Immunopathology of Omeprazole-Induced Acute Interstitial Nephritis

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Abstract

Aim

The aim of this study was to define the nature of the inflammatory infiltrate that occurs with omeprazole-induced acute interstitial nephritis (AIN) and further to compare and contrast the histology of omeprazole-induced AIN with the interstitial renal lesions seen in PR3 and MPO positive small vessel vasculitis.

Background

Omeprazole is now the most common cause of drug-induced acute interstitial nephritis. However, how omeprazole-induced acute interstitial nephritis is mediated is unknown. We observed in all patients the histological pattern is unlike the classical picture of an acute allergic drug-induced interstitial nephritis as seen with methicillin, but rather it is akin to that seen with PR3/MPO small vessel vasculitis or acute cellular rejection in renal transplant recipients. The nature of the cellular infiltrate in omeprazole-induced acute interstitial nephritis has not been characterized. Given the similarity in appearance to the renal involvement in small vessel vascultits, we postulated that the inflammatory process is predominantly a cell-mediated process involving TH1/TH17 cells.

Methods

Data from 25 biopsy-proven cases of omeprazole-induced acute interstitial nephritis (AIN) and 27 patients with renal involvement with PR3/MPO ANCA positive small vessel vasculitis, who have presented to the renal service (Dunedin Hospital, NZ) over the last 10 years, were reviewed. The lesions were graded according to the updated Banff classification of 1997 to compare the severity of the inflammatory infiltrate. The underlying immune reaction was characterized using immunohistochemical and fluoroscopic chromogenic in situ hybridization (IHC and F-CISH) to detect T-cells (and particular markers associated with Th1, Th2, Th17 and Treg effector T cells) and B-cells. The following antibodies were used CD20 for B-cells, CD4, CD8, IL17A, IL17F and Foxp3 for T-cells.

Results

All patients presented with evidence of acute kidney injury (AKI). Urinalysis demonstrated a sterile pyuria with varying amounts of proteinuria. There were no urinary eosinophils. There were no systemic symptoms to suggest a vasculitis and ANCA (PR3 & MPO titres) were negative in all cases. Histologically, all cases had

evidence of inflammatory cells crossing the tubular basement membrane and showing an active tubulitis.

The occurrence of any eosinophils in our patients was 52%. However, in contrast to previous studies only one patient has an eosinophilic infiltrate, the other cases showed only small numbers (3 – 7 eosinophils per high powerfield in some but not all views) of scattered eosinophils in the infiltrate. Similar findings were evident in the vasculitis group. The lack of a substantial eosinophilic infiltrate argues against a pre-dominant Th-2 mediated mechanism for omeprazole-induced AIN.

Our cases showed numerous CD4 positive cells, and fewer numbers of CD20 and CD8 positive cells. CD4 positive cells were present in clusters in 77% of the PPI-IN patients and 67% of the vasculitis patients. CD20 positive cells were present in clusters in 58% of all omeprazole-induced AIN patients and 37% of vasculitis patients. However CD8 positive cells were present in clusters only in 17% of omeprazole-induced AIN patients and 13% of vasculitis patients. Comparing the IL17A and CD4 double staining with the IL17F and CD4 double staining for the omeprazole-induced AIN group the grading of the staining looks very similar (for IL17A and CD4 50% of patients showing clusters, 4% intermediate, 32% of scattered and 14 % of patients without any IL17A and CD4 cells versus the staining for the IL17F and CD4 staining with 48% in clusters, 17% intermediate, 31% scattered and 4% with no staining for IL17F and CD4). Foxp3 regulatory cells were found within the interstitium and tubular epithelium of both vasculitis and omeprazole-induced AIN cases.

Conclusion

This is the largest reported biopsy series of omeprazole-induced AIN. In this series of 25 renal biopsies with omeprazole-induced acute interstitial nephritis, the predominant inflammatory cells were mainly of a Th1-Th17 lineage, suggesting this is the major type of cell-mediated inflammatory process rather than a Th2-mediated response, which has been reported with the classic allergic acute drug-induced interstitial nephritis. With the increasing prevalence of omeprazole-induced AIN, it is now crucial to identify how omeprazole might induce these presumed Th1-Th17 mediated inflammatory pathways of injury within the kidney. Once better defined, more appropriate therapy can be introduced.

Acknowlegdements

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List of Abbreviations

ANCA	Anti- neutrophil cytosplastmaic antibody
AIN	Acute interstitial nephritis
BSA	Bovine serum albumin
CD	Cluster of differentiation
CRP	C-reactive protein
DAB	3,3'-Diaminobenzidine
ESR	Erythrocyte sedimation rate
FFPE	Formalin-Fixed Paraffin Embedded
IF	Immunofluorescence
IHC	Immunohistochemistry
OIAIN	Omeprazole-induced acute intersitial nephritis
PPI	Proton Pump Inhibitor
T cell	T lymphocyte
Th1	T helper cells 1
Th2	T helper cells 2
UTI	Urinary tract infection

CHAPTER ONE LITERATURE REVIEW

1.1 Introduction

Since the introduction of omeprazole in 1989, proton pump inhibitors are amongst the most widely prescribed class of drugs. They are successful in the management of gastro-esophageal reflux, peptic ulcer disease (particularly related to *Helicobacter pylori* infection), acid-related dyspepsia and gastro-duodenal lesions associated with non-steroidal anti-inflammatory drugs.

In general they are very safe. The most commonly reported side effects (headache, dizziness and diarrhea) occur in less than 5% of patients (Geevasinga et al., 2006). However, increasing numbers of acute interstitial nephritis (AIN) secondary to omeprazole have been reported since the first case report in 1992 (Ruffenach et al., 1992); the majority of which have been from New Zealand and Australia (Simpson et al., 2006, Delve et al., 2003, Geevasinga et al., 2006). Many of these AIN patients have no specific symptoms, and do not present until they have developed advanced kidney failure (Torpey et al., 2004). The diagnosis can be confirmed by renal biopsy, which shows an active tubulitis– that is, an intense interstitial and tubular intraepithelial infiltrate in the absence of any other aetiological factor. However, over the last ten years we have observed a different histological pattern in the renal biopsies of all patients who have been admitted to Dunedin Public Hospital with omeprazole-induced AIN. The histological pattern is unlike the classical picture of an acute allergic drug-induced interstitial nephritis as observed with methicillin but rather it is similar to that seen with PR3/MPO small vessel vasculitis or acute cellular rejection in renal transplant recipients. The nature of the cellular infiltrate in omeprazole induced acute interstitial nephritis has not been previously characterized.

The aim of this study was to identify the nature of the cellular infiltrate and key inflammatory pathways mediating omeprazole-induced acute interstitial nephritis and to correlate the H&E histological features with immunohistological staining to assess the potential effector pathways. We aimed to determine which CD4+ T-cells were present, in particular, if Th1, Th2, Th17 and regulatory T cells are detectable in

the kidneys of AIN secondary to omeprazole. We also wished to analyse for the presence of B cell markers. Given the histological similarity to small vessel vasculitis, comparisons were made with biopsies from patients with both PR3 and MPO positive small vessel vasculitis where the lesions are predominantly T-cell mediated (Savage, 2011a, Wilde et al., 2011, Summers et al., 2009). We postulate a cell-mediated process involving TH1/TH17 cells mediates the inflammatory processes detected in omeprazole-induced AIN.

1.2 Drug-Induced Nephropathies

The kidneys are particularly vulnerable to drug-related toxicity. A contributing factor to this is that the kidney is the principal organ of excretion for many drugs or their water-soluble metabolites. Additionally high renal blood flow (25% of cardiac output) results in a high rate of drug delivery to the kidneys. Some drugs are present in the tubular fluid at very high concentration (1000 times the plasma concentration).

Drug-induced nephropathies can manifest as 'false' renal failure (due to competitive inhibition of creatinine handling as seen with trimethoprim), nephrotic syndrome, tubulopathies, urinary tract syndromes, renal tract tumors, chronic kidney disease, acute kidney injury and acute interstitial nephritis. Given the variety of potential drug-related renal syndromes, clinicians should always conduct a comprehensive inquiry into all drugs taken by the patient, including used or misused, prescribed or obtained over-the-counter, regular or alternative agents (Nortier et al., 2009).

In general drugs are responsible for up to 40-60% of all patients presenting with an AIN (Toto, 1990, Cameron, 1988). There are more than 100 drugs that cause this side effect including antibiotics, non-steroidals, diuretics, antiepileptics and more recently also proton pump inhibitors (PPIs). With the increasing use of PPIs they have now emerged as the single most common cause of drug-induced AIN (Simpson et al., 2006) (Torpey et al., 2004) (Geevasinga et al., 2006), Center of Adverse Reactions Monitoring (CARM) in New Zealand (as of 30 June 2011).

Acute Interstitial Nephritis (AIN)

The causes of AIN can be classified into five general categories: drug hypersensitivity reactions, infections, immune-mediated diseases, glomerular

disease and idiopathic. Historically, AIN was predominantly associated with infections but in the antibiotic era, drug hypersensitivity reactions became the most common cause of AIN (Michel and Kelly, 1998).

Clinically acute interstitial nephritis is characterized by a progressive decline in renal impairment in the absence of any specific clinical symptoms, but with mild proteinuria and sterile pyuria. Between 6 to 8% of patients undergoing renal biopsy for unexplained acute renal failure (ARF) will prove to have an acute interstitial nephritis (AIN) (Ruffenach et al., 1992) (Michel and Kelly, 1998). In the setting of inactive urine sediment, i.e. lack of heavy proteinuria, significant hematuria or presence of dysmorphic red blood cells, AIN has been reported to occur in as many as 25% of patients with ARF.

Classical Methicillin Induced Interstitial Nephritis

Methicillin (now removed from the market) was the first drug associated with AIN. With over 100 reported cases of methicillin-induced AIN, a typical clinical spectrum was observed with a triad of fever, skin rash and eosinophilia (Toto, 1990). Renal failure occured in approximately 50% of adults and 15% of children (Rossert, 2001), (Rossert and Fischer, 2007) with methicillin-induced AIN (see Figure 1). As the classical type 1 hypersensitivity drug-induced AIN is now a rare presentation, the diagnosis of drug-induced interstitial nephritis may be challenging since many patients will have non-specific symptoms and do not present until they have developed advanced renal failure, unless they happen to have a urinalysis as part of their initial review and sterile pyuria is noted and then further investigated.

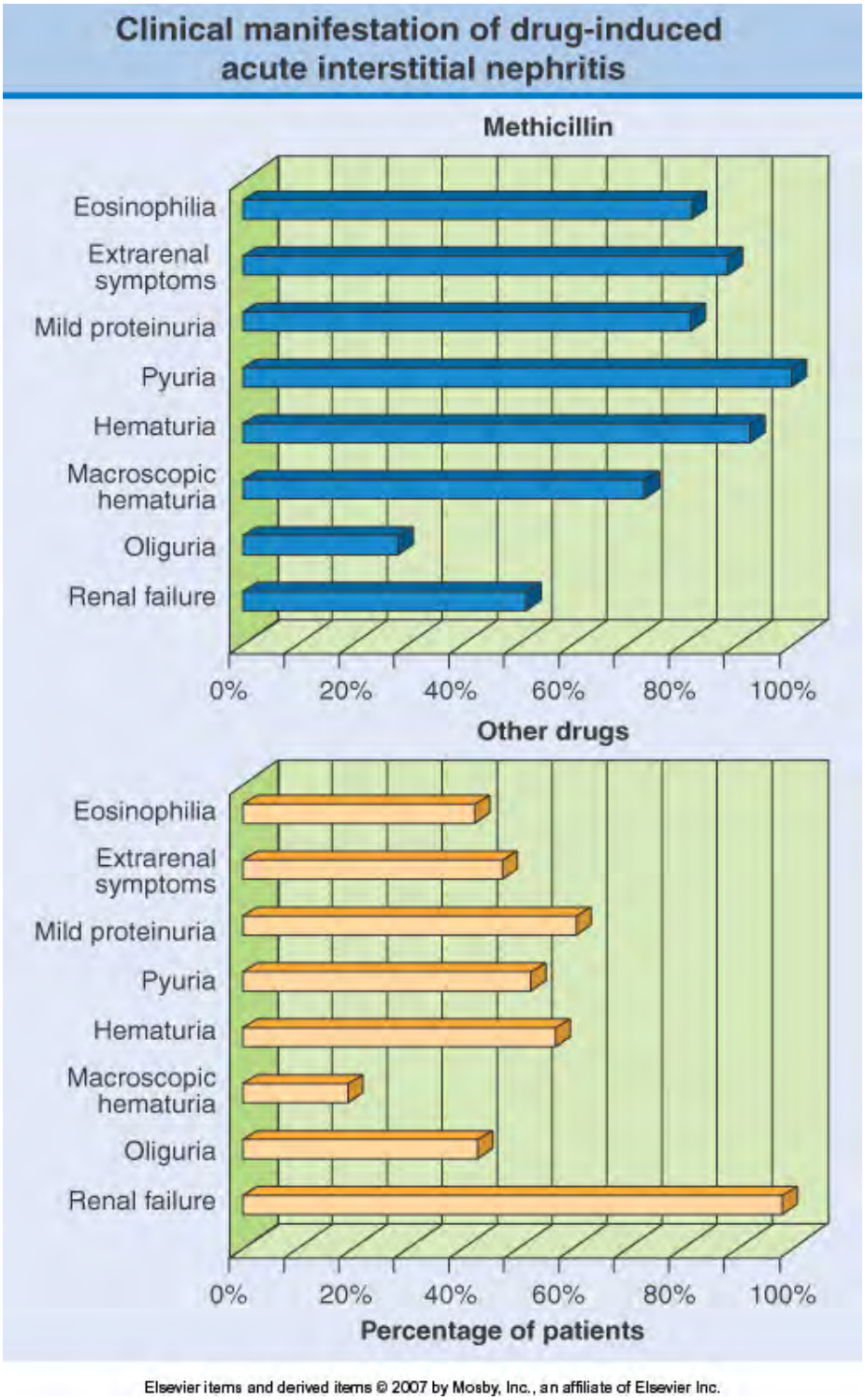

Figure 1: Clinical manifestation of drug-induced AIN (Grunfeld and Kleinknecht, 1993), permission for reprint in thesis given by the Elsevier Limited publishing group.

