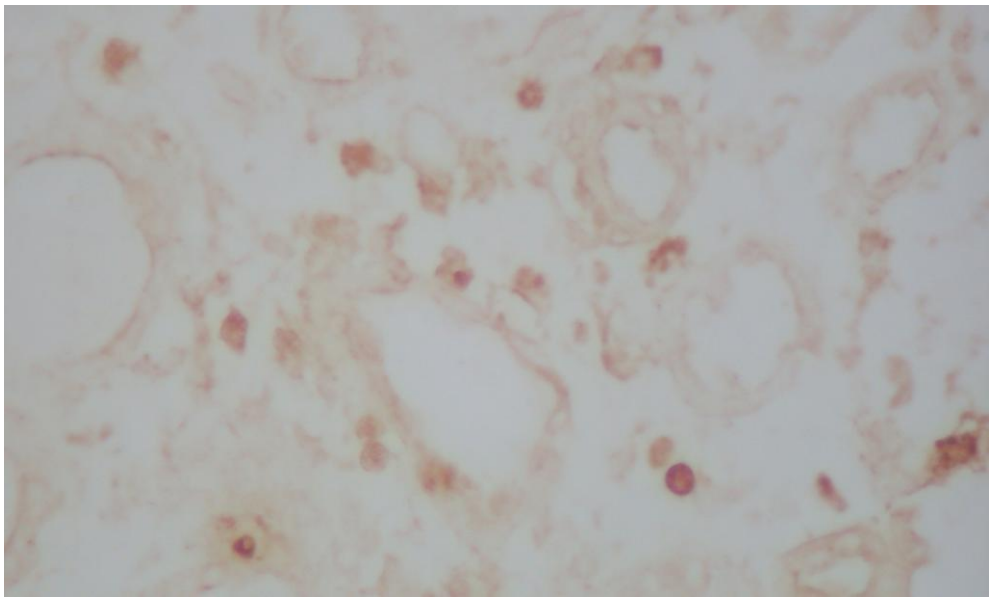
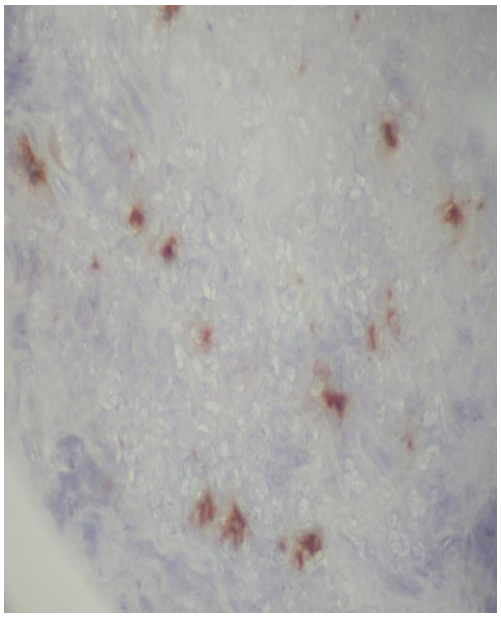


Figure 20- Immunohistochemical localization of eosinophil major basic protein (E-MBP)
A: Staining for E-MBP in nasal polyp tissue. (Positive control)
B: Staining for E-MBP Isotype control (IgG1) in acute tubulointerstitial nephritis tissue. (Negative control)
C: Staining for E-MBP in acute tubulointerstitial nephritis tissue.

A



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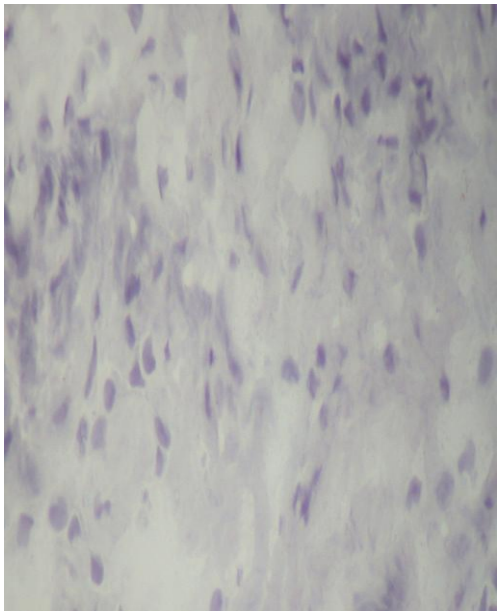

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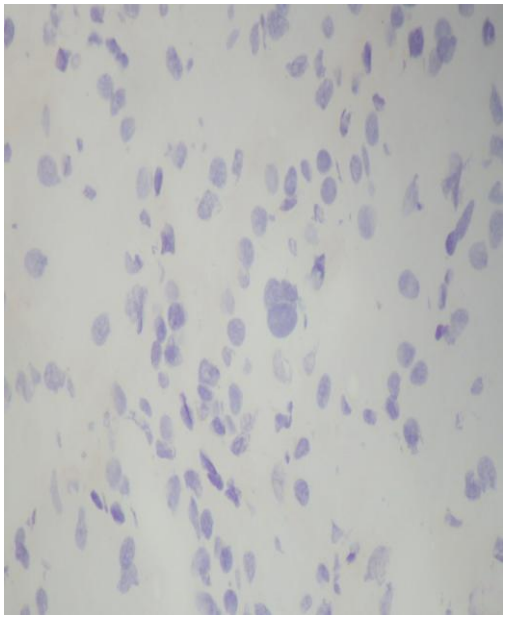

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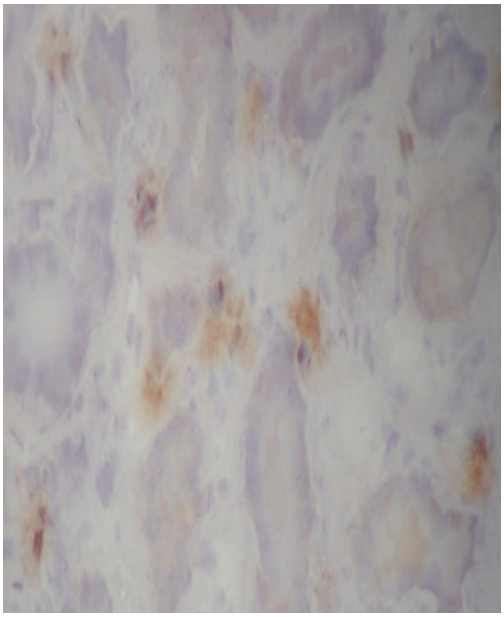


Figure 21- Immunohistochemical localization of eosinophil peroxidase (E-P)
A: Staining for (E-P) in nasal polyp tissue. (Positive control)
B: Staining for (E-P) Isotype control (IgG1) in nasal polyp tissue. (Negative control)
C: Staining for (E-P) in normal kidney tissue.
D: Staining for (E-P) in acute tubulointerstitial nephritis tissue.

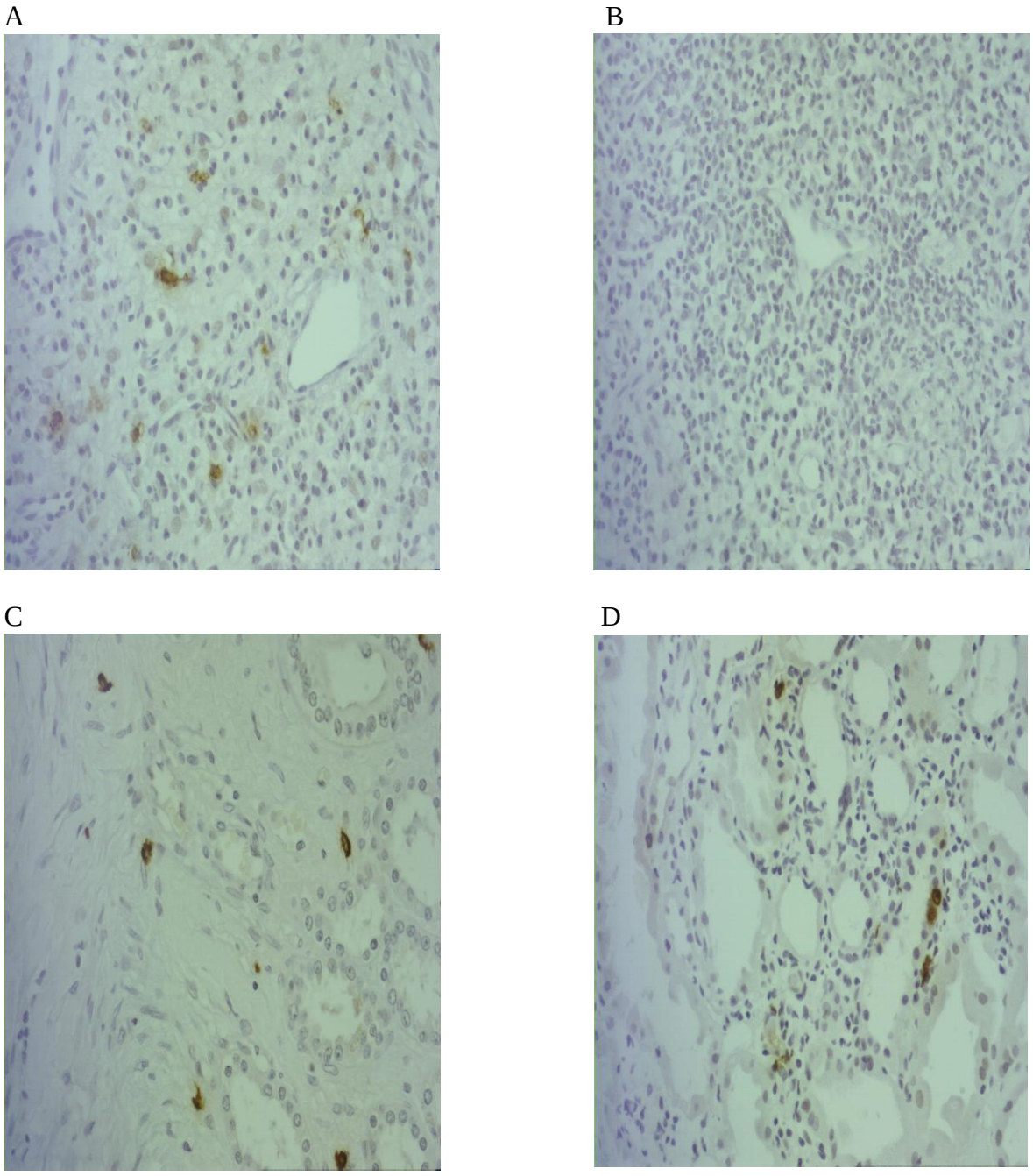


Figure 22- Immunohistochemical localization of mast cell tryptase
A: Staining for mast cell tryptase in Wegener's granulomatosis nasal tissue. (Positive control)
B: Staining for Isotype control (IgG1) in Wegener's granulmatosis nasal tissue. (Negative control)
C: Staining for mast cell tryptase in normal kidney tissue.
D: Staining for mast cell tryptase in acute tubulointerstitial nephritis tissue.

4.1.3.5- HLA-class Π and CD25:

HLA-class Π antigen can be expressed by various cell types including lymphocytes, macrophages, dendritic cells and resident tubular epithelial cells. HLA-class Π antigen was expressed by cells that had the appearance of infiltrating cells, within the interstitial tissue of ATIN (figure 24D). On the other hand, CD25 expression (interleukin-2 receptor) showed only faint staining in the interstitial areas of ATIN biopsies (figure 23D). Semiquantitative analysis for the HLA-class Π, CD25 and eosinophil peroxidase was (++) which represents 1/3- 2/3 of the tissues being stained.

4.1.4- Statistical analysis:

Quantitative analysis of the cell counts shows a big variation (Table 20), not only for CD68 positive macrophages but also for CD3, CD4 and CD8 positive T cell counts (NSAIDIATIN group analysed separately from DIATIN).

Table 20- The median and standard deviation of the infiltrating cells count among the subtypes of acute tubulointerstitial nephritis (NSAIDIATIN group analysed separately from DIATIN):

Type of cell (number of cases)	Descriptive statistic	Diagnostic groups			
		DIATIN (Cells/μm^2)	Idiopathic ATIN (Cells/μm^2)	NSAIDIATIN (Cells/μm^2)	TINU Syndrome (Cells/μm^2)
EMBP (24)	Median	4.50	2.50	6.00	5.00
	St. deviation	25.92	2.12	14.02	2.52
CD3 (23)	Median	279.00	32.50	168.00	290.00
	St. deviation	201.17	16.26	240.12	359.01
CD4 (13)	Median	98.50	-	34.00	-
	St. deviation	235.28	-	58.23	-
CD8 (30)	Median	286.00	289.00	171.50	368.00
	St. deviation	233.69	194.58	149.47	183.81
CD68 (18)	Median	359.50	-	409.00	-
	St. deviation	330.56	-	205.06	-

Where DIATIN= Drug-inducedATIN, NSAIDIATIN= non steroidal anti-inflammatory drug induced ATIN, EMBP= eosinophil major basic protein.