Proton Pump Inhibitor (PPI) induced AIN

Proton pump inhibitors are the mainstay in the treatment of acid-peptic disease especially since they are superior to H2 blockers (Shi and Klotz, 2008, Ruffenach et al., 1992, Maton, 1991). Omeprazole was the first PPI to be introduced in 1989 but five classes are now available in the US (Perazella and Markowitz, 2010). All five classes (omeprazole, pantoprazole, esomeprazole, rabeprazole and lansoprazole have been implicated in causing AIN (Geevasinga et al., 2006, Brewster and Perazella, 2007b).

1.3 Omeprazole-Induced Acute Interstitial Nephritis

In a single UK renal unit study over 4.5 years an association between tubulointerstitial nephritis (TIN) and the proton pump inhibitors (PPIs) omeprazole and lansoprozole was found in one third (8/23) of all biopsy- proven cases and PPIs featured as the most common cause of drug-induced AIN (Torpey et al., 2004).

Ruffenach et al. published the first case of omeprazole induced interstitial nephritis in a 74-year old lady with AKI in 1992 (Ruffenach et al., 1992). Since then there have been increasing numbers of mostly single case reports (Wall et al., 2000, Torpey et al., 2004, Delve et al., 2003, Geetha, 1999, Shuster, 2000, Post et al., 2000, Christensen et al., 1993). In 2004 lansoprazole and pantoprazole were also implicated with AIN (Torpey et al., 2004, Ra and Tobe, 2004), and more recently rabeprazole (Geevasinga et al., 2005a) and esomeprazole (Geevasinga et al., 2005b).

Geevasinga et al. (Geevasinga et al., 2006) reported almost two thirds (64%) of all biopsy-proven AIN to be attributed to PPI use (see Figure 2). A recent literature review (Brewster and Perazella, 2007b, Brewster and Perazella, 2007a) identified 88 cases of PPI-induced AIN, 76 of which were biopsy-proven. In 2009 a review of the English literature identified a total of 114 cases of PPI-induced AIN (with 94 biopsy-proven cases) (Chan et al., 2009).

Table 4. Patient Characteristics From the Literature Review

Authors	Age (*y*)/ sex	Prior exposure duration (*wk*)	PPI	Symptoms/signs	Serum creatinine mg/dL (*μmol/L*)				Biopsy-proven
					Baseline	Presentation	Peak	Resolution[a]	
Ruffenach et al,[3] 1992	74/F	36	Omeprazole	Anorexia, fatigue, malaise	1.02 (90)	6.11 (540)		1.28 (113)	No
Kuiper,[5] 1993	58/F	10	Omeprazole	Pruritic rash	1.50 (133)	20 (168)	3.20 (283)	1.50 (133)	No
Christensen et al,[6] 1993	86/F	8	Omeprazole	Fever/rash		9.71 (858)			Yes
Assouad et al,[7] 1994	75/M	3	Omeprazole	Asymptomatic	1.20 (106)	3.0 (265)	4.8 (424)	1.9 (168)	Yes
Gronich et al,[8] 1994	65/F	NA	Omeprazole	Fever					
Jones et al,[9] 1994	76/F	12	Omeprazole	Anorexia, lethargy	1.81 (160)	4.98 (440)		1.92 (170)	Yes
Lewis et al,[10] 1994	77/F	42	Omeprazole	Nausea, malaise/ weight loss, fever	0.94 (83)	5.18 (458)		1.36 (120)	Yes
Singer et al,[11] 1994	65/M	16	Omeprazole	Anorexia/weight loss, fever		3.40 (301)	4.84 (428)	1.63 (144)	Yes
Aust Adverse Drug React Bull,[12] 1995	51/M	12	Omeprazole	Fatigue, rigors		2.15 (190)			Yes
Fleury et al,[13] 1995	69/M	8	Omeprazole	Weight loss/oliguria					
O'Donnell,[14] 1996	69/M	2	Omeprazole	Headache, lethargy, nocturia		2.43 (215)		1.24 (110)	No
Badev et al,[15] 1997	70/F	2	Omeprazole	Anorexia, nausea, vomiting		9.73 (860)		1.92 (170)	Yes
	71/M	16	Omeprazole	Fever, night sweats		8.71 (770)		1.92 (170)	Yes
d' Amamo et al,[16] 1997	72/F	1	Omeprazole	Anorexia, fatigue/ flank tenderness	1.41 (125)	2.60 (230)	4.97 (439)	1.27 (112)	No
Yip et al,[17] 1997	50/F	8	Omeprazole	Lethargy, nausea, vomiting	1.05 (93)		4.98 (440)	1.05 (93)	Yes
Landray et al,[18] 1998	77/F	8	Omeprazole	malaise, nausea, weight loss/fever	1.39 (123)	2.29 (202)	7.74 (684)	1.6 (141)	Yes
Montseny et al,[19] 1998	69/M	12	Omeprazole	Asymptomatic	0.93 (82)	1.58 (140)	3.51 (310)	1.06 (94)	Yes
Myers et al,[24] 1999	36/F	12	Omeprazole	Nausea, vomiting, weight loss		5.87 (519)	10.54 (932)	1.26 (111)	Yes
Geetha,[20] 1999	78/F	2	Omeprazole	Fever	1.13 (100)	4.41 (390)	6.22 (550)	2.04 (180)	No
Shuster,[21] 2000	70/F	3	Omeprazole	Fatigue/fever		4.41 (390)	5.88 (520)		No
Post et al,[22] 2000	73/M	16	Omeprazole	Dry mouth, malaise/fever	1.1 (97)	5.87 (519)	6.86 (606)	1.19 (105)	Yes
Wall et al,[23] 2000	31/F	13	Omeprazole	Asymptomatic	1.58 (140)	5.44 (481)		1.6 (141)	Yes
Delve et al,[25] 2003	66/F	12	Omeprazole	Anorexia, fatigue, night sweat, weight loss/fever	1.24 (110)	3.29 (291)		1.36 (120)	Yes
	73/F	8	Omeprazole	Dehydration, nausea, lethargy, vomiting, weight loss	1.02 (90)	8.6 (760)		1.92 (170)	Yes
Ra et al,[26] 2004	77/F	8	Pantoprazole	Fatigue/fever	1.31 (100)	3.39 (300)	7.24 (640)	2.04 (180)	Yes
Moore et al,[27] 2004	23/M	6	Pantoprazole	Nausea, vomiting		17.85 (1578)		1.6 (141)	Yes

NA, not available.
[a]Follow-up varied in reported cases (range, 1 wk–6 mo).

Figure 2: Patient characteristics of 26 case reports of PPI-induced AIN (Geevasinga et al., 2006)**.** Figure from Clinical Gastroenterology and Hepatology, permission is given by the Elsevier Limited publishing group. Licence number 2885500563751.

Omeprazole-Induced Acute Interstitial Nephritis in Australasia

To date in the literature there is an increased reported incidence of proton pump inhibitor-induced AIN in Australia and New Zealand compared to world wide

reporting which probably reflects a better organised adverse drug monitoiring programme in these two countries compared to the rest of the world. Simpson and colleagues reported and analysed 15 cases in 2006 in the greater Auckland area with AIN and ARF from PPIs (Simpson et al., 2006), with biopsy confirmation in 12 of the 15 cases. Myers et al. reviewed 18 cases (Myers et al., 2001), Geevasigna et al (Geevasinga et al., 2006) reviewed records of two teaching hospitals in Australia over a 10-year period (1993 to 2003) where in 18 cases of biopsy proven AIN associated with PPI use were identified.

Adverse drug event databases in Australia and New Zealand have recorded a considerable number of PPI associated acute renal reactions. Geevasinga et al analyzed the Therapeutic Goods Administration (TGA) database in Australia over a 14-year period between 1991 and 2004 and found 34 cases of biopsy-proven AIN associated with PPI use. Of these, 28 were associated with omeprazole, 3 cases with rabeprazole, 2 cases with esomeprazole and 2 cases with pantoprazole. In New Zealand there have been 65 cases of interstitial nephritis associated with omeprazole (62) and pantoprazole (3) reported to the Centre for Adverse Reactions Monitoring (CARM) since the first reported case in September 1993 (as of 30 June 2011). The limited use of lansoprazole and esomeprazole in New Zealand may explain the lack of reports for these PPIs (Savage, 2011b).

When first introduced omeprazole was monitored as part of the Intensive Medicines Monitoring Program (IMMP). There were 2 reports of interstitial nephritis from a total cohort of 22'050 patients (Savage, 2006), indicating an incidence of one in 12'500 patient years (<0.001%). Data from the larger cohort published by Simpson et al. (Simpson et al., 2006) supported a similar incidence.

Clinical Presentation of Omeprazole-Induced AIN

In contrast to the classical acute allergic (methicillin) induced AIN, Omeprazole-Induced AIN does not manifest as a systemic "hypersensitivity type allergic reaction". The triad of fever, rash and eosinophilia associated with classical methicillin-induced AIN is absent in omeprazole-induced AIN. In the published date virtually no cases had the classic triad of hypersensitivity, less then half had a fever, fewer then 10% had a rash and approximately 33% had eosinophilia (Brewster and Perazella, 2007b). The most common presenting complaints were unspecific including fatigue, malaise, nausea and anorexia. In a review article of 18 patients

the most prominent clinical symptoms were fatigue (44%), fever (39%) and nausea (28%) (Myers et al., 2001). Symptom onset varies among cohort studies ranging from 1 week to 9 months after introduction of the PPI (Chan et al., 2009). The most common urine abnormalities were sterile pyuria (72%), haematuria (61%) and proteinuria (56%). These three in combination were present in 39% of the cases (Figure 3). Median serum creatinine level at presentation in the review of literature was 444 µmol/l (Chan et al., 2009). In the majority of cases kidney function recovers, however some will remain with some form of chronic kidney disease (Figure 4).

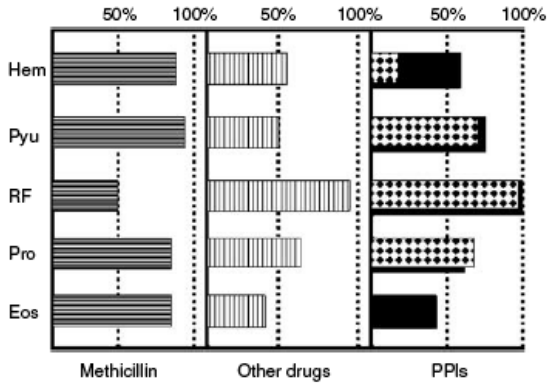

Figure 3 | Bar graphs demonstrating the clinical features of methicillin-induced AIN (longitudinal bars), non-methicillin drug-induced AIN (excluding non-steroidal anti-inflammatory drugs) (horizontal bars), and PPI-induced AIN (Geevasinga data, square pattern bars; other PPI cases combined, solid bars). (Adapted in part from;[16] with permission). Abbreviations: AIN, acute interstitial nephritis; NSAIDs, non-steroidal anti-inflammatory drugs; PPI, proton pump inhibitor, Hem, hematuria; Pro, proteinuria; RF, renal failure; Pyu, pyuria; Eos, eosinophilia.

Figure 3: Clinical features of methicillin-induced AIN, non-methicillin drug-induced AIN and PPI-induced AIN (Brewster and Perazella, 2007a**.** Figure from KI, permission is given by the nature publishing group. Licence number 2883300238370.

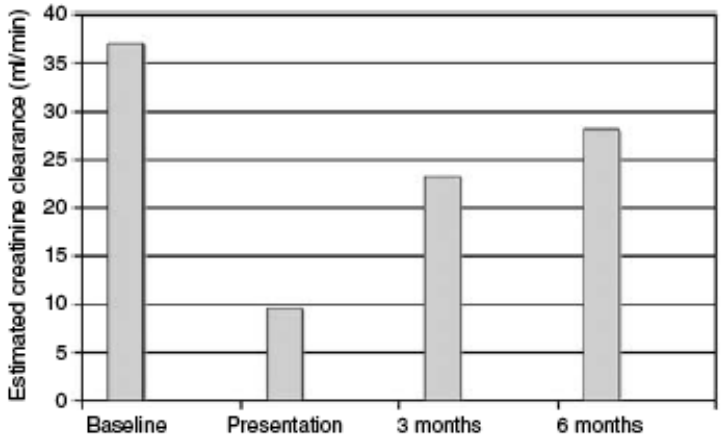

Figure 4 | Calculated creatinine clearance of 18 patients with PPI-induced AIN at various time points before, during and after exposure to PPIs. (Adapted from;[15] with permission). Abbreviations: PPI, proton pump inhibitor.

Figure 4: Calculated creatinine clearance of 18 patients with PPI-induced AIN at various time points before, during and after exposure to PPIs (Brewster and Perazella, 2007a). Figure from KI, permission is given by the Nature Publishing Group, Licence number 2883300238370.

1.4 Treatment

The mainstay of treatment in AIN is supportive therapy in conjunction with discontinuation of the potentially offending drug to avoid chronic damage (Michel and Kelly, 1998). Withdrawal of the drug results in a reversal of the AIN in 74% of cases of drug induced interstitial nephritis, and is thus an effective treatment, if discovered early enough (Bhaumik et al., 1996).

Although to this date there have been no prospective, randomized trials to assess the efficacy of corticosteroids, some reports show that the use of steroids are effective especially in the early phase, if there is no improvement in kidney function or in patients with particularly severe interstitial nephritis on renal biopsy. There has been one report where steroids were particularly beneficial during ongoing drug exposure (Kim et al., 2010).

It is very important if there is a suspected drug hypersensitivity reaction to document this well in the patient's medical record. In New Zealand such drug alerts are also notified to the Centre for Adverse Drug Monitoring (CARM). Pharmacological treatment in terms of corticosteroids is usually considered if there

is no improvement in kidney function or in patients with particularly severe interstitial nephritis on renal biopsy (Michel and Kelly, 1998).

1.5 Vasculitis

As discussed in the introduction, the histological features of omeprazole-induced AIN are very similar to the acute tubulitis seen in small vessel vasculitis. For comparison of potential immunologic pathways our omeprazole AIN cases were compared with a similar number of small vessel vasculitis cases.

Anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) including Wegener's granulomatosis (which has been renamed recently 'granulomatosis with polyangitis') and microscopic polyangitis are life threatening autoimmune diseases involving small- and medium-sized vessels. They often affect the kidneys, and renal involvement is an important factor in respect to patient morbidity and mortality (Berden et al., 2010). These vasculidities are characterized by the presence, in the majorty of cases, of ANCA directed to cell components. c-ANCA (cytoplasmic staining) is associated with proteinase 3 antibodies (PR3) and p-ANCA (perinuclear staining) is associated with myeloperoxide antibodies. PR3 antibodies have a strong association with granulomatosis with polyangiitis whereas MPO antibodies are equally associated with either forms of vasculitis (Gao and Zhao, 2009). In the past, mortality was very high with 1-year mortality being close to 50% with steroid treatment alone. Therapy with cyclophophamide improves the 1-year survival to >80% (Cattran and Hladunewich, 2011). Interstingly the incidence of the disease is dependent on geography with an increase in incidence the further North or South you go from the Equator. Especially with PR3 vasculitis there is evidence for a latitude-dependant incidence gradient for New Zealand (O'Donnell et al., 2007).

Patients with AAV are usually of older age, present with constitutional symptoms for 6-9 months before being diagnosed and may involve ENT disease, pulmonary disease, cutaneous vasculitis or rapidly progressive glomerulonephritis (RPGN). Although seropositivity for ANCA in the combination with RPGN is suggestive of ANCA-associated glomerulonephritis, the renal biopsy is still considered the gold standard for establishing the diagnosis (Berden et al., 2010). Commonly a pauci-immune necrotizing crescentic glomerulonephritis is observed.

With increased awareness of these diseases and therefore possible earlier detection with subsequent earlier renal biopsies, there may well be different patterns of inflammation. In our series the glomerular involvement was quite variable whereas the interstitial infiltrate and active tubulitis was a persistent pattern.

Pathogenesis of Vasculitis

ANCA-associated vasculitis (AAV) is an autoimmune disease where the precise aetiology remains unknown, but contributions of genetic and environmental factors seem certain (Savage, 2011a). Many of the genes described so far encode proteins that are involved in the immune response, such as human leuocyte antigen (HLA) proteins, PTPN22, CTLA4 and others (Savage, 2011a). Environmental factors such as drugs, silica, infections and geographical differences appear to contribute variously. PR3-ANCA vasculitis are more frequent in Caucasians and is far more frequent in northern areas (Europe and United States), whereas MPO-ANCA vasculitis is more frequent in southern areas and in East Asia and Japan(Kallenberg, 2011).

The pathogenetic mechanisms that are then triggered are complex and not yet fully understood. Humoral as well as the cellular immune system are involved (Wilde et al., 2011, Jennette, 2011). There is a substantial body of evidence supporting a pathogenic role for ANCA in AAV (Botermans et al., 2011, Flint et al., 2010).

The pathogenesis of ANCA disease requires at least two hits, one of which is ANCA immunoglobulin G targeting neutrophil proteinase 3 (PR3) and myeloperoxidase (MPO) usually to augment or dysregulate the expression of PR3 or MPO, promoting a proinflammatory state whereby neutrophils have the capability to damage endothelial and other cells expressing PR3 or MPO on their surface (Savage, 2011a) (Free and Falk, 2010). With subsequent cytokine release there is an intense inflammatory response with T cells being the predominant infiltrating cell in the interstitium of the kidney. Naïve T cells, once activated, develop into effector cells including Th1 and Th2 as well as Th17 and Treg cells (Flint et al., 2010, Falk et al., 1990). In a mouse model of experimental anti-myeloperoxidase (anti-MPO) glomerulonephritis Th1 and Th17 cells clearly induce a proliferative glomerulonephritis (Summers et al., 2009, Gan et al., 2010). In addition, B cells are

involved in the pathogenesis as anti-B cell therapies are highly effective, but the maner of their involvement is still unknown. Finally the role of the alternative complement pathway is under current invetigation (Savage, 2011a).

Briefly, the current understanding is, there are two pathways firstly a neutrophil pathway, secondly a T-cell pathway causing inflammation (Figure 5) (Wilde, van Paassen et al. 2011).

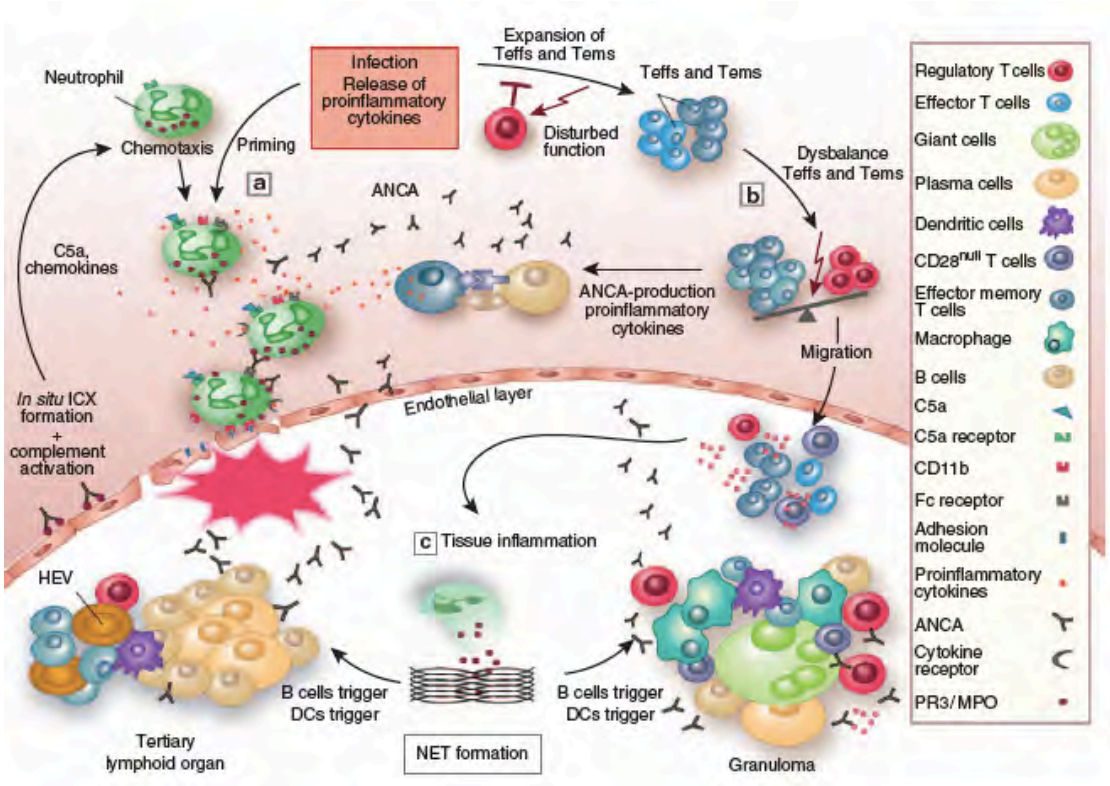

Figure 5: Two pathways contributing to disease meachanism in ANCA-associated vasculitis, a the "classic neutrophil pathway" and b the "T-cell pathway" (Wilde et al., 2011), permission is given by the Nature Publishing Group, Licence number 2886060372150.

Drug- Induced Vasculitis

There have been isolated case reports of drug- induced vasculitis. Best evidence for an association with ANCA is for cases involving propylthiouracil and hydralazine. Most cases report a p-ANCA pattern rather than a c-ANCA pattern (Jacobs-Kosmin et al., 2006). However mostly in drug-induced lesions with pANCA positivity, MPO titres are absent, and therefore the pANCA positivity is a false positive. As such this will not be discussed any further.

1.6 Overview of Immunological Mechanisms

Hypersensitivity reactions

Gell and Coombs classified hypersensitivity reactions into four types.
Type 1 allergy: immediate, associated with atopy, anaphylaxis and asthma and is mediated by IgE triggering mast cells.
Type 2 cytotoxic: antibody-dependent and is mediated by IgM or IgG.
Type 3 serum sickness like reaction, immune complex disease: IgG mediated as seen in Stevens-Johnson reaction.
Type 4 Delayed-type hypersensitivity: cell-mediated immune memory response mediated by T-cells and is antibody-independent,.
Recently there has been an additional type used as a distinction from type 2 (often used in Britain). Type 5 Autoimmune disease, antibody (IgM or IgG) binding to specific receptors, eg Grave's disease or myasthenia gravis.

Immunopathology (Idiosyncratic immune mediated reaction)

The immune system is able to combat nearly any pathogen with specialized cells and molecules. Innate immunity provides a first line of defense, whereas adaptive immunity, collectively T and B lymphocytes, provides antigen-specific second line of defense to try and keep the body pathogen-free (Free and Falk, 2010).

B cells (B lymphocytes)

B cells are involved in humoral immune response (in contrast to the cell-mediated immune response, which is governed by T cells). The principal functions of B cells are to make antibodies against antigens, perform the role of antigen-presenting cells (APCs) and eventually develop into memory B cells or plasma cells after activation by antigen interaction. Plasma cells produce large volumes of antibodies. B cells are an essential component of the adaptive immune system. The Pan-B cell marker is CD20.