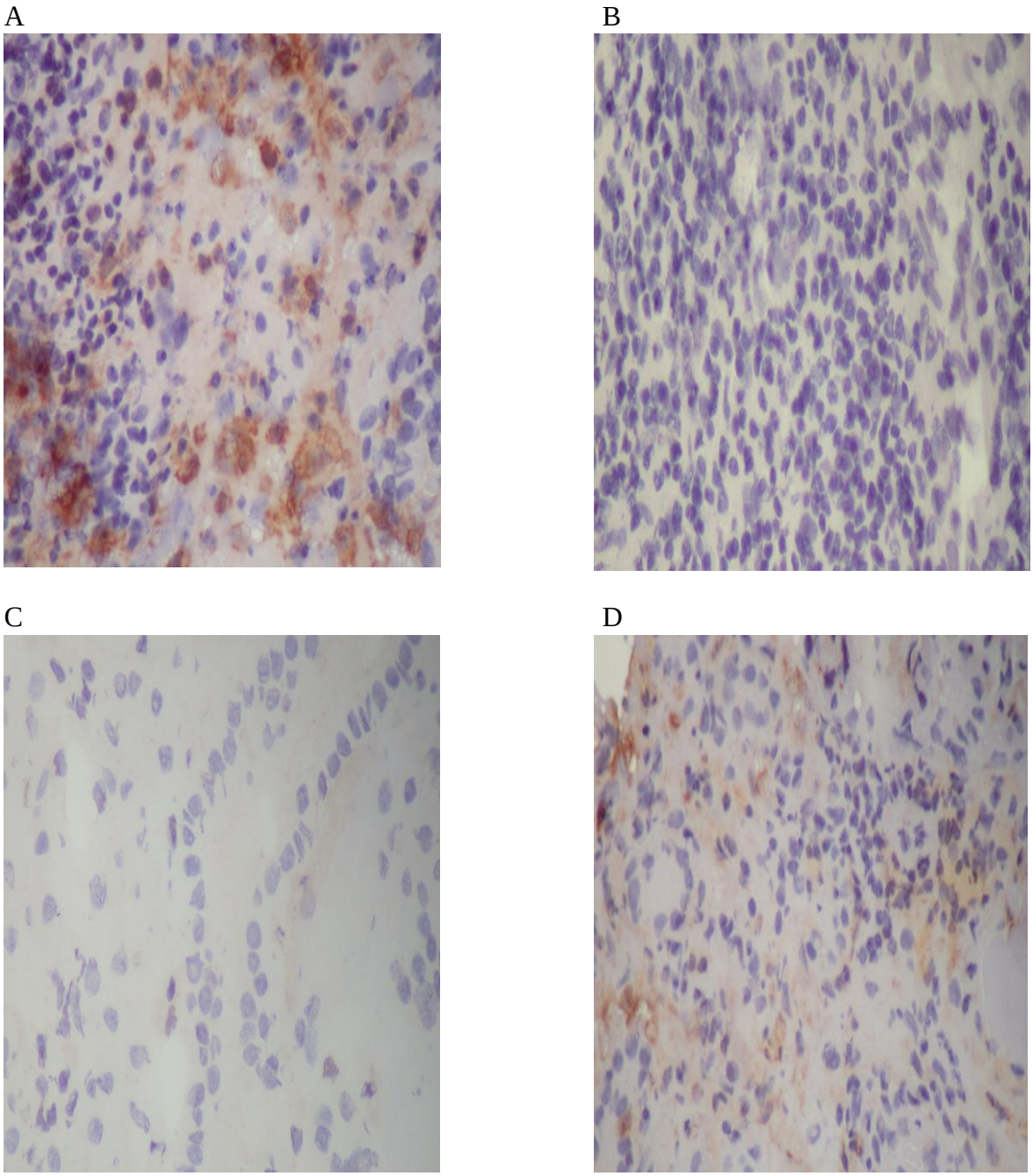
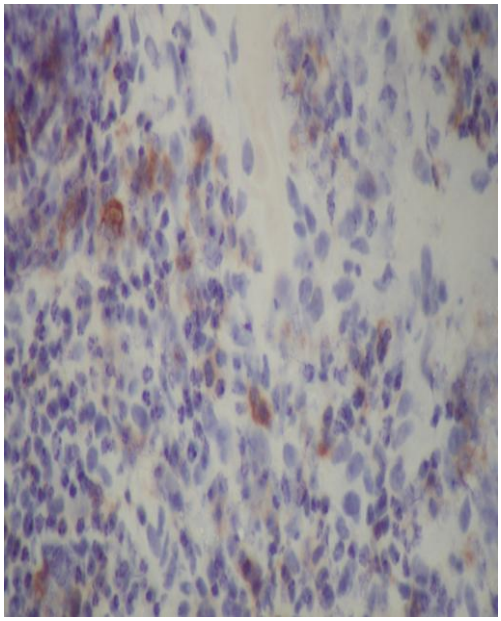


Figure 23: Immunohistochemical localization of HLA-class Π
A: Staining for HLA-class Π in nasal polyp tissue. (Positive control)
B: Staining for Isotype control (IgG1) in nasal polyp tissue. (Negative control)
C: Staining for HLA-class Π in normal kidney tissue.
D: Staining for HLA-class Π in acute tubulointerstitial nephritis tissue.

A



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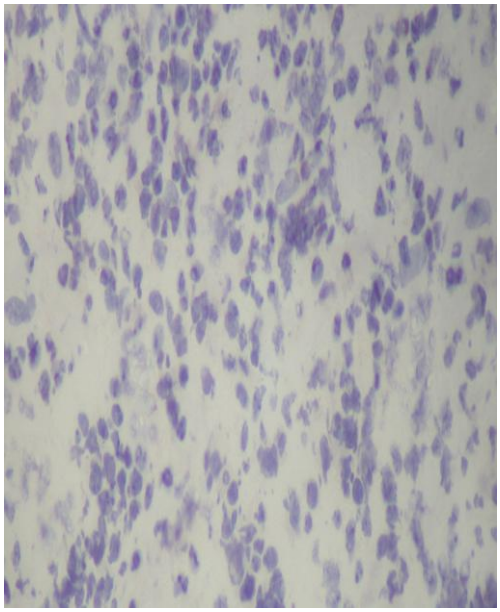

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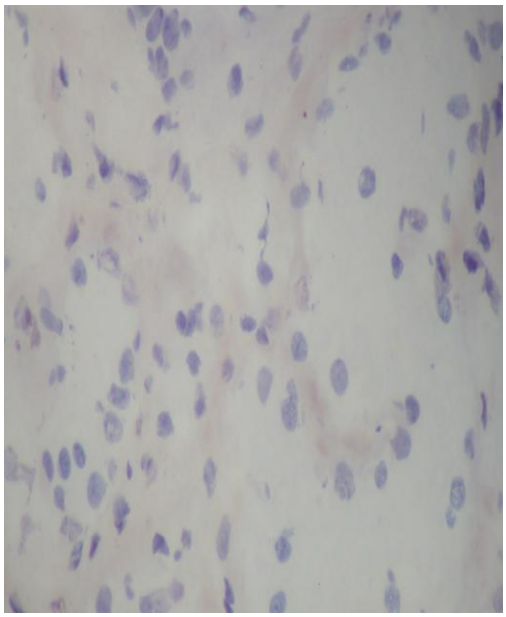

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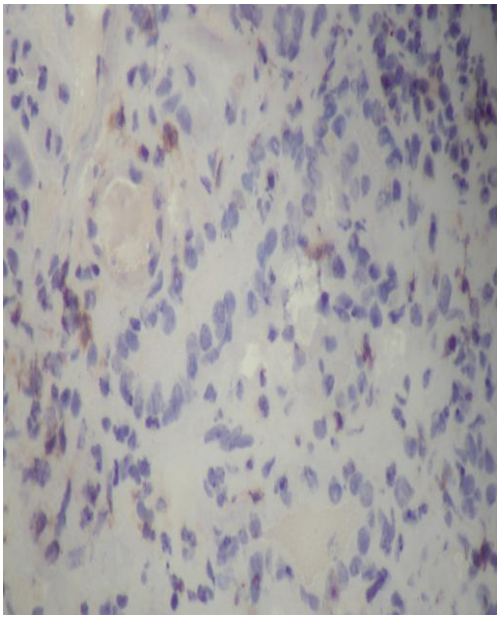


Figure 24 -Immunohistochemical localization of CD25+ cells
A: Staining for CD25 in nasal polyp tissue. (Positive control)
B: Staining for Isotype control (IgG1) in nasal polyp tissue. (Negative control)
C: Staining for CD25 in normal kidney tissue.
D: Staining for CD25 in acute tubulointerstitial nephritis tissue.

The count for cellular expression of eosinophil major basic protein (EMBP), CD3, CD4, CD8 and CD68 among the different subtypes of ATIN were shown in figures 25 and 26 respectively.

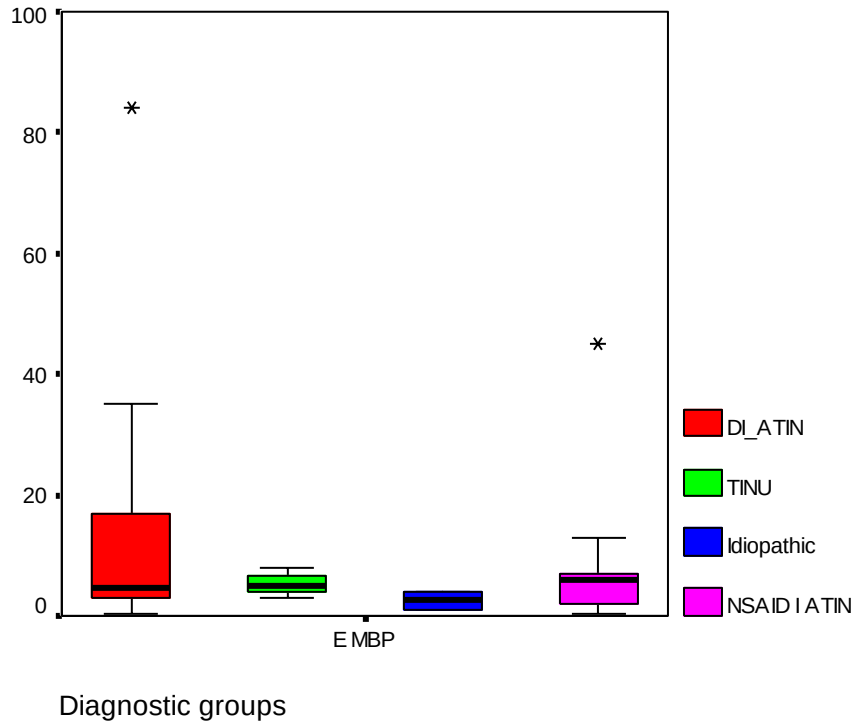


Figure 25- The cell count of infiltrating Eosinophil-major basic protein (EMBP) among the subtypes of ATIN, where red= drug-induced ATIN, green= TINU syndrome, blue= Idiopathic ATIN and pink= NSAID-induced ATIN. Stars indicate outlying values.

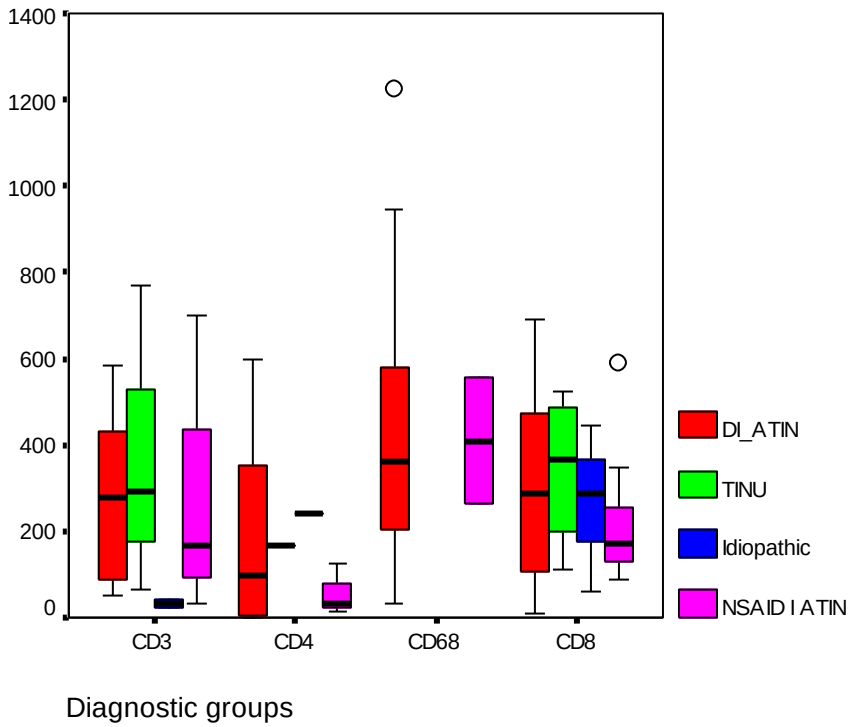


Figure 26-The infiltrating cell counts (CD3, CD4, CD8 and CD68) in the different subtypes of ATIN, where red= drug-induce ATIN, green= TINU syndrome, blue= Idiopathic ATIN and pink= NSAID-induced ATIN.

A comparison between the ATIN subtypes for the infiltrating cells (EMBP, CD3, CD4, CD8) using Kruskl-Wallis test showed no significant difference and the P value was 0.663, 0.179, 0.597 and 0.770 respectively (Table 21).

Table 21- Comparison between the infiltrating cells (EMBP, CD3, CD4 and CD8) among the ATIN subtypes, drug-induced ATIN (DIATIN), Idiopathic ATIN, NSAID-induced ATIN (NSAIDIATIN) and TINU syndrome.

Diagnosis	Number of cases	Mean rank	P value
EMBP			
DIATIN	10	13.85	
Idiopathic	2	7.25	P = 0.663
NSAIDIATIN	9	11.89	
TINU syndrome	3	13.33	
Total	24		
CD3			
DIATIN	9	12.67	
Idiopathic	2	2.00	P = 0.179
NSAIDIATIN	9	12.78	
TINU syndrome	3	14.33	
Total	23		
CD4			
DIATIN	8	6.88	
Idiopathic	1	11.00	P = 0.597
NSAIDIATIN	3	5.33	
TINU syndrome	1	9.00	
Total	13		
CD8			
DIATIN	13	16.12	
Idiopathic	3	15.17	P = 0.770
NSAIDIATIN	10	13.50	
TINU syndrome	4	18.75	
Total	30		

A non parametric correlation (Spearman's rho) between the infiltrating cells and the creatinine levels at presentation, one week, one month, three months and one year (Table 22) was undertaken and the results showed only one significant correlation between the CD68 positive cell and creatinine at presentation, which showed that there is a tendency

for higher CD68 positive cell infiltration to be associated with a higher creatinine level at presentation and P value was 0.003 (r = 0.651)(figure 27). On the other hand, no correlations were found between the other infiltrating cells, namely lymphocytes or eosinophils defined by the markers CD3, CD4, CD8 and EMBP, and the creatinine levels at any time points.

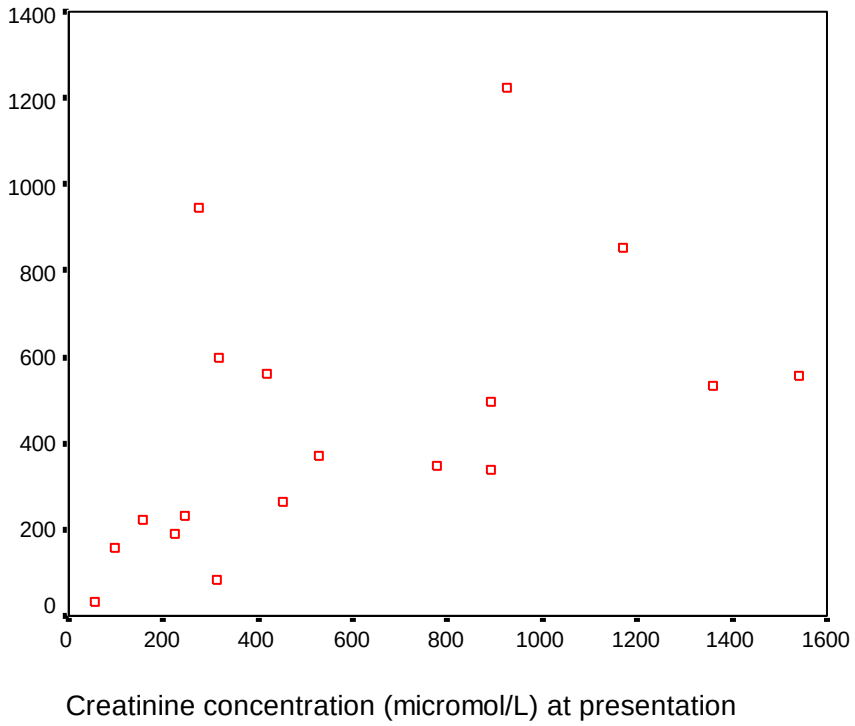


Figure 27- The correlation between CD68 positive cells and serum creatinine at presentation. (n=18). P value = 0.003, r = 0.651

Table 22- The correlation between the various infiltrating cells and creatinine levels at different time points.