T cells (T lymphocytes)

T cells play a crucial role in adaptive immunity and immunopathology in humans. Much has been learned about T cell recognition of peptide antigens and subsequent co-stimulatory pathways that instruct T cells to mature and differentiate into a number of subsets. T cells are classically divided into two subtypes the T helper cells (CD4 positive) and T cytotoxic cells (CD8 positive). Antigen-activated naive Th cells differentiate into functionally distinct subsets depending on the effector cytokines synthesized (Gan et al., 2010).

T- helper cells and autoimmune disease

Immune and autoimmune responses are regulated by a fine balance between effector and regulatory T cells. For over 35 years, immunologists have divided T-helper (Th) cells into functional subsets. Type 1 CD4 T-helper cells (Th1) drive cell-mediated immune response leading to tissue damage combating especially intracellular pathogens and they also drive antibody mediated responses in certain IgG subclasses. Type 2 CD4 T-helper cells (Th2) drive certain antibody-mediated responses, specifically those involved in allergy dominated by IgE isotype. Recently there has been a shift in the Th1/Th2 hypothesis to a newer theory, termed the Th17 hypothesis, to explain cell-mediated tissue damage in both autoimmunity and immunity triggered by microbial infection (Steinman, 2007). T-helper type 1 (Th1) cells might be involved in the initiation of tissue damage, but they do not sustain a decisive role in many commonly studied models of autoimmunity, allergy and microbial immunity. With the emergence of the cytokine interleukin 17 (IL17), which is thought to play a major role in various models of immune-mediated tissue injury, there has been an enhancement of our understanding of immune mediated tissue damage. In addition, there are regulatory T cells whose role is regulating and suppressing proliferation of effector cells and their cytokine production.

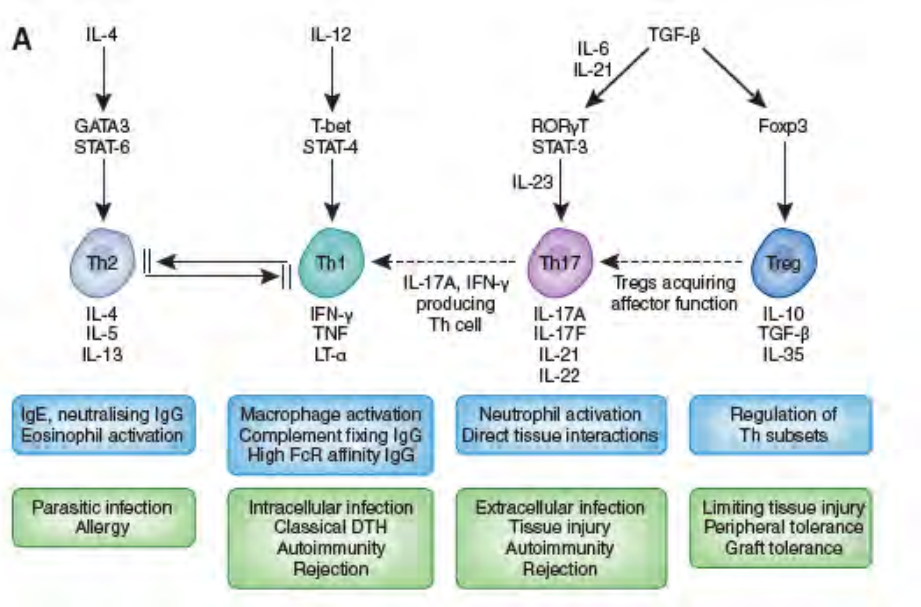

Figure 6: Simplified diagram of the Th1, Th2, Th17, and Treg subsets, showing key cytokines produced by these cells, and the cytokines and transcription factors critical to the development and expansion of these subsets (Kitching and Holdsworth, 2011). Permission 62391602 given.

Th17 cells

The new Th17 subset, first characterized in 2005 (Park 2005) correlate with autoimmune disease and are characterized by their production of IL17A. Additionally Th17 cells produce a number of other cytokines including IL-17F, IL21, IL-22 and IL-6 (Odobasic et al., 2011).

IL-17-producing Th17 effector cells are directly involved as effectors of renal injury by activating neutrophils or by participating in macrophage-mediated tissue injury (Kitching and Holdsworth, 2011).

Treg cells

Regulatory T cells (CD25+CD4+) engage in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes. Foxp3 is a key regulatory gene for the development of regulatory T cells.

Macrophages

Macrophages contribute to fibrosis and chronic kidney disease.

Monocyte-derived macrophages have a central role to repair organs after injury. Macrophages as part of the innate immune system are particularly adept at clearing debris, extracellular matrix, immune complexes and dead cell products of tissue injury. Furthermore they seem to be able to release any cytokine and growth factor described in the literature (Anders, 2011). In chronic kidney disease macrophages seem to contribute to tissue injury and fibrosis (Duffield, 2011). Macrophages, however, exhibit considerable plasticity in their phenotype and can develop into different functional states. These functional states are known as M1 for pro-injurious function or proinflammatory status and M2 for woundhealing function or anti-inflammatory status. As recently discovered various distinct phenotypes for B-cells and T-cells (Th1, Th2, Th17 and regulatory T-cells) macrophages contribute to renal pathology through functionally distinct phenotypes as well. This has been based on a working model of wound healing, where there might be at least four different types of macrophages: proinflammatory macrophages (M1-like), anti-inflammatory macrophages (M2c-like), profibrotic macrophages (M2a-like) and fibrolytic macrophages. However, surface markers have not yet been defined as clearly as with T-cells. An additional difficulty is that macrophages show a very high plasticity and also that renal biopsies display a snapshot of a dynamic process, which does not always provide an obvious clue to the underlying pathomechanism.

T helper cells and kidney disease

In immune-mediated renal disease CD4 T helper cells play a central role causing renal inflammation. T cells seem to play a major role in the pathogenesis because they are the predominant cell type in the interstitial infiltrate (Spanou et al., 2006).

Until recently, only Th1 (expressing IFN) and Th2 (expressing IL 4) T cell subsets were recognized. Most recently two further effector subsets have been discovered. Il-17-producing Th17 effector cells and T regulatory (Tregs) (Kitching and Holdsworth, 2011) (Figure 5).

In proliferative forms of GN, T cells direct adaptive immune responses that drive glomerular disease. Th1 cells predominantly produce IFN-y and activate macrophages, are important in some forms of experimental GN. Th2 cells produce IL-4 and IL5, promote humoral immunity and are important in several forms of GN, but there is little evidence that Th2 cells play a primary role in rapidly progressive GN (Summers et al., 2009). Several recent studies have shown that in most rapidly

progressive types of GN, associated with cell mediated injury, Th1 and Th17 effector cells can induce glomerular injury. Understanding how these two subsets mediate the disease may lead to targeted therapies (Summers et al., 2009). In proliferative forms of GN, T cells direct adaptive immune responses that drive glomerular disease (Summers et al., 2009).

1.7 Pathogenesis of Omeprazole-Induced AIN

How omeprazole induces AIN is unknown. Given the histological similarity to small vessel vasculits and that a number of potential pathways have been identified that mediate small vessel vasculitis (Summers et al., 2009, Gan et al., 2010, Ooi et al., 2010), our study was set up to elucidate the immunopathogenic pathways in omeprazole-induced AIN and to compare and contrast the immunopathogenic pathways with small vessel vasculitis.

Simpson et al. suggested there might be more than one pathogenic pathway involved in PPI-AIN (Simpson et al., 2006). In the reported biopsy series immunofluoroscence staining for antibody deposits is negative. This suggests a predominantly cell-mediated response that is responsible for the injury. Direct toxicity of the drug causing an interstitial inflammatory response is a further possible pathway of injury. Additional factors which may enhance the tubular injury seen on biopsy include the loss of renal autoregulation usually characteristic of the elderly population pre-disposing to compromised renal blood flow with any concurrent insult, as well as the multiple drug use that most of the population affected by AIN are on.

The classic concept of drug immunogenicity is thought to depend on their ability to form so-called hapten carrier complexes, which are newly immunogenic protein antigens that are able to stimulate both T and B cell immune responses (Perazella and Markowitz, 2010, Border et al., 1974, Toto, 1990, Cameron, 1988). For example, in methicillin-induced interstitial nephritis methicillin molecules act as haptens, which bind to the tubular basement membrane (TBM) and elicit the production of anti-TBM antibodies. However, many drugs are not chemically reactive but become so after an intermediate metabolism step, which transforms pro-haptens to haptens. Such transformations from pro-haptens to haptens occur mainly in the liver but might also occur in the kidney, because tubular epithelial cells

produce various cytochrome P450-associated metabolizing enzymes (Spanou et al., 2006). Alternatively the immunogenicity of drugs relies on a direct interaction of the drug with immune receptors of T cells such as the T cell receptor (TCR). This has been postulated as a pharmacologic interaction with immune receptors and can lead to T cell activation and expansion (Spanou et al., 2006).

Direct drug toxicity or idiosyncratic immune mediated reaction

Helsby et al. studied whether omeprazole-induced acute interstitial nephritis is an idiosyncratic immune mediated reaction or if it potentially originates from direct drug toxicity related to altered drug metabolism (Helsby et al., 2010). The time course for developing omeprazole AIN is quite variable. Some patients might be exposed up to 18 months to omeprazole before the onset of AIN, whereas others develop AIN after doubling the dose (Simpson et al., 2006). Helsby and colleagues examined the role of drug metabolism and the possibility that slow metabolisers of omeprazole may be a higher risk of AIN. Omeprazole is a substrate for CYP2C19 and homozygous individuals for the null allele are poor metabolizers of this drug. With higher exposure to the drug, especially with elderly patients known to metabolise omeprazole at a slower rate even with a normal genotype, Helsby and colleagues investigated the prevalence of the CYP2C19 poor metabolizer genotype and phenotype in patients with omeprazole-induced AIN. They genotyped twenty patients for the CYP2C19 variant alleles (*2,681 G>A and *3,636G>A) by RFLP-PCR analysis and eighteen patients for their CYP2C19 metabolizer status. Almost a third (33%) of the subjects were phenotypically CYP2C19 poor metabolizers. However, they found no difference in the CYP2C19 poor metabolizer genotype in patients with omeprazole-induced AIN compared to the normal population. It is known that elderly healthy individuals with a “normal activity” genotype have a decreased CYP2C19 activity with a poor metabolizer phenotype (Ishizawa et al., 2005). They concluded that omeprazole-induced acute intersitial nephritis is not related to CYP2C19 genotype or CYP2C19 phenotype (Helsby et al., 2010). These authors postulated that a higher plasma concentration of omeprazole may increase the chance of a drug hapten being presented to T lymphocytes resulting in an idiosyncratic immune mediated response (Helsby et al., 2010).

Direct drug toxicity via inhibition of renal H+K+ATPase

A further suggested pathway of interest is direct toxicity to the kidney with elevated concentrations of omeprazole predisposing to AIN via inhibition of the renal H+K+ATPase (Sabolic et al., 1994). Although H+K+ATPase is present on distal renal tubular cells, omeprazole administration to healthy male subjects resulted in no demonstrable effect on the renal handling of acid and did not alter the urinary electrolyte balance, the urine pH or the serum electrolytes. This study further supports the evidence for the specificity of action of omeprazole on gastric parietal cells (Howden and Reid, 1984).

Omeprazole induced AIN and positive ANCA

P- ANCA has also been linked to omeprazole induced AIN. The first case of PPI associated with p-ANCA was described by Singer et al. in 1994 (Singer et al., 1994). At that time anti-MPO titres were not available. However p-ANCA immunofluorescence is associated with a degree of false positivity and without the associated measurement of myeloperoxidase (MPO) antibodies, the significance of a positive p-ANCA is dubious. A second case was published in 2006, where in contrast to the first patient MPO-ANCA testing was undertaken and showed high levels (114, normal <20). Additionally this patient also had clinical manifestations suggestive of a concurrent vasculitis with palpable purpura, fever, malaise, weight loss, eosinophilia with confirmation of intersitial nephritis and cutanous vasculitis by biopsy. Therefore any potential association between omeprazole and the positive MPO titres is difficult to elucidate (Jacobs-Kosmin et al., 2006). Subsequent published case series have not demonstrated any significant association between ANCA positivity and omeprazole-induced AIN.

1.8 Pathology

Histology of drug induced interstitial nephritis

The gold standard for diagnosis of drug-induced interstitial nephritis is the histology demonstrated on a renal biopsy. This typically shows a normal glomerulus with a dense interstital cellular infiltrate. Histologically, drug induced interstitial nephritis can be differentiated from other types of renal failure by specific

morphologic characteristics. Characteristic biopsy findings consist of an interstitial accumulation of inflammatory cells including mostly lymphocytes, plasma cells, macrophage/monocytes, eosinophils, and/or polymorphonuclear neutrophils (PMN) with or without a tubulitis. Tubulitis is evident when inflammatory cells, predominantly lymphocytes, have crossed the tubular basement membrane and are attacking the tubular epithelial cells with subsequent cellular damage (Racusen et al., 1999). T cells seem to play a major role in the pathogenesis of the disease, because they are the predominant cell type in the interstitial infiltrate (Neilson, 1989), (Spanou et al., 2006). In a minority of cases non-caseating granulomata localized around damaged tubules are found (Wall et al., 2000, Myers et al., 2001, Montseny and Meyrier, 1998).

There has not been much data published on the cell subsets in tubulitis in acute tubulointerstitial nephritis. Furthermore it is not clear whether the tubulitis influences the outcome of the disease. A publication from 1996 looked at 6 patients with acute tubulointerstitial nephritis in ten renal biopsies. 3 were drug-induced (penicillin, amitryptiline and paracetamol, and the third case due to non-steroidal anti-inflammatory drugs), two related to Legionella infection and one was idiopathic. The inflammatory cell subsets (CD3+, CD4+, CD20+, CD45RO+, CD56+, CD57+, CD68+ and TIA-1+ cells) in the tubules and interstitium were analysed. CD8+ and CD4+ lymphocytes with a smaller number of macrophages, were the predominant cells both in the tubules and in the interstitium. The main prognostic marker was the severity of interstitial fibrosis (Ivanyi et al., 1996).

Histology of vasculitis

The renal involvement in ANCA-associated vasculitis ranges from a severe crescentic glomerulonephritis with fibrinoid necrosis and associated intense interstitial infiltrate with or without granulomata to a pattern with predominantly an interstitial infiltrate with little or no active glomerular lesions. On immunofluorescence microscopy there is little or no glomerular staining for Immunoglobulins (Igs) or complement, the so-called pauci-immune staining pattern, first described by Jennette et al. (Jennette et al., 1989). On electron microscopy immune deposits are absent, subendothelial edema, microthrombosis and degranulation of neutrophils may be present (Berden et al., 2010).

In terms of prognosis the percentage of normal glomeruli is a strong prognostic predictor regarding short- and long-term renal outcome (Berden et al., 2010). Interestingly the percentage of active cellular crescents is related to recovery of renal function independent of baseline GFR (Hauer et al., 2002). Patients with more severe disease activity, but not so much fibrosis in the renal biopsy are likely to present with very impaired renal function, but have a good chance of renal recovery (Allison, 2010). However the percentage of fibrous crescents adversely affects long-term renal outcome. Patients with sclerotic ANCA-associated glomerulonephritis had a higher risk of death then others (Berden et al., 2010).

Although the current schema for ranking the severity of ANCA-associated glomerulonephritis is based purely on the presence of glomerular lesions the tubulointerstitial lesions may also be of prognostivc value in ANCA-associated vasculitis. There has been a standardized scoring system developed where the dominance of any cell type in the infiltrate is noted (plasma cells or eosinophils), along with a high number of interstitial granulomas or extensive arteriosclerosis (Bajema et al., 1996).

Initial histological review of the PPI biopsies reveals features of the interstitial infiltrate that are similar to that seen in PR3/MPO ANCA small vessel vasculitis.

Evaluation of clinical and histological prognostic markers of Drug- induced acute interstitial nephritis

Clinically there are no reliable prognostic markers of outcome for drug-induced acute interstitial nephritis due to the lack of substantial prospective observational studies. In older case series published before the recognition of omeprazole-induced AIN, neither urine volume nor peak serum creatinine concentration serve as a clinical parameter for prognosis. Similarly eosinophilia, eosinophiluria, hypertension and allergic manifestations fail to provide any prognostic clue. In the study by Bhaumik et al. overall 14/19 (74%) of the patients recovered normal renal function within 6 weeks following withdrawal of the offending drug (Bhaumik et al., 1996). Only a minority of patients (17-36%) with drug-induced AIN required dialytic support (Grunfeld and Kleinknecht, 1993). Of the published series of omeprazole AIN, subsequent to this, none of the cases have required dialysis support.

Histologically in the earlier series of AIN, there was no correlation between the extent of the interstitial infiltrate and the severity of the renal failure (Buysen et al., 1990). Furthermore the severity and type of cellular infiltrates (mainly looking at Pan-T-cells (CD3+), T helper cells (CD4+), T cytotoxic cells (CD8+), Pan-B cells (CD20+), Memory CD4 and CD8 cells (CD45RO+), Natural killer cells (CD56+), Large granular lymphocytes (CD57+), presence of tubular necrosis and interstitial edema did not affect the outcome (Bhaumik et al., 1996). Only tubular atrophy and interstitial fibrosis are known to affect renal recovery adversely (Bhaumik et al., 1996, Ivanyi et al., 1996). Both Bhaumik and Ivanyis studies indicate that interstitial fibrosis is crucial in the prognosis of drug-induced interstitial nephritis.

1.9 Aim of this study

The aim of this study was to define the nature of the inflammatory infiltrate that occurs with omeprazole-induced acute interstitial nephritis in order to establish the probable immunopathogenesis of the lesion. The second aim was to compare and contrast the histology and immunopathology in the omeprazole-induced AIN group with the renal lesions seen in PR3 or MPO positive small vessel vasculitis.

We investigated 25 patients with biopsy-proven omeprazole (PPI) induced acute interstitial nephritis (AIN) and compared them with 27 patients with PR3/MPO small vessel vasculitis. To assess the severity of interstitial nephritis we initially graded these renal biopsies according to the Banff-criteria 1997. Then using immunostaining with various antibodies to T-cell surface markers (CD8, CD4, IL17A, IL17F, Foxp3) and B-cell surface markers (CD20) we characterized the predominant cell type of the inflammatory response and compared this to the inflammatory response evident in small vessel vasculitis.

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CHAPTER TWO METHODS

2.1 Patients

This study encompassed a clinical audit of all biopsy proven omeprazole-induced acute interstitial nephritis cases that have presented to the Dunedin Public Hospital since the first documented cases in 2000 (Delve 2003). The Dunedin Public Hospital provides renal service to a population of 350,000 in the lower South Island of New Zealand. The reports of all kidney biopsies carried out over a 11 year period from 2000 to 2011 with the diagnosis of interstitial nephritis secondary to omeprazole were selected from an online database and the case notes and histological reports were reviewed. We included patients who presented to our renal service with the diagnosis of interstitial nephritis secondary to omeprazole on the discharge summary which was confirmed by renal biopsy. Exclusion criteria were interstitial inflammation as stated on the histopathological diagnosis secondary to other drugs such as non-steroidal anti-inflammatory drugs, antibiotics, lithium, N- benzylpiperazine (BZP), viral infections and leptospirosis.

To compare with a potentially similar cell-mediated process we selected a vasculitis group. We included patients presenting to our renal unit over a similar time period of 12 years from 1998 to 2011 with the diagnosis of ANCA positive vasculitis with a crescentic glomerulonephritis and florid interstitial infiltrate on renal biopsy.

Collection of patient demographic and medical history data

The medical notes of our patients with the diagnosis of omeprazole-induced acute interstitial nephritis and the ANCA positive vasculitis group were reviewed for the details of their presentation, medical history, investigations, treatment and outcome. Specific details noted for each patient were: age at presentation, gender, all medications (omeprazole with duration and dose of treatment, where possible), investigations, including pre-morbid serum creatinine before presentation, serum creatinine at the time of renal biopsy,

and the final recovery serum creatinine, urinalysis (microscopy) and culture, urine protein creatinine ratio (or albumin creatinine ratio where possible), the presence or absence of eosinophilia, haemoglobin, ESR, CRP, weight and blood pressure. We did not calculate or measure creatinine clearance. In addition anti-neutrophil cytoplasmic antibodies (cANCA and pANCA) and PR3 and MPO titres (where measured), was noted in both study cohorts.

2.2 Renal biopsies

All renal biopsies were obtained using a standard percutaneous approach under ultrasound guidance with a 'Tru-cut' renal biopsy needle (18 gauge, Bard, Crawley, UK) following informed consent by the patient. The biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. 2 µm thick sections were cut from each paraffin block for histology and immunostaining and put onto superfrost Plus slides (Lomb scientific). Segments were also processed for immunofluorescence (frozen tissue) and electron microscopy (4% glutaraldehyde fixed tissue).

Of the total 25 patients with biopsy-proven omeprazole-induced acute interstitial nephritis and the 27 patients with PR3/MPO ANCA positive small vessel vasculitis 24-25 underwent immunopathology staining in the omeprazole-induced acute interstitial nephritis group and 23-25 in the vasculitis group due to limited availability of tissue.

2.3 Histological grading of the interstitial infiltrate

Berden and colleagues (Berden et al., 2010) designed a classification system for ANCA-associated glomerulonephritis based on glomerular pathology as assessed by light microscopy that groups biopsy samples with ANCA-associated glomerulonephritis into four categories- focal, crescentic, mixed and sclerotic- according to glomerular pathology as assessed by light microscopy (Table 1).

Table 1: Classification schema for ANCA-associated glomerulonephritis

Class	Inclusion Criteria[a]
Focal	≥50% normal glomeruli
Crescentic	≥50% glomeruli with cellular crescents
Mixed	<50% normal, <50% crescentic, <50% globally sclerotic glomeruli
Sclerotic	≥50% globally sclerotic glomeruli

[a]Pauci-immune staining pattern on immunofluorescence microscopy (IM) and ≥1 glomerulus with necrotizing or crescentic glomerulonephritis on light microscopy (LM) are required for inclusion in all four classes. See Figure 1 for hierarchical structure.

This classification system does not score the interstitial infiltrate adequately and therefore did not allow comparison between the vascilitis group and the omeprazole acute interstitial nephritis group. As mentioned previously there has been a standardized scoring system developed by Bajema et al. where the dominance of any cell type in the infiltrate is noted (plasma cells or eosinophils), along with a high number of interstitial granulomas or extensive arteriosclerosis (Bajema et al., 1996). However, this scoring system was not detailed enough for our study.

The Banff criteria 1997 was used for grading the severity of the inflammatory infiltrate and used on H and E sections and on special stained sections (see appendix 3 Banff criteria (Racusen et al., 1999)). This criteria was originally developed to standardise the histological scoring for acute kidney transplant rejections. The histological appearances of the omeprazole-induced interstitial nephritis could be mistaken for an acute cellular rejection of a renal transplant if there was no associated history provided. We wished to use a recognized semi-quantitative scoring process to define the histological injury and correlate this with immunohistological findings on renal biopsies of our two cohort groups. Haematoxylin and eosin stained slides were imaged using standard light microscopy (Leica DM 2000 microscope, Leica DFC 295 camera, and Leica Version 3.5.0 Application Suite software, Leica, Solms, Germany).

2.4 Immunostaining techniques

Immunohistochemistry (IHC) and immunofluorescence (IF) are molecular techniques combining principles from both immunology and biochemistry to study pathology. This technique identifies proteins in tissue

sections exploiting the principle of antibodies specifically recognizing their antigens in biological tissue. See Table with the list of proteins which were detected in this study and conditions used for IHC and IF.

Double staining immunohistochemistry is used to reveal two distinct antigens in a single tissue. We double stained for CD20 and CD8 on the renal biopsy sections. The antigen can be identified using two different primary antibodies from mouse (or rat) and rabbit (or guinea pig) species. Slides used for IHC were imaged using standard light microscopy (Leica DM 2000 microscope, Leica DFC 295 camera, and Leica Version 3.5.0 Application Suite software, Leica, Solms, Germany).

Double staining immunofluorescence (IF) uses fluorescence to detect two antigens and can be used to easily view antigens in the same cell. We used IF to stain IL17A and CD4, IL17F and CD4 together, and FoxP3 as a single stain.

2.5 Optimized tissues, negative controls

Staining was optimized on control tissue first, which contained T- and B-cells. Optimization for staining was initially done on tonsil (CD8 and CD4 positive tissue) (Figure 7), kidney (Figure 8) and placenta to look at background staining and additionally it was a readily available source of tissue in the laboratory). Tissues with inflammatory cells similar to AIN were also used as positive controls and included Crohns disease (Figure 9, 10 and 11B), spleen (Figure 11A) and appendicitis. The optimized conditions are described in Table 2.