Nonparametric Correlations

Correlations

			E_MBP	CD3	CD4	CD8	CD68	Cr present	CR_1WEEK	Cr 1month	Cr 3months	Cr 1 year
Spearman's rho	E_MBP	Correlation Coefficient	1.000	.195	.267	.361	1.000	.157	.034	-.232	-.377	-.336
		Sig. (2-tailed)	.	.372	.455	.099	.	.465	.875	.274	.092	.136
		N	24	23	10	22	2	24	24	24	21	21
	CD3	Correlation Coefficient	.195	1.000	.455	.657**	-1.000	-.325	-.051	-.136	.226	.265
		Sig. (2-tailed)	.372	.	.187	.001	1.000	.130	.816	.535	.339	.259
		N	23	23	10	22	2	23	23	23	20	20
	CD4	Correlation Coefficient	.267	.455	1.000	.792**	1.000**	-.377	-.300	-.252	.064	.018
		Sig. (2-tailed)	.455	.187	.	.001	.	.204	.320	.406	.852	.958
		N	10	10	13	13	3	13	13	13	11	11
	CD8	Correlation Coefficient	.361	.657**	.792**	1.000	1.000**	-.177	-.133	-.019	.080	.109
		Sig. (2-tailed)	.099	.001	.001	.	.	.350	.482	.921	.656	.597
		N	22	22	13	30	3	30	30	30	27	26
	CD68	Correlation Coefficient	1.000**	-1.000	1.000**	1.000**	1.000	.651**	.452	.350	.344	.309
		Sig. (2-tailed)	.	1.000	.	.	.	.003	.060	.155	.192	.244
		N	2	2	3	3	18	18	18	18	16	16
	Cr present	Correlation Coefficient	.157	-.325	-.377	-.177	.651**	1.000	.839**	.625**	.388*	.297
		Sig. (2-tailed)	.465	.130	.204	.350	.003	.	.000	.000	.011	.060
		N	24	23	13	30	18	47	47	47	42	41
	CR_1WEEK	Correlation Coefficient	.034	-.051	-.300	-.133	.452	.839**	1.000	.757**	.548**	.502**
		Sig. (2-tailed)	.875	.816	.320	.482	.060	.000	.	.000	.000	.001
		N	24	23	13	30	18	47	47	47	42	41
	Cr 1month	Correlation Coefficient	-.232	-.136	-.252	-.019	.350	.625**	.757**	1.000	.756**	.739**
		Sig. (2-tailed)	.274	.535	.406	.921	.155	.000	.000	.	.000	.000
		N	24	23	13	30	18	47	47	47	42	41
	Cr 3months	Correlation Coefficient	-.377	.226	.064	.090	.344	.388*	.548**	.756**	1.000	.965**
		Sig. (2-tailed)	.092	.339	.852	.656	.192	.011	.000	.000	.	.000
		N	21	20	11	27	16	42	42	42	42	40
	Cr 1 year	Correlation Coefficient	-.336	.265	.018	.109	.309	.297	.502**	.739**	.965**	1.000
		Sig. (2-tailed)	.136	.259	.958	.597	.244	.060	.001	.000	.000	.
		N	21	20	11	26	16	41	41	41	40	41

**. Correlation is significant at the .01 level (2-tailed).

*. Correlation is significant at the .05 level (2-tailed).

A comparison of the different infiltrating cell types (detected via EMBP, CD3, CD4, CD8 and CD68 staining) between the diagnostic groups (drug-induced ATIN and NSAID-induced ATIN) using Mann-Whitney test showed no significant difference between these two groups and P values were between 0.549 and 1.00 (Table 23 shows these results). This was despite a worse renal outcome in patients with NSAID-induced ATIN (figure 15).

Table 23- The comparison of the infiltrating cells (EMBP, CD3, CD4, CD8 and CD68) between drug-indued ATIN and NSAID-induced ATIN using Mann-Whitney test.

Diagnosis		No. of cases	Mean rank	P value
EMBP	DIATIN	10	10.75	
	NSAID IATIN	9	9.17	0.549
	Total	19		
CD3	DIATIN	9	9.44	
	NSAID IATIN	9	9.56	1.00
	Total	18		
CD4	DIATIN	8	6.25	
	NSAID IATIN	3	5.33	0.776
	Total	11		
CD8	DIATIN	13	12.77	
	NSAID IATIN	10	11.00	0.563
	Total	23		
CD68	DIATIN	16	9.44	
	NSAID IATIN	2	10.00	0.941
	Total	18		

EMBP= eosinophil major basic protein, DIATIN= drug-induced ATIN and NSAID IATIN= non-steroidal anti-inflammatory drug-induced ATIN.

To determine if there was a relationship between the infiltrating cells and the outcome of renal function, a comparison between the infiltrating cell numbers and creatinine levels at three months was done using the Mann-Whitney test (where reversible renal impairment was defined as a Cr <150 μmol/l and, irreversible renal impairment as a Cr > 150 μmol/l). The results showed no significant relationship between any type of infiltrating cell and the reversibility of renal function (Table 24).

Table 24- The relationship between the infiltrating cells (EMBP, CD3, CD4, CD8 and CD68) and the outcome of renal function at three months.

Infiltrating cell		No. of cases	Mean rank	P value
EMBP	0.00	9	10.22	
	1.00	15	13.87	0.238
	Total	24		
CD3	0.00	9	13.00	
	1.00	14	11.36	0.60
	Total	23		
CD4	0.00	5	5.70	
	1.00	8	7.81	0.354
	Total	13		
CD8	0.00	12	16.92	
	1.00	18	14.56	0.491
	Total	30		
CD68	0.00	7	10.86	
	1.00	11	8.64	0.425
	Total	18		

Where 0.00 = irreversible and 1.00= reversible.

With regard to the presence of granulomas, there were four biopsies with granulomas, three of them were associated with drug-induced ATIN and the fourth one was related to NSAID.

4.2- Immunohistochemical analysis of Th 2 cytokine, chemokine, and chemokine receptors in renal tissue of ATIN:

4.2.1-Background:

Leukocyte traffic is highly coordinated and a breakdown of the underlying control mechanisms might contribute to immune dysregulation or autoimmune disease.

The migration profiles vary greatly among individual types of leukocytes but are known to directly affect leukocyte retention and relocation at sites of immune defense and inflammation.

It is now well recognized that chemokines, together with adhesion molecules, are master controllers of leukocyte migration.

4.2.2-Experimental methods and materials:

Frozen sections of renal biopsy specimens derived from histopathologically confirmed cases of ATIN were analysed for the expression of IL-4 (figure 28D), eotaxin (figure 29D), CCR3 (figure 30D), CCR5 (figure 31D) and VCAM-1 (figure 32D).

Tissues from patients with nasal polyps were analysed for the expression of IL-4 (28A and B), Eotaxin (29A and B), CCR3 (30A and B), CCR5 (31A and B) and VCAM-1 (figure 32A and B) as a positive and negative control for each antibody respectively.

A three stage indirect immunoperoxidase method was used to stain the sections for Eotaxin, CCR3 and VCAM-1, while for IL-4 and CCR-5 a two stage indirect immunoperoxidase method was used because it gave lower background staining.

4.2.3- Results of staining:

4.2.3.1-Interleukin-4 (IL-4):

In ATIN, there was a clear staining of the Th 2-associated cytokine IL-4 (figure 28D) compared with normal kidney tissue (figure 28C). This IL-4 expression was accompanied by positive staining for the eosinophil-derived proteins (eosinophil peroxidase and eosinophil major basic protein), as well as for CD3 positive cells.

4.2.3.2- Eotaxin:

Eotaxin chemokine (an eosinophil chemoattractant that is produced by T lymphocytes) was detected with clear staining in the renal tissue of ATIN (figure 29D) and the staining was located in the same area as CD3+ cells within the interstitium. Figure (29B) shows isotype control (IgG1) staining in nasal polyp slides.

4.2.3.3-CCR3:

CCR3 is the receptor for eotaxin, and is expressed by eosinophils and a subset of T lymphocyte cells, mainly of Th 2 type. It is often expressed on Th 2 T cells local to eosinophil populations. In ATIN, CCR3 (figure 30D) was mainly located near the eosinophil cell infiltrates and T lymphocytes within the interstitium. Figure 30B shows isotype control (IgG2a) staining in nasal polyp slides.

4.2.3.4- CCR5:

CCR5 is expressed by activated T lymphocytes mainly of the Th 1 subtype and monocytes. CCR5 is a receptor for RANTES, MIP 1α and MIP 1β.

The expression of CCR5 (figure 31D) in ATIN was located in interstitial and peritubular sites.

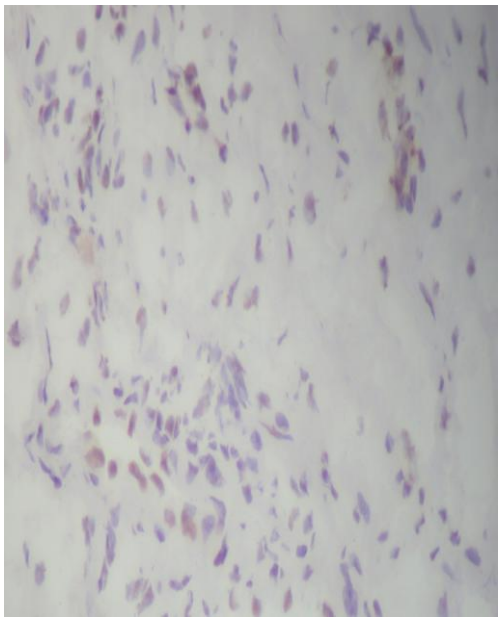
4.2.3.5- VCAM-1:

VCAM-1 (an adhesion ligand for leukocyte integrins, VLA-4) (figure 32D) expression was found in tubular epithelium and in renal vascular endothelium.

4.2.3.6- Statistical analysis:

As the staining was not sharply localized, a semiquantitaive analysis of the immunostaining of the chemokines and chemokine receptors was used, and it was (++), which represent a 1/3-2/3 of the tissues stained.

A



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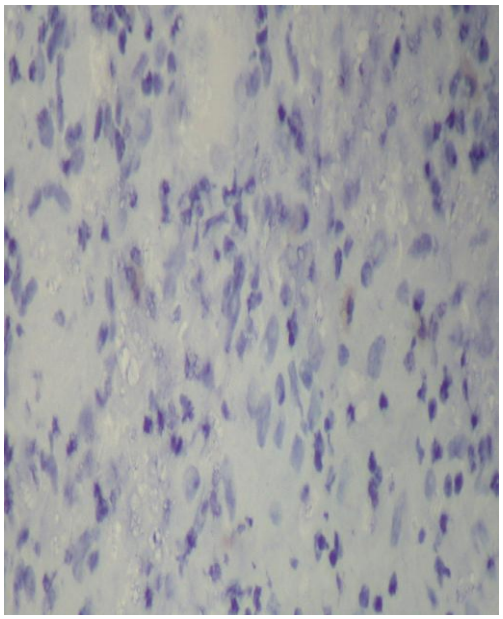

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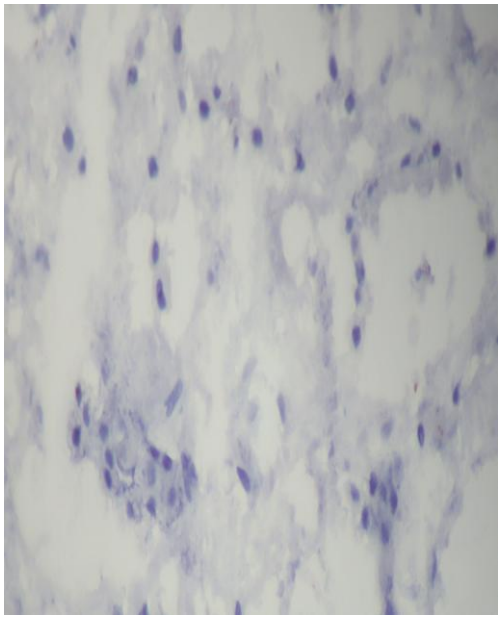

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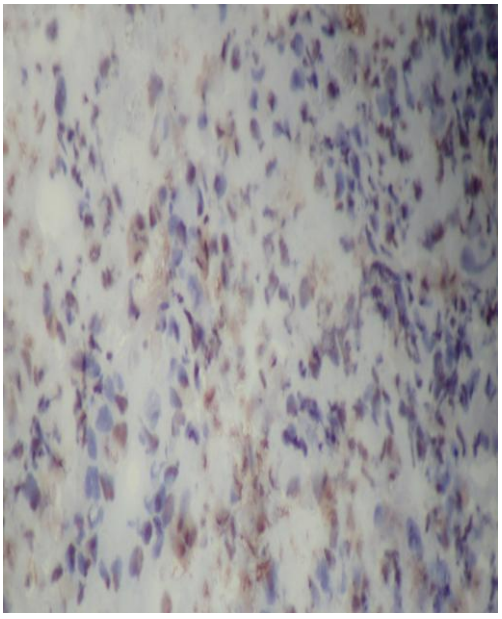
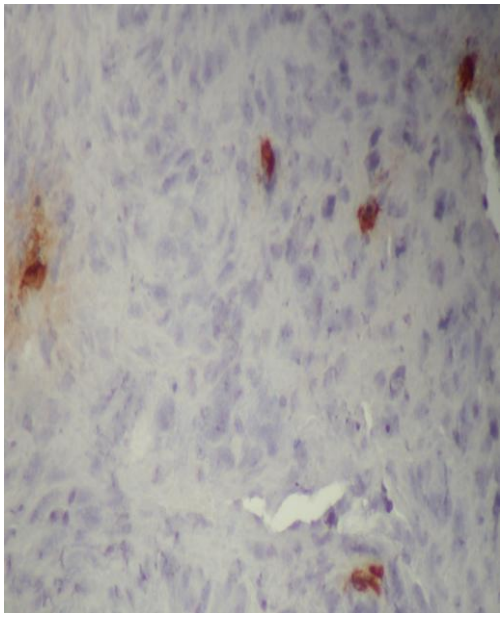


Figure 28:- Immnohistochemical localization of Interleukin-4 (IL-4)
A: Staining for IL-4 in nasal polyp tissue. (Positive control)
B: Staining for Isotype control (IgG2a) in nasal polyp tissue. (Negative control)
C: Staining for IL-4 in normal kidney tissue.
D: Staining for IL-4 in acute tubulointerstitial nephritis tissue.