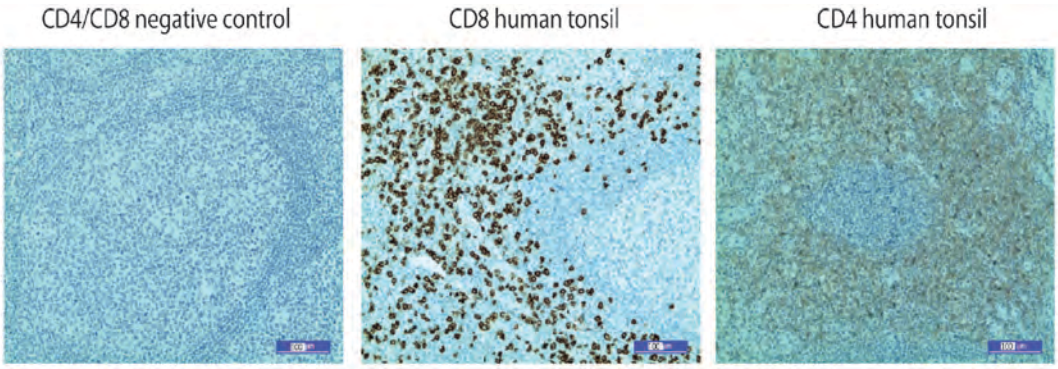


Figure 7: Optimisation of CD4 and CD8 staining on tonsil tissue.

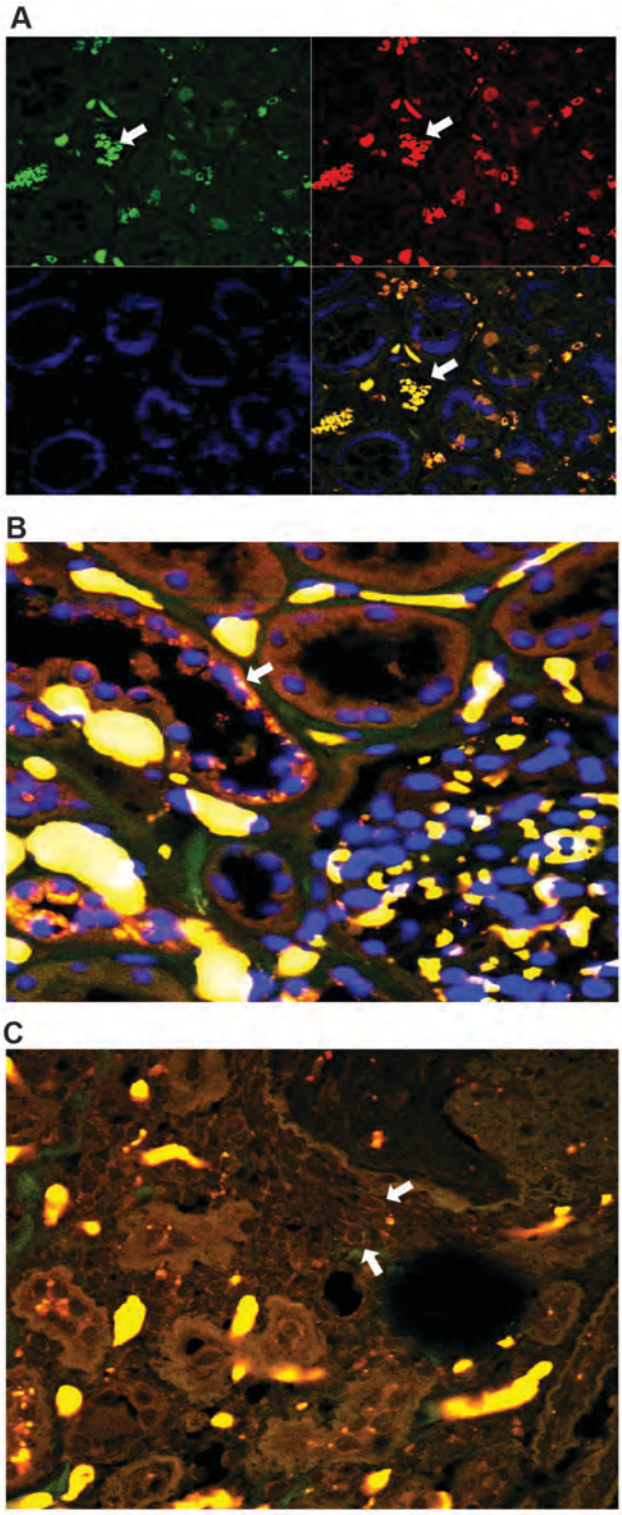


Figure 8: Negative controls for CD4 (green) and IL17A (red). A Example of autofluorescence, neg control in crohns disease patient sample. Autofluorescence of RBC (highlighted by the white arrow) and mastcells showing up in the CD4 (green), IL17A (red) and DAPI (blue) merged image bottom right. **B** Negative kidney control with unspecific tubular uptake highlighted by the white arrow. **C** Negative kidney with 2 unspecific plasma cells highlighted by the white arrows.

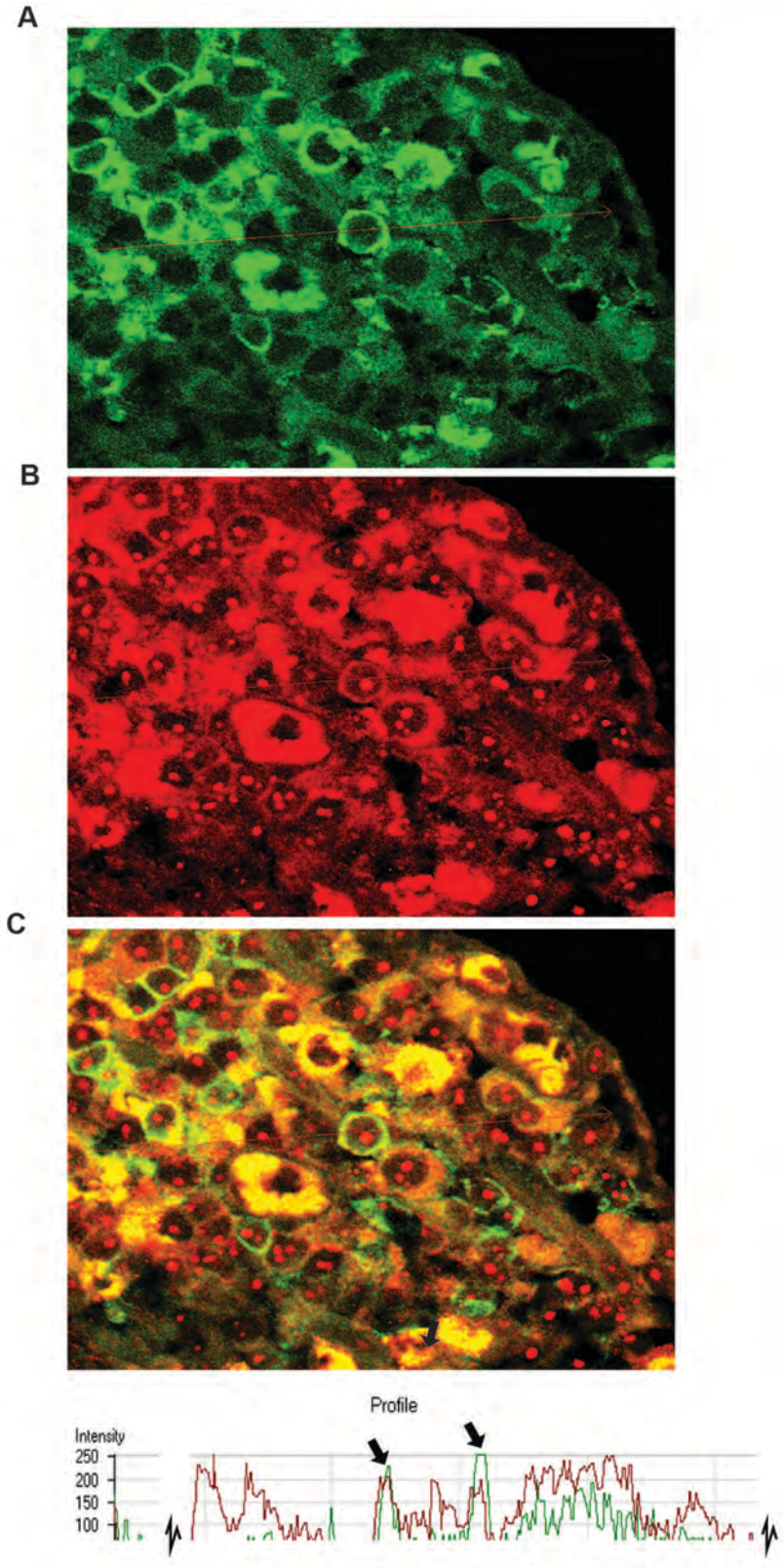


Figure 9: Crohns positive control CD4 (green) and IL17A (red). A CD4 (green), **B** IL17A (red), **C** overlap of CD4 (green) and IL17A (red) with confocal profile. Fluorescence confocal profile, measuring fluorescence intensity of the green channel (CD4) and red channel (IL17F), a tool to show double staining. Double stained cell indicated by black arrows on the profile.

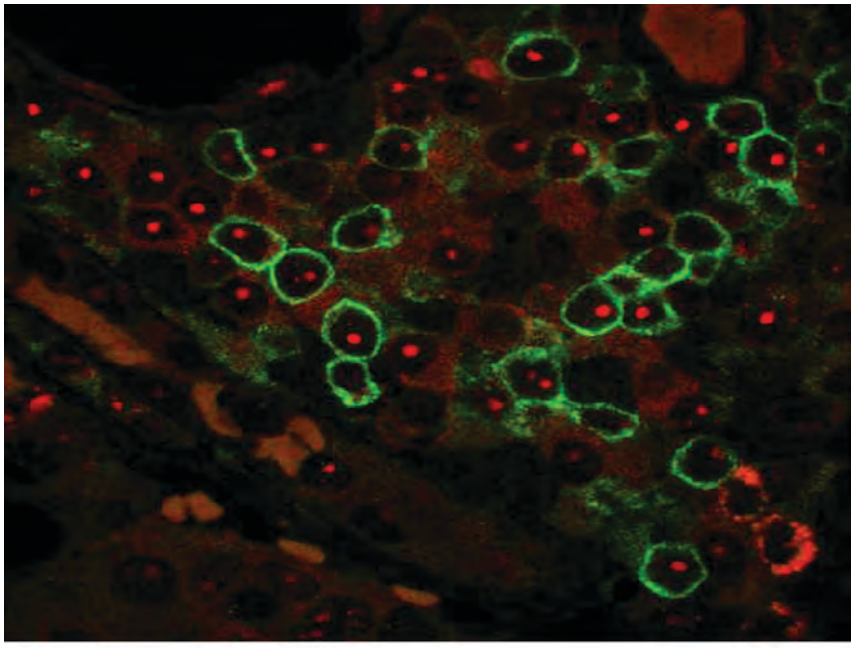
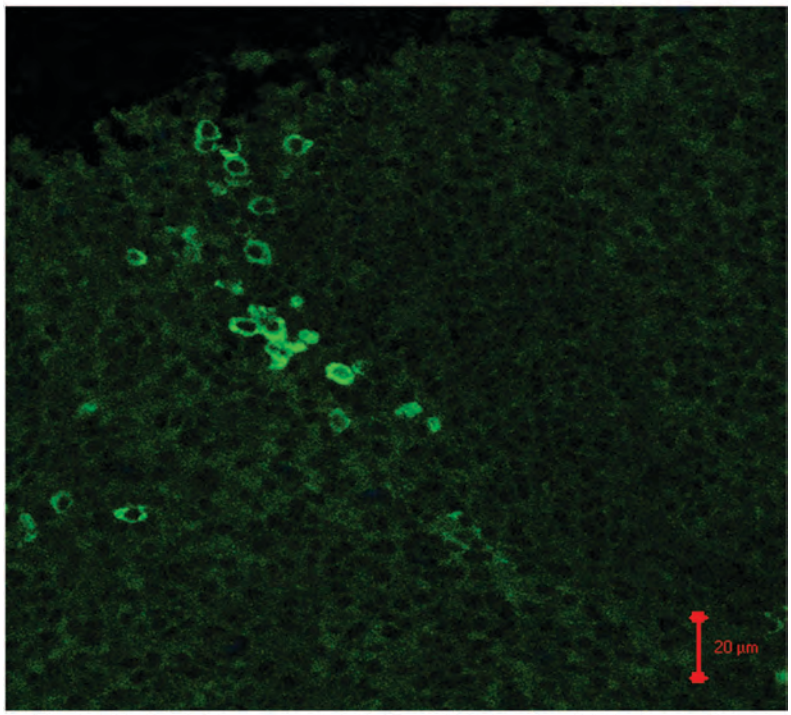


Figure 10: Crohns positive control for CD4 (green) and IL17A (red). There are mainly CD4 single stained cells.