A



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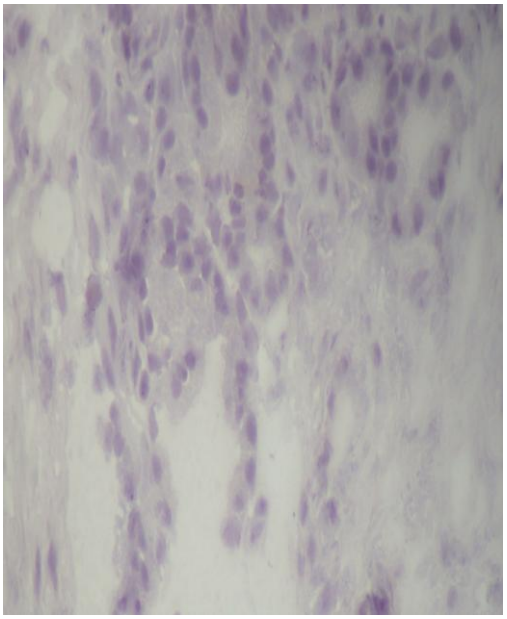

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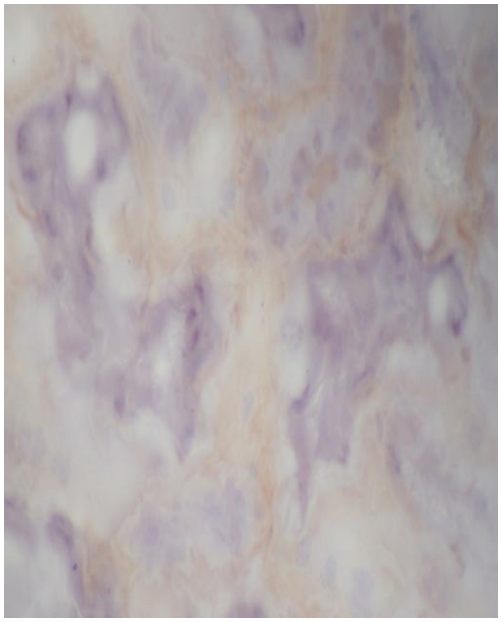

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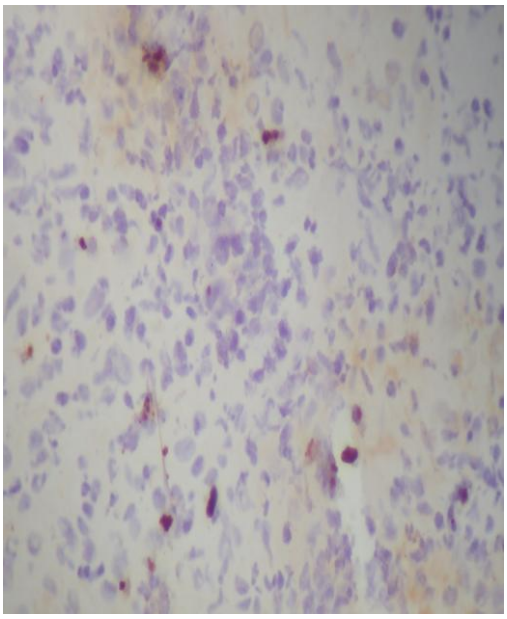
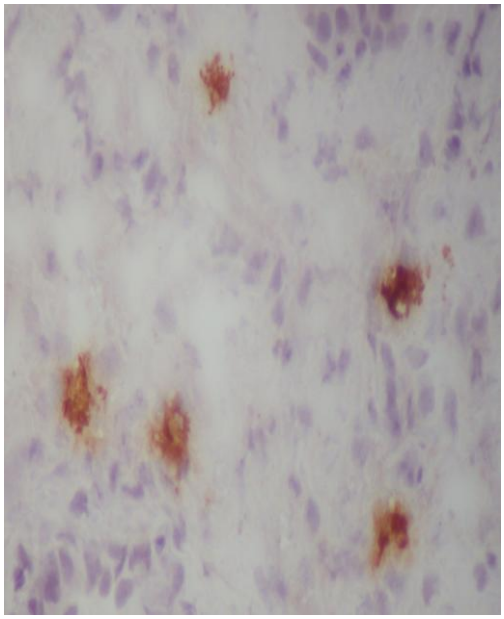


Figure 29:- Immunohistochemical localization of Eotaxin
A: Staining for Eotaxin in nasal polyp tissue. (Positive control)
B: Staining for Isotype control (IgG1) in nasal polyp tissue. (Negative control)
C: Staining for Eotaxin in normal kidney tissue.
D: Staining for Eotaxin in acute tubulointerstitial nephritis tissue.

A



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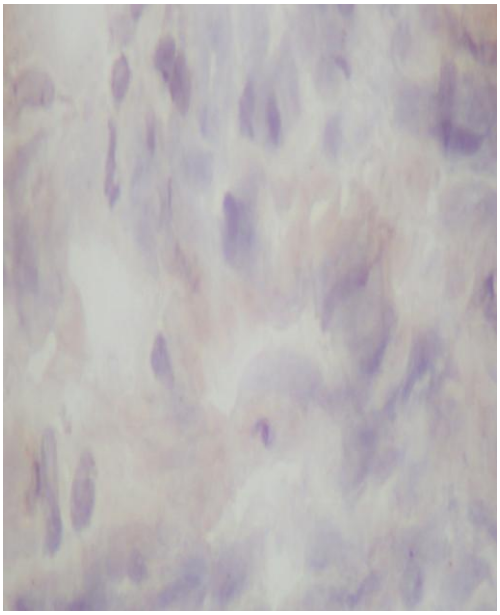

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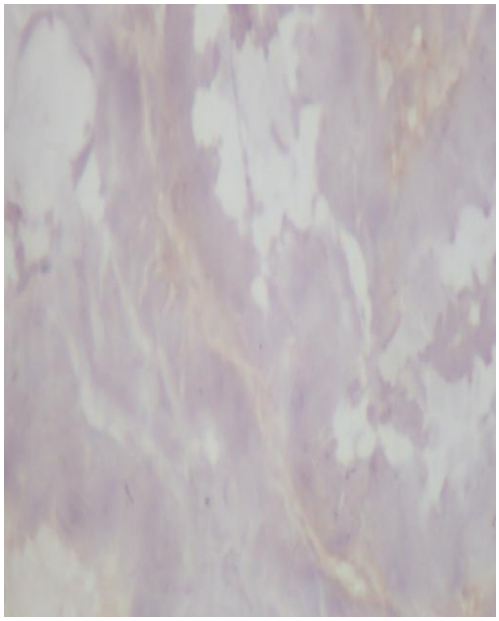

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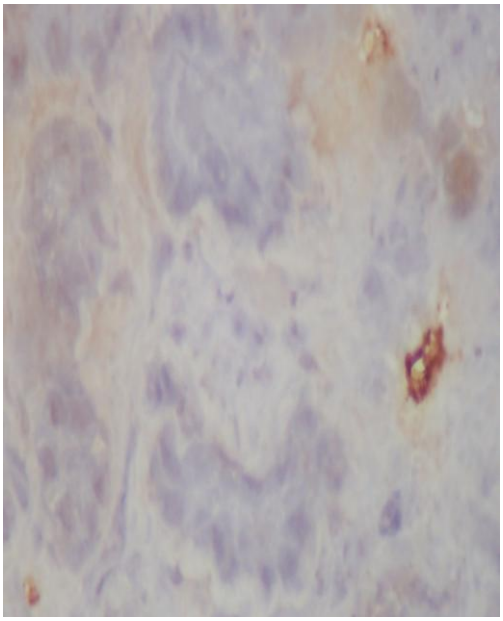
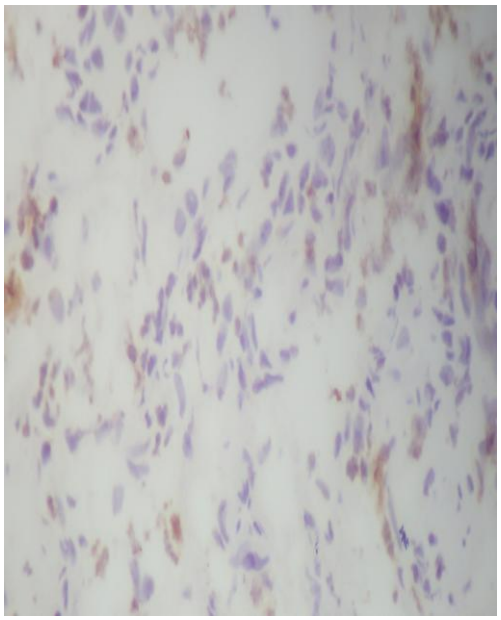


Figure 30:- Immunohistochemical localization of CCR3
A: Staining for CCR3 in nasal polyp tissue. (Positive control)
B: Staining for Isotype control (IgG2a) in nasal polyp tissue. (Negative control)
C: Staining for CCR3 in normal kidney tissue.
D: Staining for CCR3 in acute tubulointerstitial nephritis tissue.

A



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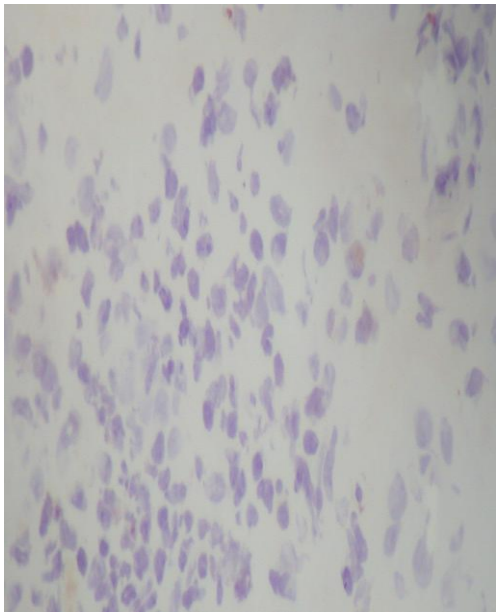

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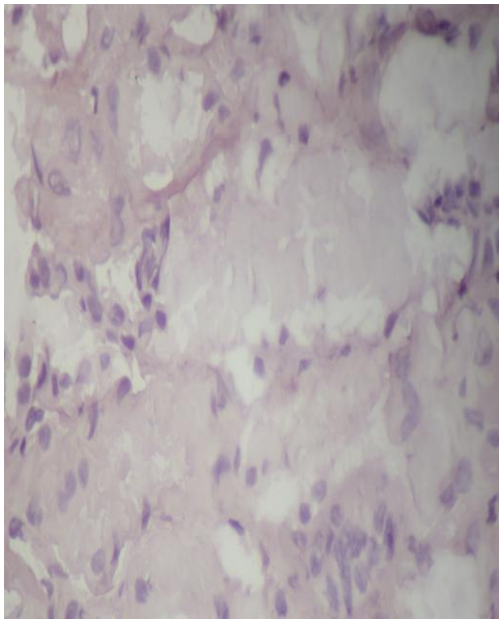

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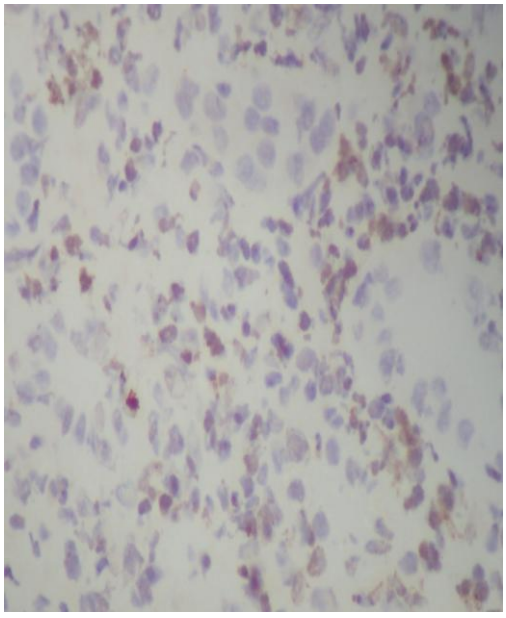
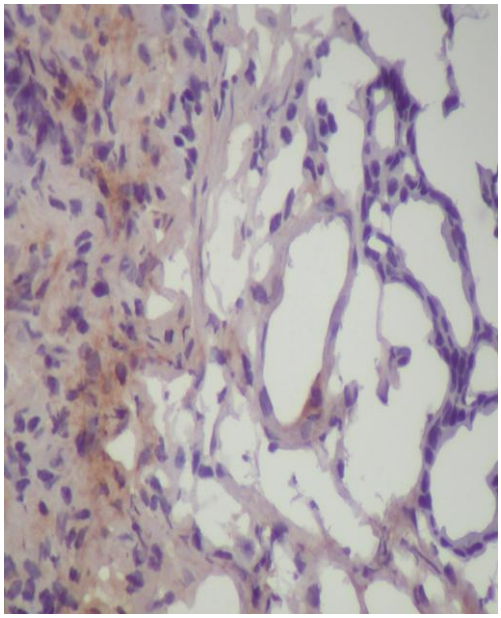


Figure 31:- Immunohistochemical localization of CCR5
A: Staining for CCR5 in nasal polyp tissue. (Positive control)
B: Staining for Isotype control (IgG2B) in nasal polyp tissue. (Negative control)
C: Staining for CCR5 in normal kidney tissue.
D: Staining for CCR5 in acute tubulointerstitial nephritis tissue.

A



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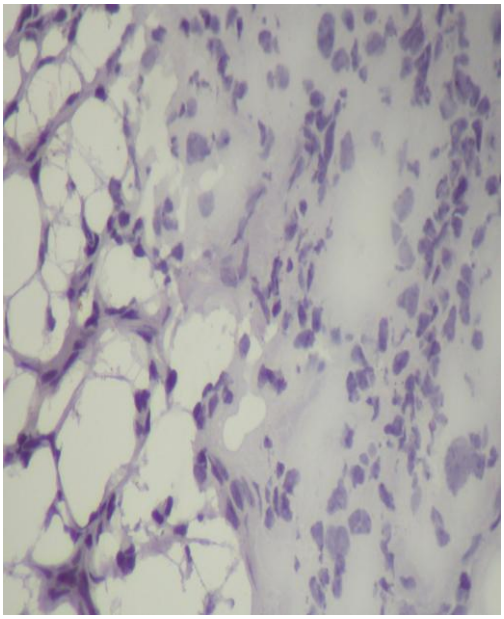

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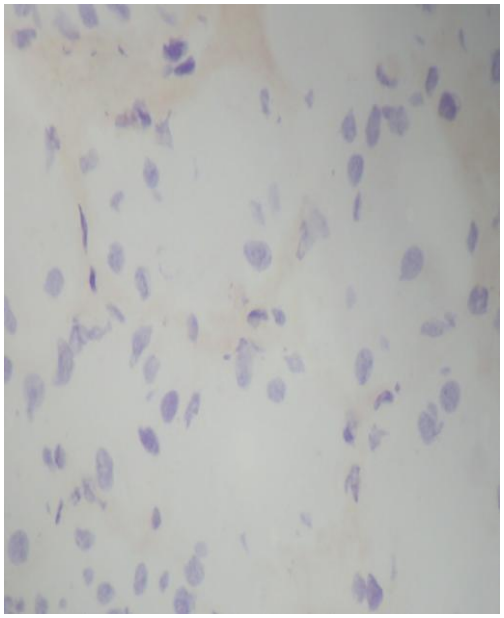

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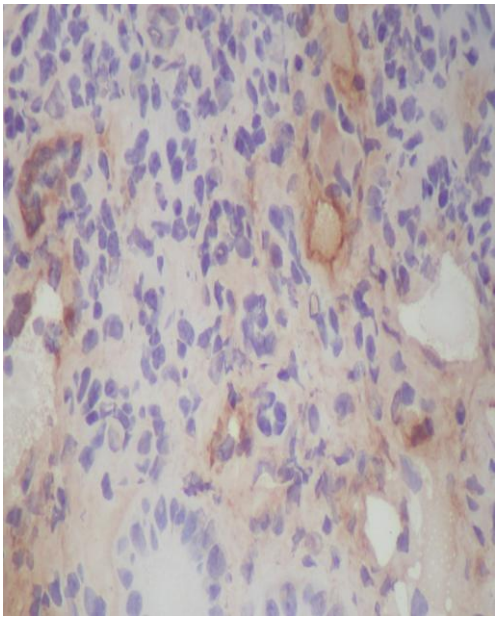


Figure 32:-Immunohistochemical localization of VCAM-1
A: Staining for VCAM-1 in nasal polyp tissue. (Positive control)
B: Staining for VCAM-1 Isotype control (IgG1) in nasal polyp tissue. (Negative control)
C: Staining for VCAM-1 in normal kidney tissue.
D: Staining for VCAM-1 in acute tubulointerstitial nephritis.

4.3- Immunohistochemical analysis of double staining method:

Double immuno-staining is a very difficult method for staining, especially when the two primary antibodies are raised in same species as this raises the possibility of cross-reactivity between the two antigen-antibody systems.

In this study, double staining for CD3+ cells and eosinophil peroxidase was successful (figure 33). However, colocalization of eotaxin to eosinophil proteins and T lymphocyte cells, or CCR3 to eosinophil proteins, all failed to show any co-localization. Different methods were tried for double staining (including a Dako protocol for double staining) and different isotypes were used from three different companies to try to make it work, but unfortunately with no success.

Co-localization staining is a difficult method, especially in this situation where all primary antibodies were mouse monoclonal in origin (i.e from same species) and this caused cross-reactivity between these antibodies and their isotypes. Even with this difficulty, clear individual staining could be detected for all of these antibodies. On the other hand, the isotype controls for these antibodies also gave positive staining which meant that there was no specificity to the results.

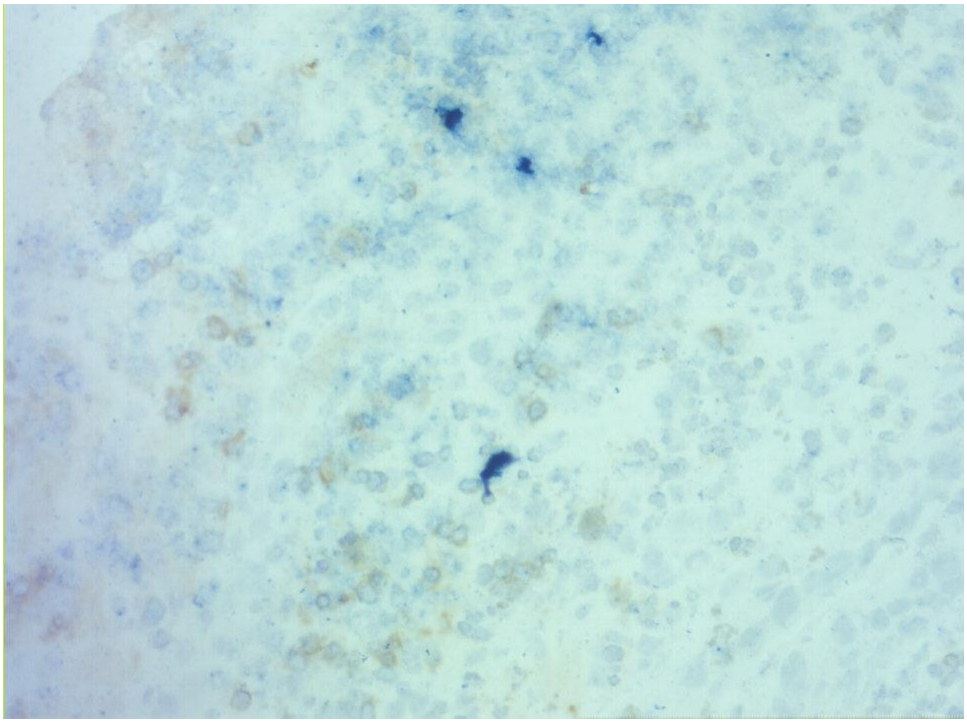


Figure 33– Immunohistochemical localization of CD3+ cells and Eosinophil peroxidase (Double staining) in nasal polyp tissue (Eosinophils in blue and CD3+ cells in brown colour).

4.4- Analysis of EBV markers in acute tubulointerstitial nephritis:

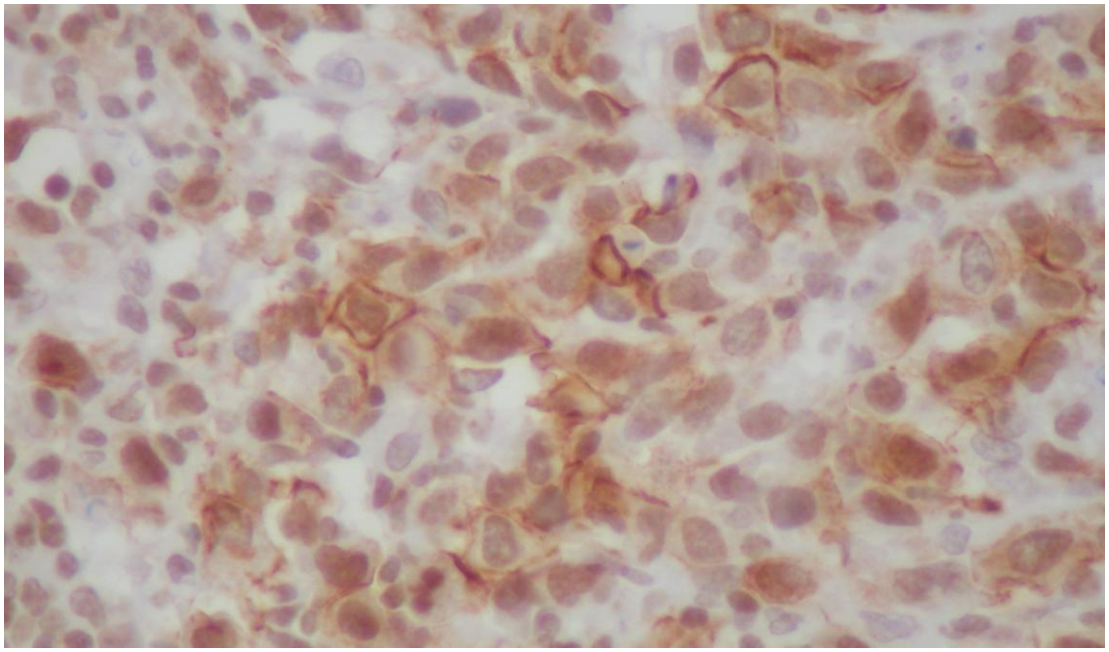
The presence of EBV was analysed in paraffin fixed sections using immunohistochemistry for EBV-encoded latent membrane protein (LMP)-1 and RNA/RNA in situ hybridization for EBV-encoded small RNAs (EBERs). EBER in situ hybridization is highly sensitive and is the method of choice for the histological detection of EBV. Hodgkin's disease lymph node tissues were used as a positive control for LMP-1 (figure 34A), ISH (figure 35A) and as a negative control for ISH (figure 35B).

Nearly 80 renal paraffin fixed slides were stained for latent membrane protein (LMP)-1 using a mouse monoclonal antibody and around 34 paraffin fixed slides were stained for EBERs using RNA/RNA in situ hybridization.

EBV-infected cells were not detected using either EBER RNA/RNA in situ hybridization (Figure 35C) or LMP-1 immunohistochemistry (Figure 34B).

To summarize, there is no relation between primary acute tubulointerstitial nephritis and Epstein-Barr virus in this study. It is possible that when a patient is acutely infected with the EBV virus, an acute tubulo-interstitial nephritis may develop.

A



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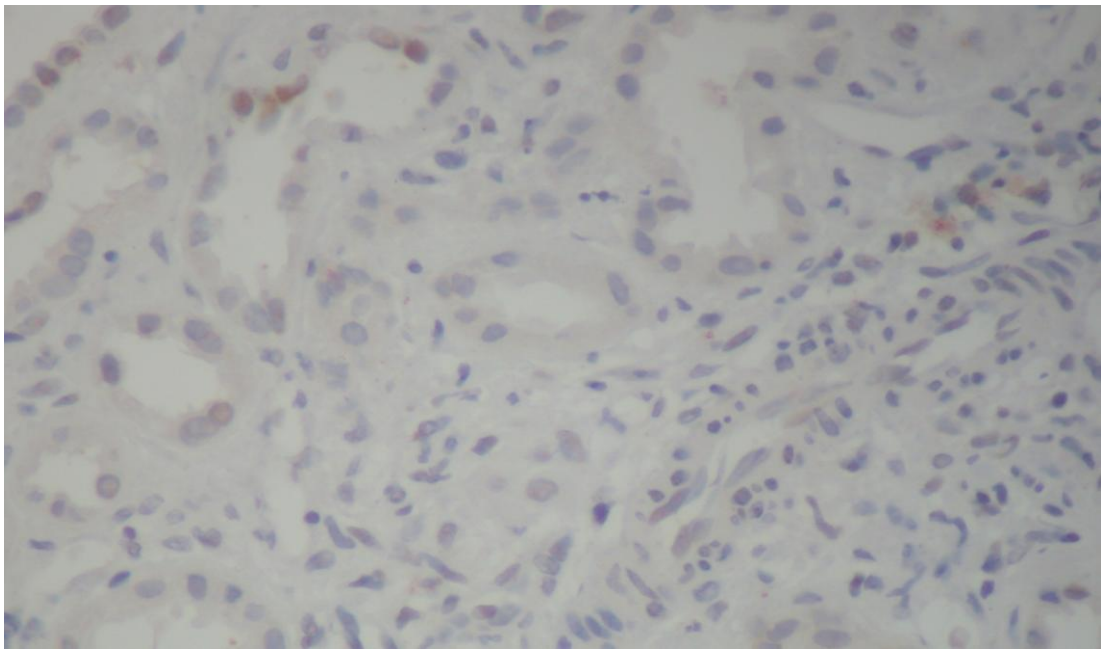
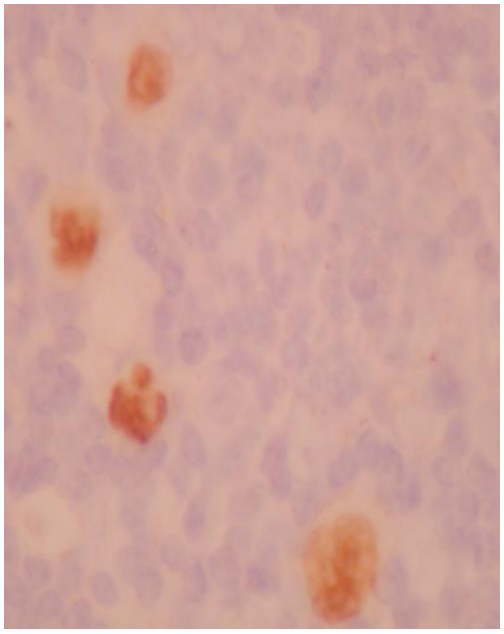


Figure 34:-Immunohistochemical localization of LMP-1+ cell

A: Staining for LMP-1 in Hodgkin lymph node tissue. (Positive control)

B: Staining for LMP-1 in acute tubulointerstitial nephritis tissue.

A



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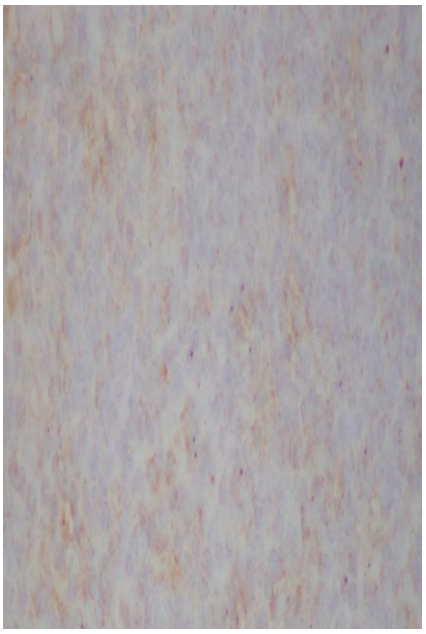

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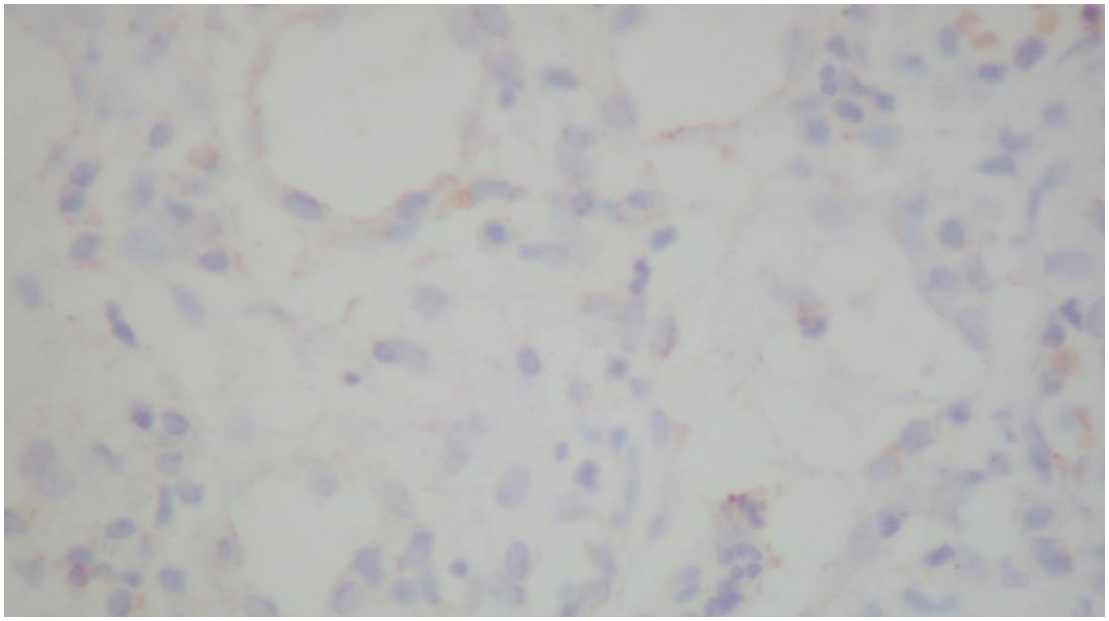

Figure 35:- In situ hybridization

A: Staining for (AS) anti-sense in Hodgkin lymph node tissue. (Positive control)

B: Staining for (S) sense in Hodgkin lymph node tissue. (Negative control)

C: Staining for (AS) anti-sense in acute tubulointerstitial nephritis tissue.