A



B

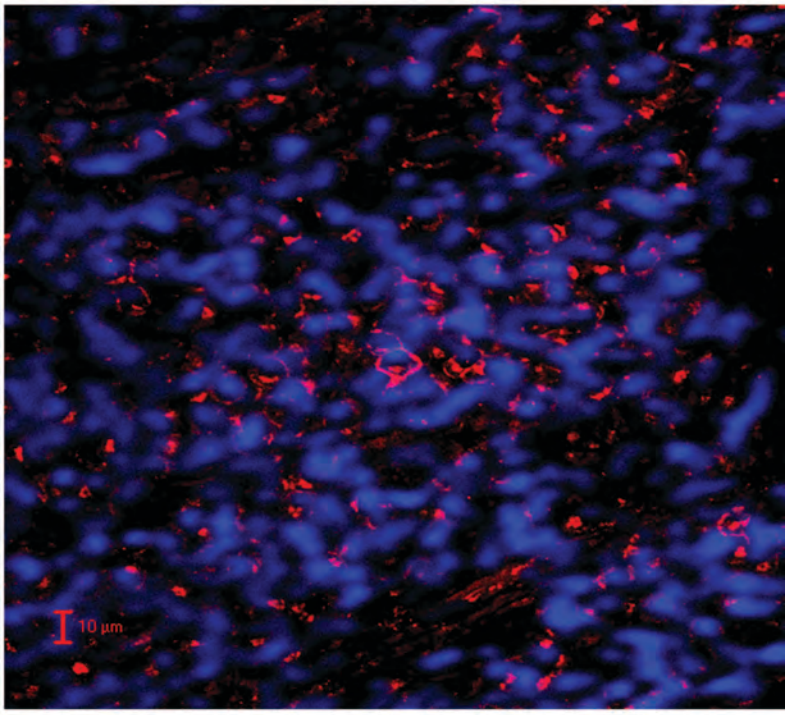


Figure 11: Macrophage marker optimisation. A MMR (green) in the spleen, B INOS (red) in crohns tissue.

Preparation of tissue

The patient's kidney sections were deparaffinized in xylene and alcohol (see appendix-staining protocol). Antigen retrieval was done with Tris EDTA pH 9 or citrate pH 6 in the microwave (Milestone KOS) at 95℃ for 25 minutes with 20 minutes cooling in a waterbath.

2.6 Immunohistochemistry

Immunohistochemistry CD8 and CD20

The CD20 and CD8 antibodies used, dilutions and optimized conditions are described in Table 2. Initially we used the Novolink Max Polymer Detection system (Novocastra) staining protocol for single staining. However in order to preserve tissue we changed to double staining using the Invitrogen Staining kit. For the negative control, sections of the kidney with no primary antibody were added, and instead antibody dilutant (2% BSA in 1xPBS) was added. Additionally to the Invitrogen staining protocol we used a DAB enhancer solution (DAB Enhancer, Zymed Laboratories Inc.) for 3 minutes after the DAB chromogen had been rinsed off in distilled H_2O. Additionally we replaced the Hematoxylin in the kit with Hematoxylin Ready-to-use (Ref 008011, Invitrogen) and used Scott's blue made "in house" (1 l distilled H_2O, 2 g potassium bicarbonate, 20 g magnesium sulphate, pH 7.78) to counterstain the slides. For mounting Immuno-Mount (Thermo scientific) was used and slides dried overnight at room temperature. To avoid air bubbles under the cover slip the slides were sealed with nail varnish.

CD20 was stained a double stain together with CD8 using optimized conditions as described in Table 2 and using the staining protocol outlined in the kit by Invitrogen. Goat anti-Mouse IgG-HRP was used with DAB to produce one stain **(brown and detected CD8)**; Goat anti-Rabbit IgG-AP is used with Fast-Red to produce a second stain **(red and detected CD20)** according to the manufacturers description (Invitrogen-Description).

2.7 Immunofluorescence

Immunofluorescence CD4, IL17A and IL17F

Initially we tried to optimize CD4 clone SP35 rabbit from Cell Marque 1 in 50 dilution, which had faint staining. Additionally because we wanted to use it in double staining together with IL17A (being a rabbit antibody) we preferred to switch to a mouse CD4 antibody and used clone 4B12 mouse (Dako). The CD4 (Dako) antibody was detected using a goat anti-mouse antibody labelled with Alexa Fluor 488, Invitrogen.

The IL17A antibody was detected using goat anti-rabbit labelled with Alexa Fluor 555, Invitrogen. Because of problems with autofluorescence we tried to reduce the background using Sudan black (see below) and then further also used a second antibody to IL17 IL17F to compare and support our staining results.

IL17F (goat) was stained as double stain together with CD4 (mouse) using the optimized conditions in Table 2 and a more detailed protocol is in the appendix. We then graded those results and compared them with our IL17A and CD4 double staining results. The IL17F antibody was labelled with Alexa Fluor 555 (Invitrogen) donkey anti-goat.

Immunofluorescence FOXP3

Foxp3 is a key regulatory gene for the development of regulatory T cells. We chose to stain for Foxp3 only (without CD25/CD4) because Foxp3 represents a more specific marker than currently used cell-surface molecules (such as CD25, CD45RB, CTLA-4, and GITR), which are unable to completely discriminate between regulatory T cells and activated, effector, or memory T cells (Hori et al., 2003). CD25 (clone 4C9, mouse from CellMarque, 1 in 100 dilution) was optimized initially but because as described Foxp3 being a more

specific marker, CD25 was not further used for staining of our patients slides. The Foxp3 antibody was labeled with Alexa Fluor 488 goat anti-mouse.

Immunofluorescence INOS (M1)

INOS (M1) a macrophage 1 marker was optimized using described optimized conditions in table 1 with the aim to double stain with CD163.

Immunofluorescence MMR (M2)

MMR (M2) a macrophage 2 marker was optimized using described optimized conditions in Table 2 with the aim to double stain with CD163.

2.8 Staining protocols

1) Immunohistochemical Technique 1-DAB (also see appendix 1)

The PicTureTM-DOUBLE STAINING KIT used according to the manufacturer's instructions using a commercial kit from Invitrogen Polymer Detection System, Invitrogen Corporation, Camarillo, CA, USA, CAT. NO 87-9999. (See staining protocol in appendix 1).

2) Immunofluorescence human leukocyte staining for formalin fixed paraffin embedded tissue (see appendix 2)

Table 2- Monocloncal and polyclonal antibodies used in the present study

Antigen	Clone	Manufacturer	Dilution	Secondary Label	Antigen retrieval
CD4	4B12 mouse	Dako, Glostrup, Denmark	1 in 40	Alexa Fluor 488	Microwave (pH 6)
CD8	C8/144B mouse	Cell Marque, Rockin, CA	1 in 100	IgG-HRP DAB	Microwave (pH 9)
CD20	SP32 mouse	Cell Marque, Rockin, CA	1 in 100	IgG-AP DAB	Microwave (pH 9)
IL17A	H 132 rabbit	SantaCruz Biotechnology, CA	1 in 20	Alexa Fluor 555	Microwave (pH 6)

FOXP3	150D mouse	BioLegend, San Diego, CA	1 in 50	Alexa Fluor 488	Microwave (pH 6)
IL17F	HYZ03 goat	R&D systems, Minneapolis, MN	1 in 20	Alexa Fluor 555	Microwave (pH 6)
INOS (M1)	Ab3523	Abcam, Cambridge, UK	1 in 100	Alexa Fluor 555	Microwave (pH 6)
MMR	15-2	BioLegend, San Diego, CA	1 in 500	Alexa Fluor 488	Microwave (pH 6)

The optimized conditions are summarized in Table 2. The secondary labels were used in a 1 in 250 dilution.

Microscopic analysis

With IHC, CD20 and CD8 positive cells were detected using light microscopy. The CD20 (red) and CD8 (brown) positive cells were graded semi quantitatively by two different observers, blinded to the tissue of origin (1 anatomical pathologist, 1 physician) by using "+" equaling few scattered positive cells, "++" intermediate amount of positive cells and "+++" large numbers of cells in clusters.

With IF, CD4, IL17 A & F, FoxP3 positive cells were detected using a Zeiss Confocal LSM510 Axioplan 2 Upright Epifluoroscence microscope (Carl Zeiss, GmbH, Germany). Cellular nuclei were stained using DAPI. At least 100 and up to 1000 lymphocyte nuclei were examined for positive staining. For double staining, cells positive for one marker were further investigated for positivity for the second marker. Positive cell counts were made based on cells being positive for both markers. The number of cells with positive staining were counted semiquantitatively as described above. Cells were identified by a consultant pathologist (NH). Photography was performed by digitally overlaying sequential excitations, to prevent "bleed-through" (Slatter et al., 2012).

2.9 Staining challenges

A new emerged antibody IL17A and IL17F

To date staining for IL17A and IL17F in human kidney tissue is still experimental and not in routine laboratory practice. Initially it was difficult to see if we had true staining. Autofluorescence in the kidney tissue especially with IL17A was a challenging problem, which initially made it difficult differentiating the inflammatory process from the kidney tissue. To eliminate or reduce autofluorescence, Sudan black was used on the IL17A stains. Slides were immersed in 0.1% Sudan Black B made up in 70% ethanol (Sigma-Aldrich cat #199664-25g) for 30 minutes, then washed in a stream of PBS out of a squeezy bottle, and then immersed for a further 10 minutes in PBS before placing the coverslips on the slides (Baschong et al., 2001). The washing step is important to prevent stain deposit on the sections stained.

The immunohistochemical and immunofluoresence studies were carried out with ethics approval from the Multi-regional Ethics committee (MEC/07/05/065).

2.10 References

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CHAPTER THREE RESULTS

3.1 Clinical data

We identified 25 patients with biopsy-proven omeprazole-induced acute interstitial nephritis and compared them with 27 patients with PR3/MPO ANCA positive small vessel vasculitis and renal involvement.

The demographics and other features for the omeprazole-induced AIN group are similar to previous published studies and are shown in Table 3.

The patients ranged in age from 55-82 years (median patient age was 73 years), with a gender ratio of 16:9 (female/male). The mean baseline (pre-morbid) serum creatinine concentration was 91.8μmol/l (range 56μmol/l to 140μmol/l), peak serum creatinine concentration at the time of biopsy ranged from 130μmol/l to 758 μmol/l (mean 411 umol/l). The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were elevated at the time of diagnosis in 19 out of 25 patients (in 6 patients they were normal or unknown). The duration of PPI therapy prior to diagnosis, where known, ranged from 3 weeks to 2 years. Blood pressure ranged from 101/49 to 243/107mmHg (143/74mmHg) at admission.

Table 3: Clinical characteristics and the ANCA status of patients with omeprazole- induced acute interstitial nephritis. Definition of eosinophilia: an upper limit of 0.4 or 0.6 x 10^9/l depending upon what laboratory range is accepted.