4.5- The index of chronic damage:

The index of chronic damage (chronic damage in renal biopsies) of ATIN was compared among the diagnosis groups (Drug-induced ATIN, NSAID-induced ATIN, Idiopathic ATIN and TINU syndrome) using Kruskal-Wallis test and the results showed a significant difference between these groups (P value = 0.024). Figure 36 shows this comparison.

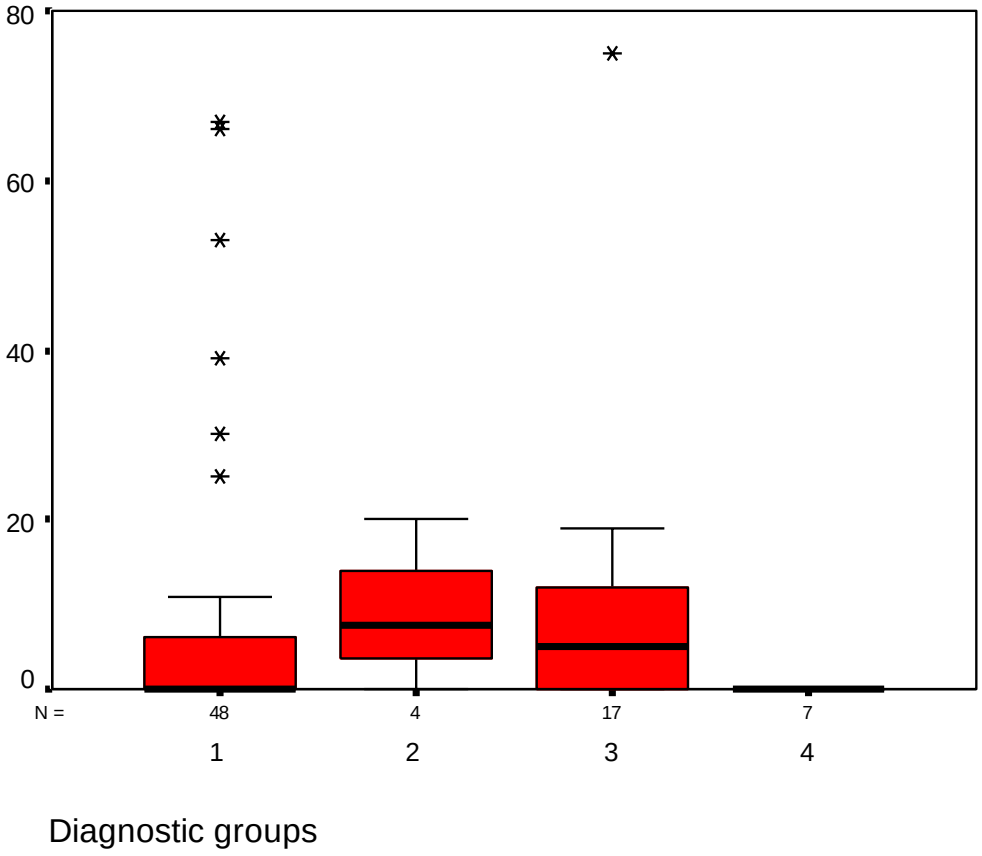

Figure 36:- The comparison of index of chronic damage among the four diagnosis groups, where 1= DIATIN group, 2= Idiopathic ATIN group, 3= NSAIDI ATIN and 4=TINU syndrome group. Stars show outlying values.

Furthermore, using a Mann-Whitney test comparison between the four groups on a pair basis, there was a significant difference (Table 25)

.

Table 25-Comparison of the index of chronic damage on paired groups using Mann-Whitney test.

Diagnostis Groups	P value
Groups 1 and 4	0.040
Groups 2 and 4	0.013
Groups 3 and 4	0.042

Where 1=DIATIN group, 2= Idiopathic ATIN group, 3= NSAIDIATIN group and 4= tinu syndrome group.

To analysis these findings further, group 4 (TINU syndrome) was excluded as it had a 0% index of chronic damage in all the patients, and a comparison between the other three groups (DIATIN, NSAIDIATIN and Idiopathic ATIN) using Kruskal-Wallis test was performed. The results showed no significant difference between these groups (P value = 0.204).

Although patients in the NSAID IATIN group had a poorer outcome at one year compared to DIATIN (Figure 15), there was no significant difference between the indices of chronic damage although the index of chronic damage tended to be high for patients with NSAID- IATIN.

For further analysis, the relationship between the index of chronic damage and the reversibility of renal function was explored at presentation, three months and one year, where Cr < 150 μmol/l was considered reversible and Cr > 150 μmol/l irreversible.

At presentation, there was no relation between the index of chronic damage and reversibility of renal function and the P value was 0.626 (figure 37).

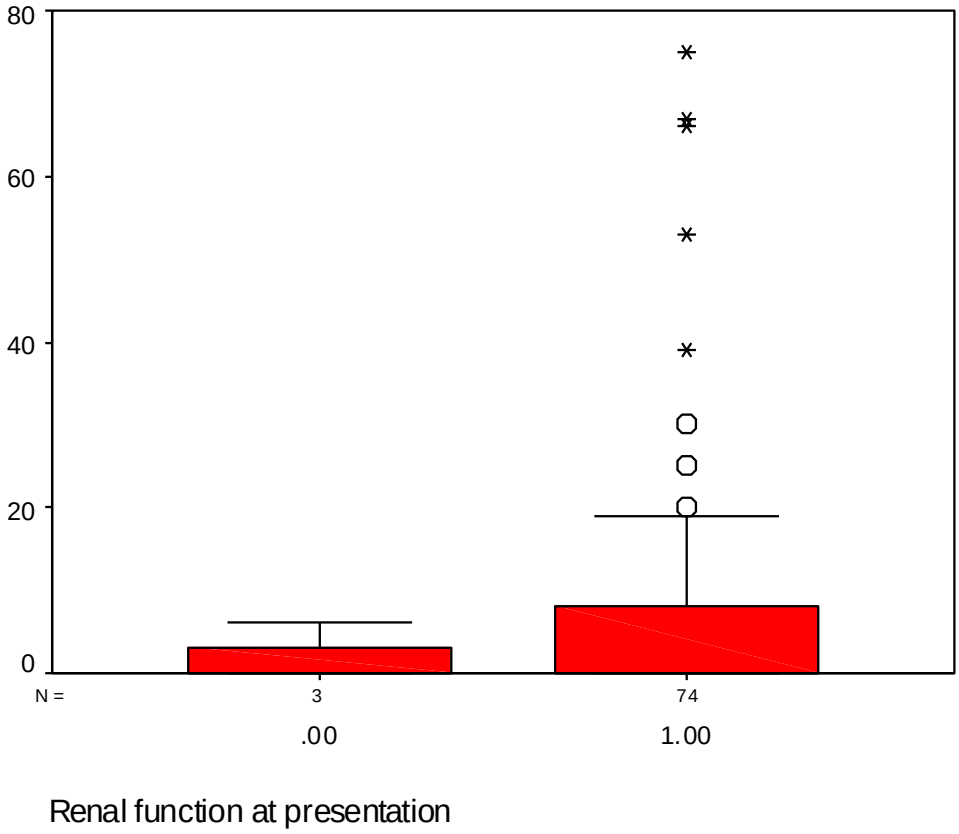

Figure 37**:-** The comparison between the index of chronic damage and renal outcome at presentation, where .00= reversible renal function and 1.00= irreversible renal function.

On the other hand, at three months (figure 38) and one year (figure 39) there was a significant relation between the index of chronic damage and reversibility of renal function and the P values were 0.002 and 0.001 respectively (a low chronic damage index was associated with a low creatinine level).

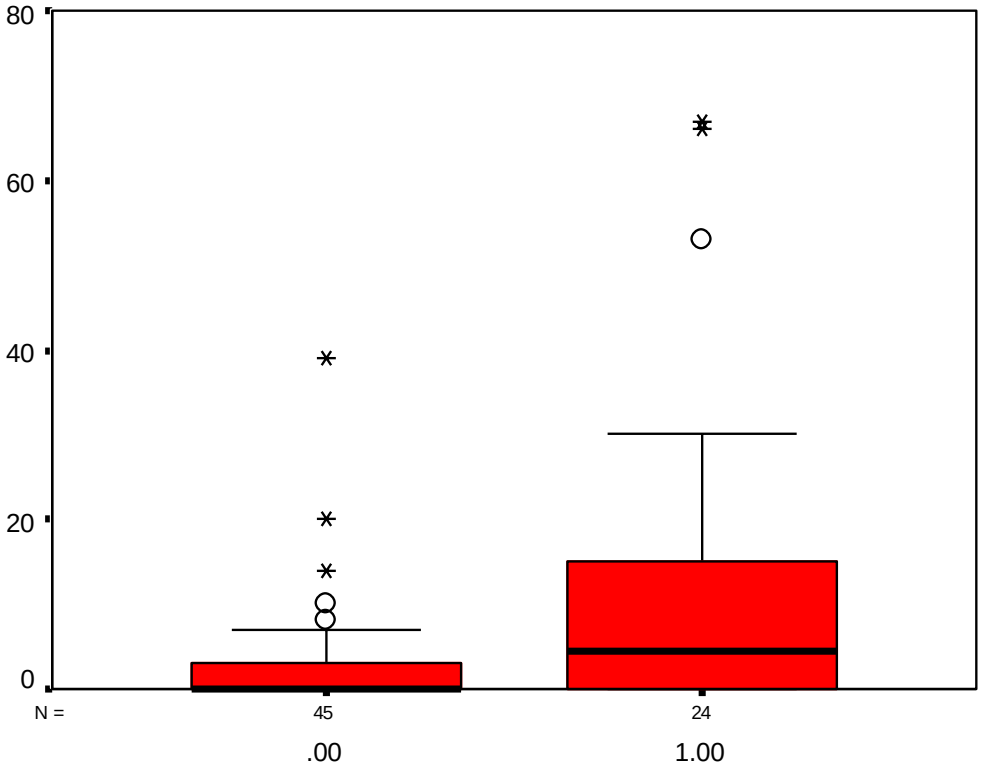

Figure 38- The comparison between the index of chronic damage and renal outcome at three months, where .00 =reversible renal function and 1.00 = irreversible renal function

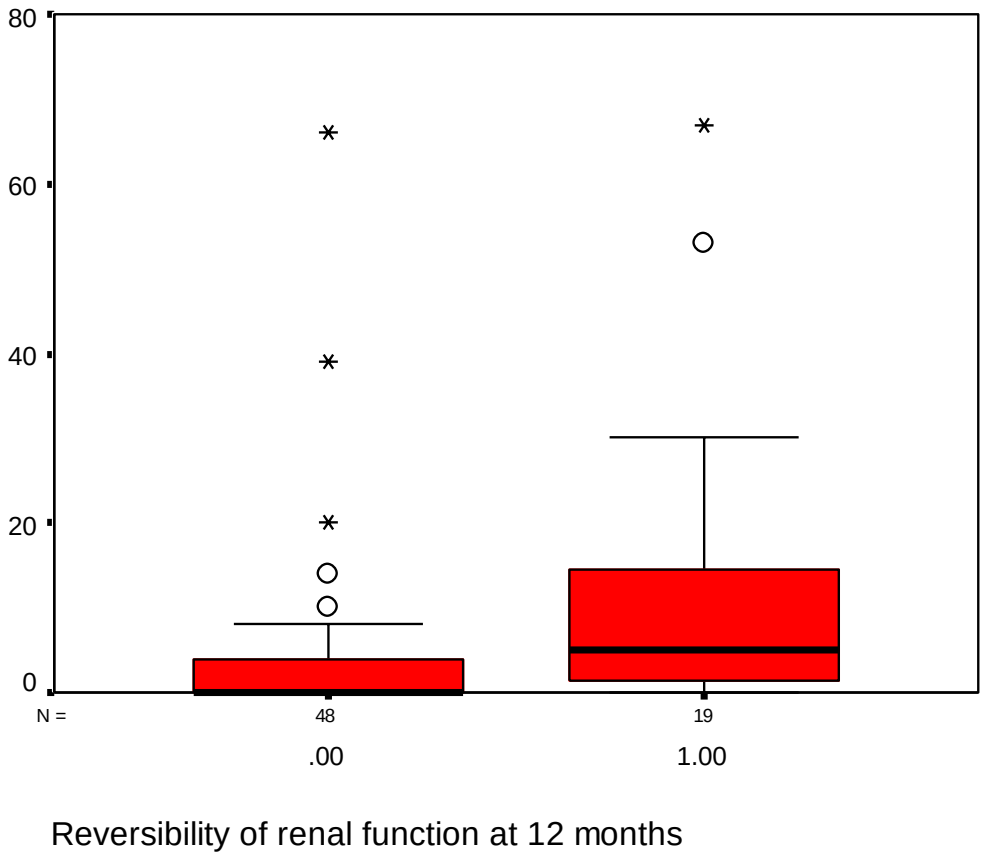

Figure 39:- The comparison between the index of chronic damage and renal outcome at one year, where .00 =reversible renal function and 1.00 = irreversible renal function.

To confirm these result and because the index of chronic damage was a continuous variable and in most biopsies it was 0%, the statistical analysis was repeated using Kendall's tau-b test and the result showed a significant relationship between the index of chronic damage and renal function at three months and one year and the P values were 0.028 and 0.013 respectively. However, it is worth noting that for an individual patient, it may be difficult to predict outcome at 3 months or one year unless the biopsy shows 0% damage or shows a very high damge index.

Chapter 5:

Discussion

5.1- The clinical features of acute tubulointerstitial nephritis:

5.1.1-Epidemiology:

Acute tubulointerstitial nephritis is a preferred term to acute interstitial nephritis because it emphasizes that tubular and interstitial changes are always associated features, whatever the primary process may be (13).

The incidence of ATIN in the biopsy material available for this study was 1%, which lies within the range of generally reported figures (1%-11%, mean 1.8%) (12, 90, 319, 322). ATIN in the present study accounts for approximately 8% of all the lesions associated with acute renal failure, which is smaller than that generally reported (15%-27%) (14, 405). The diagnostic approach of a department to patients with progressive renal disease is more important than true regional differences (14, 15). Not all patients with acute renal failure including those with ATIN are biopsied, since the causative event is often transient and undiagnosed or diagnosed retrospectively. Thus, the true incidence of ATIN is underestimated.

ATIN was originally thought to be exclusively infection-induced but it is now recognized to be mainly drug-induced. In the series of biopsy–proven acute tubulo-interstitial nephritis described here, 85% of the cases were drug-induced, compared to 35% of those reported by Cameron et al. (12) and to a mean of 49% reported in studies from 1974 to 1990 in the study by Schwarz, A et al (405).The diagnosis of TINU syndrome in this study was dependent on the diagnosis of uveitis by a consultant ophthalmologist prior to the presentation of ATIN in six patients and after ATIN in the seventh one. So, the drug history in the TINU syndrome was either a coincidence or a trigger factor. In the literature, TINU syndrome has not been associated with any specific drug and there are documented cases in the literature which have clearly occurred in the absence of any drug usage (339,

406). For idiopathic ATIN, there was no history of drug usage prior the presentation with ATIN. Renal insufficiency was always reversible in infection-induced ATIN (12) and the same was true for idiopathic ATIN and TINU syndrome in this study, but drug-induced ATIN resulted in permanent renal insufficiency in 25% of the cases after one year follow-up, which equates to the outcome of ATIN reported in the literature (26%) (405).

The patients' mean age was 55 years (15-85 years), and 53% were men compared to 46.6 years and 56.3% in Baker's study(407) and to 44 years and 56% in Schwarz's Series (405), thus the patients in the present study were older.The percentage of male patients was slightly higher than that for females in the three series. Acute symptoms were noted in 50% of the cases reported here, compared to 40% in Schwarz series (405) and some patients had several symptoms: rash in 16%, fever in 35% and eosinophilia in 15 % in our study, compared to 50%, 75% and 80% respectively in the study reported by Neilson et al (9). Recently Baker in his series (407) showed that the symptoms of rash, fever and eosinophilia were present in 14.8%, 35% and 23.3% of patients at presentation, respectively (Table 26).

Table 26- Percentage of some parameters in different studies.

Studies	Parameters			Year of report
	Rash	Fever	Eosinophilia	
Apple's study	50%	75%	80%	1983
Baker's study	14.8%	35%	23.3%	2004
Present study	16%	35%	15%	2006

The findings of this study were very similar to those of Baker and both are in contrast to an earlier series where allergic-type features dominated the clinical picture, which may be explained either by type of drugs (in earlier studies antibiotics were the main causative agents, for example methicillin-induced ATIN, while now a wide range of drugs is associated with ATIN), or may be due to increased recognition of the disease and earlier discontinuation of the drug. The triad of fever, arthralgia and rash was present in only seven of 78 (9%), which is similar to the 10% in Baker's series.

5.1.2-Treatment of ATIN:

The mainstay of treatment in ATIN is supportive therapy. After a presumptive (or biopsy-proven) diagnosis of ATIN has been made, any potentially offending drugs should be discontinued, or underlying infections treated. Treatment of ATIN would be much more straightforward if it could be diagnosed with a sensitive and specific non invasive test, and if one could reliably determine which agent, in the case of drug-induced ATIN, is responsible. In some patients, the degree of renal insufficiency may be quite significant, and dialytic therapy may be required as a supportive measure. In patients in whom drug discontinuation is not followed by a rapid improvement in renal function one must consider pharmacologic therapy for ATIN.

In addition, many nephrologists favour early pharmacologic therapy in patients who have a particularly severe interstitial nephritis, as manifested by either a rapidly rising serum creatinine or diffuse cellular infiltration on renal biopsy, or both. In forms of interstitial nephritis associated with systemic autoimmune disease and glomerulonephritis, pharmacologic therapy is usually appropriate.

Although corticosteroids are the most commonly used immunosuppressive drugs for ATIN, there have been no prospective, randomized trials performed to assess the

efficacy of this treatment. Evidence for efficacy has come from anecdotal case reports and small, uncontrolled, nonrandomized studies (9). In a retrospective analysis of 14 patients with methicillin-induced ATIN, eight of 14 patients received prednisolone therapy, with an average daily dose of 60 mg for a total mean duration of 9.6 days. Prednisolone therapy was associated with a higher percentage of patients returning to their previous serum creatinine level, a lower average serum creatinine at follow-up, and a shorter time between the peak serum creatinine and its return to a new base line (9.3 versus 54 days) (308). Pusey et al, (301) retrospectively examined seven patients with biopsy-proven ATIN treated with high-dose IV methylprednisolone. All responded with onset of diuresis or a spontaneous fall in serum creatinine within 72 hours. In all treated patients, renal function returned to near normal, with creatinine clearances 60 to 90 ml/min. Of the two patients not treated, one recovered renal function slowly, and one progressed to chronic renal insufficiency. There were no detectable adverse effects from the short courses of steroids used in either study (318). There have been no trials that establish the optimum dosing or duration of corticosteroid therapy. Neilson et al, have recommended a therapy with Prednisolone in an oral dose of approximately 1mg/kg daily, and treatment should be maintained for a period of approximately 4 weeks. If there has been no significant response by that time, there probably will not be and the drugs should be discontinued (9).

In this study, 70% of the patients had stopped the causative agents (mainly drugs) on admission to hospital, but there was no significant difference in terms of renal outcome between the patients who stopped their medications (causative agents) on admission and those who had not. Also, 62% of these patients received steroid therapy in a dose of 45-60 mg/day for a period of 4-12 weeks, and 28% of them steroids and dialysis together and the median creatinine concentrations at three months for the two groups was 129 µmol/l and

126 μmol/l respectively. There was no significant difference in renal outcome between those patients who received dialysis in addition to prednisolone and those who did not. Overall, 90% of this study's patients received prednisolone making any evaluation of the efficacy of this treatment difficult.

Patients with TINU syndrome and idiopathic ATIN were all treated with steroids and their median creatinine concentrations at 3 months were 118 and 130 μmol/l respectively. In almost all cases of TINU syndrome, the nephropathy responds to steroid treatment (345). The uveitis follows its course independently from the nephropathy and sometimes tends to relapse (344). Patients with drug-induced ATIN and NSAID-induced ATIN had median creatinine concentrations of 127μmol/L and 154μmol/L at 3 months respectively.

Some have recommended adjunctive therapy with cyclophosphamide at 1 to 2 mg/kg per day, if there is no improvement in serum creatinine after a trial of steroid therapy (9). Usage of both cyclophosphamide and cyclosporine A to treat ATIN is supported by investigations in experimental models of interstitial nephritis (78, 408), but there are no clinical trails in man. Plasmapheresis may be considered as adjunctive therapy along with prednisolone or cyclophosphamide in those in whom anti-TBM antibodies are demonstrable in the renal biopsy. There were no patients in this category in this cohort.

5.1.3-Prognosis of ATIN:

Because ATIN is associated with diverse aetiologies, it is difficult to establish a general prognosis for all causes of ATIN. Most of the available information on outcomes is derived from patients with probable drug-induced ATIN. In general, if drug-associated ATIN is detected early (within 1 week of the rise in serum creatinine), and the drug is

promptly discontinued, the long-term outcome is favourable for a return to baseline serum creatinine.

The inflammatory lesion of ATIN can become a lesion characterized by fibrosis and tubular atrophy, hallmarks of chronic interstitial nephritis, if the inciting factors persist. Laberke and Bohle compared clinical and morphological findings in 30 cases of ATIN, all of which had been confirmed by renal biopsy , to determine whether histological findings could provide conclusive information regarding the course and prognosis of ATIN (11). This was a retrospective study and serum creatinine values were used as clinical criteria for evaluating course and prognosis of disease. The findings suggested that it is important to differentiate histologically between ATIN cases with diffuse infiltration and those with patchy and/or incompletely diffuse infiltration. Prognosis is significantly better for the latter. The presence of 1 to 6% neutrophils in the infiltrate also correlated with an adverse prognosis. Patients with ATIN accompanied by acute renal failure of more than three weeks duration had a poorer prognosis for complete recovery of renal function.

The present study shows that renal insufficiency was reversible (Cr < 150 μmol/l) in 47 patients with ATIN (60%), irreversible (Cr > 150 μmol/l) in 23 patients (30%), 4 patients died (5%) and no data was available for the remaining 4 patients (5%) at 3 months. Patients, who did not have malaise and tiredness and who had fever, normal or high level of haemoglobin, lower or normal potassium level and those with low or normal phosphate tended to have reversible renal function. These parameters (haemoglobin level, serum potassium and serum phosphate) are probably indirect measures of impaired renal function, while malaise, tiredness and fever are non specific symptoms. Other indices such as blood pressure, arthralgia, initial renal symptoms, and gender had no significant relationship with the outcome.

Kaplan-Meier functional survival curves to determine the renal functional outcome showed that 75% of our patients had an improvement in renal function by one year (i.e Cr level < 150 μmol/l), and this agrees with the reported studies in the literature which showed permanent renal insufficiency in 26% of the cases (319, 409). This study also shows that ATIN due to non-steroidal anti-inflammatory drugs carries a worse prognosis in comparison to other causes of ATIN, and this finding broadly agrees with the published reports, where chronic renal insufficiency occurred in 10%-67% after NSAID-induced ATIN (12, 20, 410, 411), and this can be explained by delay in the diagnosis of renal disturbance due to NSAID intake due to the absence of acute symptoms reflecting hypersensitivity (412-414), and longer period of drug intake, which however, may lead to permanent renal insufficiency (405).

In a previous study of lupus nephritis, the index of chronic damage was highly correlated with the degree of renal impairment at the time of biopsy and at the end of the period of observation (415). This study also shows a significant relationship between the index of chronic damage (chronic damage in renal biopsies) of ATIN and the reversibility of renal function at three months and one year time points, and P values were 0.002 and 0.001 respectively (a low index of chronic damage, or a 0% score, was associated with a low serum creatinine level), and this measurement can be used to predict the outcome for renal function. The difference between lupus disease and ATIN in the correlation between the index of chronic damage and the degree of renal impairment can be explained as, in lupus, disease may have been present for longer before becoming clinically overt, thereby leading to more chronic damage, while there may have been a greater proportion of acute lesions in ATIN. There was a tendency for patients with NSAID-induced ATIN to have a higher index of chronic damage but this was not statistically significant.

In other studies, the phenotype of infiltrating cells, degree of tubulitis, and tubular expression of Vimentin (as an assessment of tubular damage) were not found to predict outcome, instead, the severity of interstitial fibrosis was the most important prognostic factor (416).

Kida et al. examined the long-term prognosis of 14 patients with biopsy proven ATIN by analyzing laboratory data, histological changes, and clinical features both early and late in the disease course. They noted two phases to the recovery from ATIN: an initial phase of rapid improvement in glomerular filtration rate (the first 6 to 8 weeks) followed by slow improvement in glomerular filtration rate over the following year. In their series, half of the patients studied ultimately displayed a higher baseline serum creatinine. Final glomerular filtration rate correlated with the degree of early improvement, suggesting that the latter may be a reliable predictor of long-term prognosis. Age at onset of ATIN correlated inversely with final glomerular filtration rate, whereas other indices such as extra renal manifestations, initial renal symptoms, and gender had no significant relationship to outcome. Severity of the interstitial lesion was noted to correlate closely with final glomerular filtration rate (409).

5.2-The histopathological findings in acute tubulointerstitial nephritis:

5.2.1-Lymphocytes:

The hallmark of ATIN is the presence of inflammatory infiltrates within the interstitium. These infiltrative lesions may be patchy or diffuse, predominating in the deep cortex and in the outer medulla. They are composed mostly of T lymphocytes, as well as monocytes/macrophages, plasma cells, eosinophils and mast cells. Among T cells present within the interstitium, the relative number of CD4+ cells and CD8+ cells varies from one patient to another (97, 416-418). The relative representation of T cells is probably influenced by the type of noxious drug, and the genetic background of the patient (419).

This study showed a variable number of infiltrating cells within the renal biopsies and the quantitative analysis of these cells showed no significant difference between them across the different types of ATIN. It also showed that there is no correlation between the type of infiltrating cell and the serum creatinine level at various time points. A comparison between the diagnostic groups (drug-induced ATIN and NSAID-induced ATIN) and the different infiltrating cell types showed no significant difference, furthermore, comparison between the infiltrating cell and the reversibility of renal function showed no significant association between any type of infiltrating cells and the reversibility of renal function, and this is in agreement with published reports (419).

Presentation of antigen by MHC class Π- bearing cells results in the activation of helper/inducer T cells and the propagation of the immune response (12). Within the normal human kidney, MHC class Π antigen is displayed only upon vascular endothelium, and the dendritic cells in close relationship to the peritubular capillaries of the interstitium (420). Renal tubules express increased amounts of MHC class Π specificities during allograft rejection (421) and in human tubulointerstitial nephritis of all types (305). This study

demonstrated the presence of MHC class Π expression by the interstitial infiltrating cells and to lesser extent by tubular cells, which is in agreement with previous studies (305, 420)

IL-2 receptors (CD25) are considered to be transiently expressed in recently activated T lymphocytes (422). This study demonstrated the presence of T lymphocytes expressing IL-2 receptors (CD25) in the renal interstitium, which is in agreement with other studies (97, 423, 424). However, neither MHC class Π expression nor CD25 expression correlated with type of ATIN.

5.2.2-Macrophages:

Macrophage infiltration is an important feature of glomerular and tubulointerstitial disease, and the degree of mononuclear cell infiltrate has been used to predict subsequent progression (425). A large number of infiltrating macrophages may be present in many renal diseases, for example, in antineutrophil cytoplasmic antibody (ANCA)- positive glomerulonephritis, lupus nephritis, cryoglobulinemia, mesangioproliferative glomerulonephritis, and IgA nephropathy (426). Interstitial macrophages are a common feature of most forms of progressive renal damage including those not generally considered to be inflammatory such as diabetic nephropathy (427).

In IgA nephropathy, plasma creatinine at time of biopsy was significantly greater in patients with an increased number of interstitial monocytes/macrophages ($P < 0.05$) (428). Furthermore, glomerular function at presentation was significantly correlated with a higher number of CD14 +ve monocytes/macrophages in lupus nephritis (415). This study showed a variable number of infiltrating macrophages in the biopsies of patients with ATIN and the quantitative analysis of these cells showed no significant difference across the different types of ATIN. However, it did show a correlation between the number of infiltrating CD68+ macrophages and the serum creatinine concentration at presentation. This indicates

that there is a tendency for a higher CD68+ cell infiltration to be associated with a higher serum creatinine concentration at presentation (i.e more severe renal damage) and the P value was 0.003 (r=0.651), which is in agreement with the findings of others, in lupus nephritis, IgA nephropathy and glomerulonephritis respectively (412, 393, 394).

5.2.3-Eosinophils:

Eosinophils are implicated in the pathogenesis of numerous inflammatory processes, especially allergic disorders (131) (429). More important, extensive deposition of eosinophil "specific" or "secondary" granule proteins is present in the inflamed tissue of allergy patients. These granule proteins include several cationic proteins: eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), and eosinophil major basic protein (EMBP). Major basic protein (EMBP) is the most abundant cationic eosinophil granule proteins (429). Futhermore, MBP and EPO can directly induce tissue damage and dysfunction (228). Eosinophils are occasionally demonstrated within a mixed cellular infiltrate producing interstitial injury (430) which agrees with the findings of this study . Regarding the pathogenetic role of eosinophils, the toxic cationic molecules that they produce probably participate in tubular damage, while lipid mediators produced by eosinophils probably contribute to the inflammatory process (429). The prognostic value of eosinophilia has not been carefully studied in patients with drug-induced ATIN (416), but in patients with acute renal allograft rejection, renal eosinophilia has been associated with an increased risk of graft loss (431). However, their importance in ATIN has probably been overemphasized as this study shows no relationship between numbers or presence of infiltrating eosinophils and the reversibility of renal function in ATIN.

5.2.4-Mast cells:

Mast cells were mainly detected in fibrotic lesions of tubulointerstitial nephritis, rather than in oedematous areas. Furthermore, mast cells could not be detected in any damaged glomeruli as found previously (432). Mast cells express high-affinity IgE receptors in contrast to eosinophils which express low-affinity IgE receptors (433). Activation of mast cells can be induced by multiple mechanisms, including IgE- and complement-mediated pathways. Mast cells synthesize and secrete histamine, heparin, and various proteases and chemical mediators, such as prostaglandins, leukotrienes, and platelet-activating factors (434). Mast cell tryptase is a major protease and has mitogenic effects on various types of cultured cells, such as smooth muscle cells and bronchial epithelial cells (435). Mast cell tryptase is stabilized as an enzymatically active tetramer by association with heparin and dissociates to inactive monomers in the absence of heparin, suggesting that heparin is an essential component for the induction of tryptase activity on cultured renal fibroblasts (101). Tryptase has been shown to mediate effects on cells primarily by Protease Activated Receptors-2 (PAR-2). The PAR receptors have an interesting mechanism of activation since the protease cleaves an amino-terminal peptide to unmask a new receptor amino terminus which serves as a tethered peptide ligand (436). This study shows sporadic distribution of mast cells in the interstitium of ATIN sections, which when compared under light microscope to the normal kidney sections showed no differences.

5.2.5-Interleukin 4 (IL-4):

IL-4 is the prototypic Th 2 cytokine (437) that has multiple effects on activated T lymphocytes, including directing the development of Th 2 cells and blocking the development of Th1 cells (438, 439). IL-4 has been used successfully as an anti-

inflammatory agent in Th1-type animal models of autoimmune disease, such as rodent models of crescentic glomerulonephritis (440), anti-glomerular basement membrane antibody-mediated glomerulonephritis (441), experimental autoimmune encephalomyelitis (442), arthritis (443), and diabetes (444). It has been suggested that the anti-inflammatory effect of IL-4 in these models is due to induction of a Th2 phenotype combined with direct inhibition of the Th1 response. IL-4 is known to cause specific phenotypic as well as functional inhibition of CD8+ Th1 cells. CD8+ cells activated in the presence of IL-4 stop expressing interferon-γ and perforin (445), and are thus rendered noncytotoxic. IL-4 also inhibits macrophage activation and their production of cytotoxic molecules, including TNF-α, and nitric oxide (203, 446, 447).

In this study, IL-4 was localized to the infiltrating cells in the renal interstitium of ATIN biopsies. Its role in the pathogenesis of ATIN may be attributable to its Th 2 cytokine properties, although this remains unproven (437). In addition, IL-4 may induce eotaxin mRNA expression in resident renal cells as it does in human lung epithelial and dermal fibroblast cell lines (233) and, as discussed next, eotaxin is a chemoattractant for eosinophils. IL-4 also induces endothelial VCAM-1 expression (297), which may help in eosinophil recruitment to the inflammatory site.

To further delineate the role of IL-4 in ATIN would require an animal model that mimics human disease as closely as possible where the function of IL-4 is prevented, e.g. by anti-IL-4 blocking antibodies or by use of IL-4 knockout animals.

5.2.6-Eotaxin:

Eotaxin was originally described as a potent and selective stimulus for eosinophil leukocytes, inducing eosinophil migration in vitro and accumulation in vivo (224, 448). These effects were shown to be mediated via chemokine receptor 3 (CCR3) that is highly

expressed on eosinophils, basophils, and Th 2 lymphocytes (227, 267, 449-451) and, thus, mainly involved in allergic inflammation (452, 453). Eotaxin mRNA and protein is present in both human lung epithelial and dermal fibroblast cell lines (233), and in normal endometrium and in endometriosis biopsies (454).

In this study, eotaxin was localized in the infiltrating cells in the interstitium of ATIN biopsies, which seems to be the first time that eotaxin expression has been demonstrated using renal tissue. The role of eotaxin in ATIN pathogenesis may therefore relate to the recruitment of eosinophils to the interstitium and a Th 2 immune response. Eotaxin may also promote the release of preformed IL-4 from eosinophil granules (455).

5.2.7-Chemokine receptor 3 (CCR3):

A primary role of chemokine receptors is to mediate activation and migration of leukocytes in the periphery. CCR3 is expressed on human eosinophils, basophils, T helper cells, and dendritic cells (227, 451, 456, 457). In one study, there was a significant correlation between CCR3 mRNA and protein expression and eosinophils in the airway mucosa in asthmatic patients (236). Balding et al, has described CCR3 expression by infiltrating cells in nasal and renal tissues of patients with Wegener's granulomatosis vasculitis (458). No CCR3-positive cells were detectable in the normal kidney in this study, which is in agreement with other studies (459).

The present study localizes the expression of CCR3 to the interstitial infiltrating cells in ATIN, which is in agreement with other studies (458)

5.2.8-Chemokine receptor 5 (CCR5):

The chemokine receptor CCR5 is one member of a family of structurally and functionally related seven-transmembrane-spanning, G-protein-coupled receptors.

Irrespective of the type of kidney disease (membranous glomerulonephritis, IgA nephropathy, lupus nephritis, membranoproliferative glomerulonephritis, ATIN, chronic tubulointeratitial nephritis, and acute and chronic transplant rejection), CCR5 expression is only found on infiltrating mononuclear cells. No expression of CCR5 could be detected on intrinsic cells of the glomerulus, tubuli, or vasculature (460)

The present.study demonstrates the presence of CCR5-positive cells in the interstitial infiltrates and no CCR5-positive cells in normal kidney, which is in agreement with previous studies (277, 460).

5.2.9-VCAM-1:

VCAM-1 expression in the normal kidney was restricted to Bowman's capsule epithelium, some proximal tubular epithelial cells and interstitial vascular endothelium (461). Expression of VCAM-1 was not seen in glomerular endocapillary cells of the normal biopsy specimens studied by Pall et al (462), and this in agreement with the observation in this study.

In vasculitis, the expression of VCAM-1 in glomerular endocapillary cells was associated with a more severe renal lesion, as assessed by the proportion of glomeruli affected by a segmental necrotising glomeulonephritis (462). In addition to transplant rejection, enhanced tubular expression of VCAM-1 has been described in human drug-induced ATIN, in experimental models of lupus nephritis, and in cultured cells (303, 463, 464)

In this study, VCAM-1 expression was seen in tubular epithelium and in renal vascular endothelium, and this in agreement with other studies (465). In addition to its role in the recruitment of leukocytes to the sites of inflammation, VCAM-1 can produce co-stimulatory signals that are essential for the activation of T lymphocytes (466).

If an antigenic challenge is “allergic” in nature and involves IgE antibody, mast cells can release IL-4 (296). Although both TNF-α and IL-4 induce endothelial VCAM-1 expression, IL-4 unlike TNF-α , does not upregulate E-selectin or ICAM-1 (297, 298). This would remove adhesion molecule support for most neutrophil and some monocyte extravasation, and result in a predominantly eosinophilic infiltration. While such a purist approach does not apply to the infiltrates observed in ATIN in this study, it is interesting to note that, when neutrophils are present, the prognosis is worse (256), and may indicate that other mechanisms, possibly driven by intercurrent infection, are causing escalation of injury. None the less, VCAM-1 expression by tubular epithelium and renal vascular endothelium may be important in selectively recruiting eosinophils, mononuclear cells and macrophages that express VLA-4 (CD49d/CD29) and it is possible that this adhesion molecule is important in the development of the ATIN.

In this study, the picture that emerges is that the tubulointerstitial lesion in ATIN comprises variable numbers and variable proportions of infiltrating T cells, eosinophils, and macrophages, with detectable expression of eotaxin, VCAM-1 and IL-4, as well as presence of CCR3 and CCR5, particularly on infiltrating cells. The presence of eotaxin, IL-4 and CCR3 suggests that there may be an allergic type of immune reaction in ATIN. However, eotaxin and eosinophils are likely to make a relatively small contribution to tissue injury, given the poor correlation with renal outcome. Overall, macrophages appear to be the major determinant of injury (as discussed above). No particular differences were found between the types of ATIN studied with respect to infiltrating cells and inflammatory mediators, suggesting similar pathogenic pathways are present irrespective of the type of ATIN.

5.3-The relevance of Epstein-Barr virus to acute tubulointerstitial nephritis:

The evidence for direct viral infection of renal tissue comes from studies of chronic tubulointerstitial nephritis. Bao et al. 1996 demonstrated the presence of EBV DNA by PCR in renal biopsy tissue in 8 of 12 patients with idiopathic chronic tubulo-interstitial nephritis, compared to 0 of 10 control patients with minimal change disease (P value < 0.01)(467). Using both ISH and PCR techniques Becker et al detected EBV DNA in 9 of 17 cases of chronic tubulo-interstitial nephritis and in 0 of 11 various pathologic control cases, 5 were due to ATIN (2 were drug-induced, 2 idiopathic ATIN and one was infection related) (468). Moreover, detection of EBV in the kidney is not necessarily disease-specific; in addition to being implicated in tubulo-interstitial nephritis (399), the EBV genome has been found in biopsy specimens from patients with IgA nephropathy, membranous nephropathy, and focal and/or segmental lesions (469).

The above findings encouraged us to seek a relationship between ATIN, especially idiopathic ATIN and EBV, using immunohistochemical analysis of LMP-1 in renal tissue biopsies and a sensitive EBER-ISH method (386) in 80 and 34 specimens respectively. This constitutes a substantial number of cases but, despite this, no relationship was found between primary ATIN due to any cause and the EBV. This would suggest that EBV as a cause of ATIN is extremely rare. It is possible that when a patient is acutely infected with the EBV, ATIN (subclinical) may develop (398).

5.4-Conclusions:

This study, the largest single centre study to date, confirms that ATIN remains an important cause of acute renal failure and that it is mainly drug-induced, with non-steroidal drugs being particularly implicated. The clinical spectrum has changed since the time when

methicillin was the commonest culprit with less overt hypersensitivity responses occurring. Renal biopsy remains an essential tool in diagnosis. Continuing vigilance is required to identify the emergence of new toxic compounds that cause ATIN. Renal outcome will usually be good but in a significant minority the outcome may be poor. There is suggestive evidence that steroids lead to a more rapid and more complete recovery of renal function, and may reduce the likelihood of development of chronic inflammation with associated interstitial fibrosis but this evidence is from retrospective uncontrolled studies. Analysis of the index of chronic damage showed that this correlated with reversibility of renal function at 3 months and one year, suggesting that it is a useful clinical tool.

A better understanding of the mechanisms responsible for the interstitial infiltration by inflammatory cells, and for the increased production of extracellular matrix within the interstitium, should help define new therapeutic agents. This study did not define a pathologic mechanism that was unique to ATIN or that was unique to individual subtypes of ATIN, as defined within this study. Cellular and inflammatory markers suggested there was a trend towards development of Th 2 responses, at least during the early phase of disease. However, there was a strong correlation between macrophage infiltration and serum creatinine at presentation, suggesting that macrophage-dependent immunological damage occurring during ATIN parallels other forms of renal disease that target both tubules and interstitium (415, 428). This is important to define since new therapeutic agents may be applicable across a wide spectrum of renal diseases.

Further work:

Work presented in this thesis could logically be extended by using the immunohistochemical technique of dual staining in order to determine which cells are

contributing to the cytokine and chemokine receptor profiles in renal sites. By using ISH, one could assess the presence of IL-4 in ATIN renal biopsy material and analyse its potential ability to stimulate renal proximal tubular epithelial cell production of eotaxin and other chemokine targets for CCR3. Further functional studies could be performed using cultured renal cell lines (e.g. proximal tubular cells) in transwell migration assays, along with specific blocking peptides for CCR3, or other chemokine receptors to analyse a role for eotaxin or other chemokines in chemoattracting particular leukocyte subsets. Also by using neutralizing mAb against IL-4 we can examine the effect of this cytokine on the development of ATIN and on the production of Th1 and Th 2 cytokines.

This study shows that a high number of CD68+ cells were infiltrating in the interstitium during ATIN and there was a significant correlation with renal function at presentation. So, further studies could be performed to analyse a role for macrophages in causing ATIN, as well as defining processes that are common to other renal diseases. In addition to prospective correlative studies using human renal biopsies, this question could be addressed using murine models of interstitial nephritis, such as the spontaneous model of interstitial nephritis in kdkd mice (470).

Clinically, this retrospective study explores the ATIN features and their relationship to the renal outcome, and these findings could be applied in a prospective study to predict the renal functional survival and the benefits of steroid treatment compared to other treatments. Multivariate analysis could be carried out to determine how different factors (creatinine at presentation, macrophage infiltration, index of chronic damage, and use of steroids) collectively impact on outcome.

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Chapter 6:

APPENDIX

APPENDIX A

-The preparation of Tris Buffer Saline (TBS) PH 7.6

- Fill a plastic jar with 10 litres of distilled water.

- Add the correct amount of the following components to the distilled water

Sodium chloride	31.88 grams.
Hydrochloric acid (HCl) concentrated	32.63 ml.
TRIS-TRIZMA BASE	60.50 grams.

- Mix well the solution.

- If necessary, adjust final PH to 7.6 with either 1 M HCl or 0.2 M TRIS solution.

N.B:

1 M HCl= 8.4 ml/100 ml distilled water.

0.2 M TRIS = 2.4 g TRIS in 100 ml distilled water.

APPENDIX B

The preparation of TRIS/EDTA solution PH 8.0:

- Fill the flask with 500 ml of distilled water.

- Add the correct amounts of the following components to the distilled water

TRIS	12 grams.
EDTA	1 gram (Sigma- UK)
1 M HCl	10 ml.

- Mix well the solution.

- Dilute 1/10 for use.

APPENDIX C

The preparation of Phosphate Buffer Saline (PBS) PH 7.4

- Add 0.74 gram Sodium di-hydrogen phosphate ($Na\ H_2\ PO_4$) and 2.88 gram Di-sodium hydrogen phosphate (Na_2HPO_4) to 500 ml of distilled water and mix well.

APPENDIX D

- The preparation of DAB (concentrated frozen aliquots)

- Dissolve 500 mg DAB- 3,3'-diaminobenzidine (Sigma-UK) in 20 ml of the phosphate buffer. As soon as DAB has dissolved, prepare 1 ml aliquots in plastic vials.

N.B- Weigh with care and with magnetic stirrer in weighing bottle with a stopper. Freeze and store plastic aliquots at -20°c.

1 ml aliquot (25 mg) diluted to 10 ml phosphate buffer= 2.5 mg/ml.

For use:

Take one aliquot from freezer, allow to thaw and dilute with 9 ml of the phosphate buffer. Immediately before use, add 100 µl Hydrogen peroxide. Mix and use.

APPENDIX E

The preparation of DEPC water (Diethyl Pyrocarbonate)

Add 500 µl DEPC (Diethyl Pyrocarbonate) to 500 ml distilled water and autoclave.

APPENDIX_ F

The preparation of Standard Saline Citrate buffer (SSC)

-Standard saline citrate buffer stored as 20x concentrate.

- Make up 2x SSC by diluting 20 ml stock with 180 ml DEPC water.

- Make up 0.1x SSC by diluting 10 ml 2x SSC with 190 ml DEPC water.

-Put some of both SSC dilutions into sterile squeezy bottles for use.