Patient	Age (years)	Gender	ANCA plus PR3, MPO titres	Pre-morbide Crt (umol l-1)	Biopsy Crt (umol/l-1)	Recovery Crt (umol/l-1)	Smoker	Dose (mg/d)	PPI Duration on PPI
1	73	F	pANCA 4+ (PR3, MPO neg)	80	758	154	Ex	-	3 months
2	80	M	cANCA 2+ (PR3, MPO not known)	106	484	159	Ex	20	Not known
3	66	F	neg	69	290	100	Non	10	5-6 months
4	70	F	neg	62	194	71	Ex	20	2 years
5	76	F	?pANCA+(Ethanol fix positive)	88	340	116	Non	20	Not known
6	80	M	neg	119	545	185	Ex	20	Not known
7	78	F	neg	130	509	160	Ex	40	recently
8	59	M	N/A	120	430	190	not known	-	Not known
9	73	M	neg	115	276	210	Ex	20	6 months
10	77	F	pANCA not conclusive	56	462	140	Ex	-	Not known
11	78	F	neg	98	387	160	Non	20	"some time"
12	82	F	N/A	73	196	150	Ex	80	4-5 months
13	69	F	neg	62	298	99	Ex	20	4-5 months
14	81	M	N/A	114	565	136	Ex	40	"longterm"
15	78	F	ANCA pos. PR3 31, MPO neg (repeat neg)	76	562	125	Non	20	3 weeks
16	69	F	ANCA pos (PR3, MPO neg) (PR3 25 bb)	84	363	79	Non	-	2 months
17	55	M	ANCA pos, (PR3,MPO neg)	83	271	107	Non	20	1-2 months
18	68	M	neg	90	602	240	Ex	20	1 month
19	64	M	neg	97	477	160	Ex	-	3 weeks
20	65	F	pNACA not excluded	95	407	87	Non	20	Not known
21	76	F	N/A	78	130	118	Non	40	3-4 weeks
22	65	F	ANCA not excluded (PR3, MPO neg)	84	737	103	Non	20	Not known
23	82	F	neg	140	470	220	Non	20	9 months
24	70	F	N/A	86	353	119	Non	40	6 weeks, higher Dose 1/52 ago
25	69	M	neg	89	181	136	Ex	-	3 months

Table 4: Clinical characteristics and the ANCA titres of the patients with biopsy proven vasculitis. PR3 ANCA vasculitis is synonymous with Wegeners granulomatosis and MPO ANCA vasculitis for microscopic polyangitis.

Patient	Age	Sex	Diagnosis	ANCA plus PR3, MPO titres	Pre-morbide Creat	Biopsy Creat	recovery Creat	Smoker
1	62	M	pANCA vasculitis	pANCA Ethanol fix + (titres not known)	79	393	136	ex-smoker
2	69	M	?PR3 ANCA vasculitis, gran GN	pANCA pos, MPO, PR3 neg	115	615	not known	non-smoker
3	29	F	ANCA neg vasculitis	ANCA neg, MPO, PR3 neg	not known	318	157	ex-smoker
4	70	M	PR3 ANCA vasculitis	cANCA pos, PR3 81, MPO neg	not known	119	95	ex-smoker
5	44	F	MPO ANCA vasculitis	not known	not known	not known	114	non-smoker
6	78	F	MPO ANCA vasculitis	pANCA 4+ pos, MPO 109, PR3 neg	78	91	86	not known
7	51	M	MPO ANCA vasculitis	pANCA pos, MPO 110, PR3 neg	106	295	165	ex-smoker
8	45	F	PR3 ANCA vasculitis	pANCA 2+ pos, MPO 21, PR3 146	77	116	82	non-smoker
9	66	M	PR3 ANCA vasculitis	c-ANCA 4+ pos, PR3 strong pos (titres not known)	104	95	102	non-smoker
10	69	M	ANCA neg vasculitis	ANCA neg, MPO, PR3 neg	not known	213	168	ex-smoker
11	54	M	PR3 ANCA vasculitis	pANCA 4+ pos, PR3 23, MPO 583	106	321	137	smoker
12	80	F	PR3 ANCA vasculitis	cANCA 2+ pos, MPO 209, PR3 neg	77	74	62	not known
13	63	M	MPO ANCA vasculitis	pANCA 2+ pos, MPO 613, PR3 neg	108	321	dialysis	ex-smoker
14	71	F	PR3 ANCA vasculitis	cANCA 4+ pos, PR3 925, MPO neg	100	901	dialysis	ex-smoker
15	44	F	PR3 ANCA vasculitis	cANCA pos, PR3 1097, MPO neg	62	230	148	ex-smoker
16	63	M	PR3 ANCA vasculitis	pANCA pos, MPO 64, PR3 neg	106	238	253	not known
17	79	M	PR3 ANCA vasculitis	cANCA 4+ pos, PR3 1172, MPO neg	94	185	87	non-smoker
18	58	M	PR3 ANCA vasculitis	ANCA pos, PR3 4480	not known	94	114	ex-smoker
19	77	F	PR3 ANCA vasculitis	ANCA pos, PR3 483	CKD	720	dialysis	ex-smoker
20	79	M	PR3 ANCA vasculitis	ANCA pos, MPO 348, PR3 neg	not known	349	336	ex-smoker
21	78	M	MPO ANCA vasculitis	ANCA pos, MPO 130, PR3 neg	115	156	114	ex-smoker
22	60	F	MPO ANCA vasculitis	ANCA pos, MPO 321, PR3 neg	116	460	418	ex-smoker
23	61	M	PR3 ANCA vasculitis	ANCA pos, PR3 >200, MPO neg	115	214	175	non-smoker
24	70	M	MPO ANCA vasculitis	ANCA pos, MPO 70, PR3 neg	88	796	400	ex-smoker
25	58	M	MPO ANCA vasculitis	ANCA pos, MPO 85, PR3 neg	110	216	230	non-smoker
26	75	F	PR3 ANCA vasculitis	ANCA pos, PR3 20, MPO neg	65	256	dialysis	ex-smoker
27	63	F	MPO ANCA vasculitis	ANCA pos, MPO 134, PR3 neg	75	333	489	smoker

The demographics for the vasculitis group are shown in Table 4.

The patients ranged in age from 29-80 years (mean age 63.6 years) and on average were 10 years younger than the omeprazole-induced AIN group, with a gender ratio of 11:16 (female:male). The baseline (pre-morbid) serum creatinine concentration, where available) ranged from 62 µmol/l to 116 µmol/l (mean 94.8 µmol/l), peak serum creatinine concentration at the time of biopsy ranged from 74µmol/l to 901 µmol/l (mean 312 µmol/l). As expected, the ESR and/or CRP were elevated at the time of diagnosis in 26 out of 27 patients (1 patient unknown).

Clinical features of the omeprazole-induced interstitial nephritis and vasculitis group

As expected, the majority (70%) of patients with vasculitis had extrarenal manifestations, 19/27 presenting with fever, rash, arthralgia, pneumonia or polyneuropathies with only 5/27 having renal limited disease at the time of diagnosis. All the omeprazole-induced AIN cases had no extrarenal manifestions or systemic inflammatory symptoms and none had features of the classic acute allergic interstitial nephritis, namely no rash, fever or arthralgia. Urinary abnormalities were similar in both groups with sterile pyuria and proteinuria. No patients were found to have eosinophiluria. Haematuria was less frequent in the omeprazole group. Four patients in the omeprazole-induced AIN group were found to have a concurrent ascending urinary tract infection, but did not have specific urinary tract symptoms.

The majority of the omeprazole-induced AIN patients were treated empirically with steroids for 3 to 6 months, where as the vasculitis group were treated with cyclophosphamide and steroids initially and subsequently had 6 to 12 months maintenance therapy with either azathioprine or mycophenolate mofetil and low dose prednisone. Of interest was the fact that 88% of the omeprazole group were left with impaired kidney function with a persistently elevated serum creatinine compared to their pre-morbid values. 76% (20/26) (1 patient unknown) of the vasculitis group had impaired renal function post therapy with 4 patients who remained dialysis-dependent.

Eosinophilia and tissue eosinophils

Peripheral blood film eosinophilia has traditionally been a stated feature of drug-induced acute interstitial nephritis. Depending upon what laboratory range is accepted (an upper limit of 0.4 or 0.6 x 10^9/l) 4 patients had evidence of eosinophilia, however, only 1 out of the 4 could possibly be classed as drug-induced eosinophilia as the other 2 had normal values over the next 2 to 3 days in the absence of any steroid treatment and one individual with eosinophilia (5.0 x 10^9/l) had a known background history of a myelodysplastic syndrome with persistent eosinophilia, even after stopping omeprazole. This is striking because in many other drug-induced AIN eosinophilia can be present in up to 80% (see Figure 1).

Histologically most patients with omeprazole-induced AIN had no evidence of any eosinophils. Where eosinophils were detected, numbers were very small and there was only one case with clusters of eosinophils (Figure 11A).

However, in both the omeprazole and vasculitis groups, with the most severe interstitial infiltrate there were scattered eosinophils with 3 to 7 eosinophils evident per high powerfield.

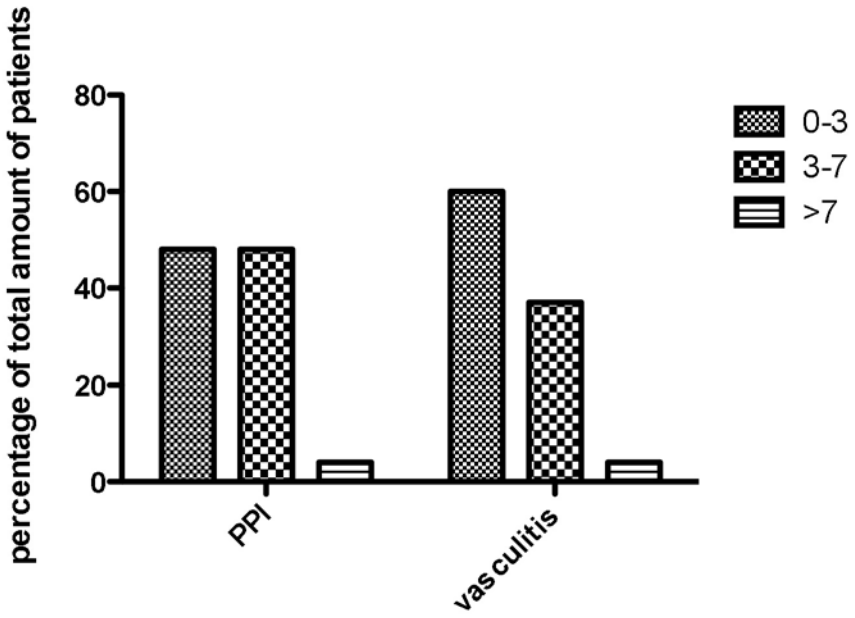


Figure 11A: Patients in the omeprazole-induced AIN group show smiliar

amount of eosinophils in the interstitial infiltrate compared to the vasculitis group. Three categories: No (0-2) eosinophils per high powerfield, scattered (3-7) eosinophils per high powerfield and clusters of (more than 7) eosinophils per high powerfield.

In our study we only had one omeprazole-induced AIN case where we truly found clusters of eosinophils on the HE stain of the renal biopsy as well eosinophilia in the serum.

Concomitant medications

Multiple drug use may confound the aetiology of drug-induced AIN. Non-steroidals, frusemide and bendrofluazide are reported in the literature as a cause of AIN (Toto, 1990). In only 2 patients was there concomitant NSAIDS use at the same time as the PPI. Classically NSAID induced AIN presents with nephrotic range proteinuria and usually normal renal function. In both patients there was no significant proteinuria or any other features to suggest NSAIDS as a possible cause for the AIN.

Four patients were on a diuretic either bendrofluazide or furosemide. Classically these drugs produce the typically allergic type AIN with an associated skin rash. These agents were not stopped and the AIN resolved with withdrawal of the omeprazole and steroid therapy.

ANCA status

There have been isolated case reports of ANCA-positive cases with omeprazole (Singer et al., 1994, Jacobs-Kosmin et al., 2006), however almost all these cases were prior to the availability of PR3 and MPO assays and are clearly false positive results. With the exception of one case, all of our cases of omeprazole-induced AIN were ANCA negative (Table 4). We had only one cANCA positive case on peripheral blood film immunofluorescence (presentation was prior to the advent of PR3 and MPO titers) but there was no clinical evidence to support any other

diagnosis. As expected in the majority of the vasculitis group the PR3-ANCA and the MPO-ANCA titers were strongly positive.

3.2 Histological findings

Inflammation in the omeprazole induced AIN and the vasculitis group

Summary of H&E findings

In the omeprazole induced AIN and the vasculitis group the overall inflammation was semi-quantified by scoring the type of main infiltrate. In both groups the predominant pattern of cellular infiltrate was a mononuclear infiltrate where lymphocytes, macrophages and plasma cells were the pre-eminent cell type, with only occasional neutrophil and eosinophil present (76% (AIN) versus 78% (vasculitis)). To a much lesser extent, some biopsies demonstrated a mixed inflammatory cell picture where there was a similar degree of mononuclear and polymorphonuclear infiltrate present within the lesion (19% (AIN) versus 18% (vasculitis)). There was one case in both groups (omeprazole-induced AIN and vasculitis) where the pattern was a predominant granulomatous infiltrate. In 3 patients there was a predominant polymorphonucleocyte pattern evident. When the patients with a concurrent urinary tract infection were excluded, there were no substantial differences in the characteristics of the interstitial infiltrate in either the omeprazole-induced AIN or vasculitis groups (Figure 12 A, B&C). Representative histological features are demonstrated here (Figure 13-18).

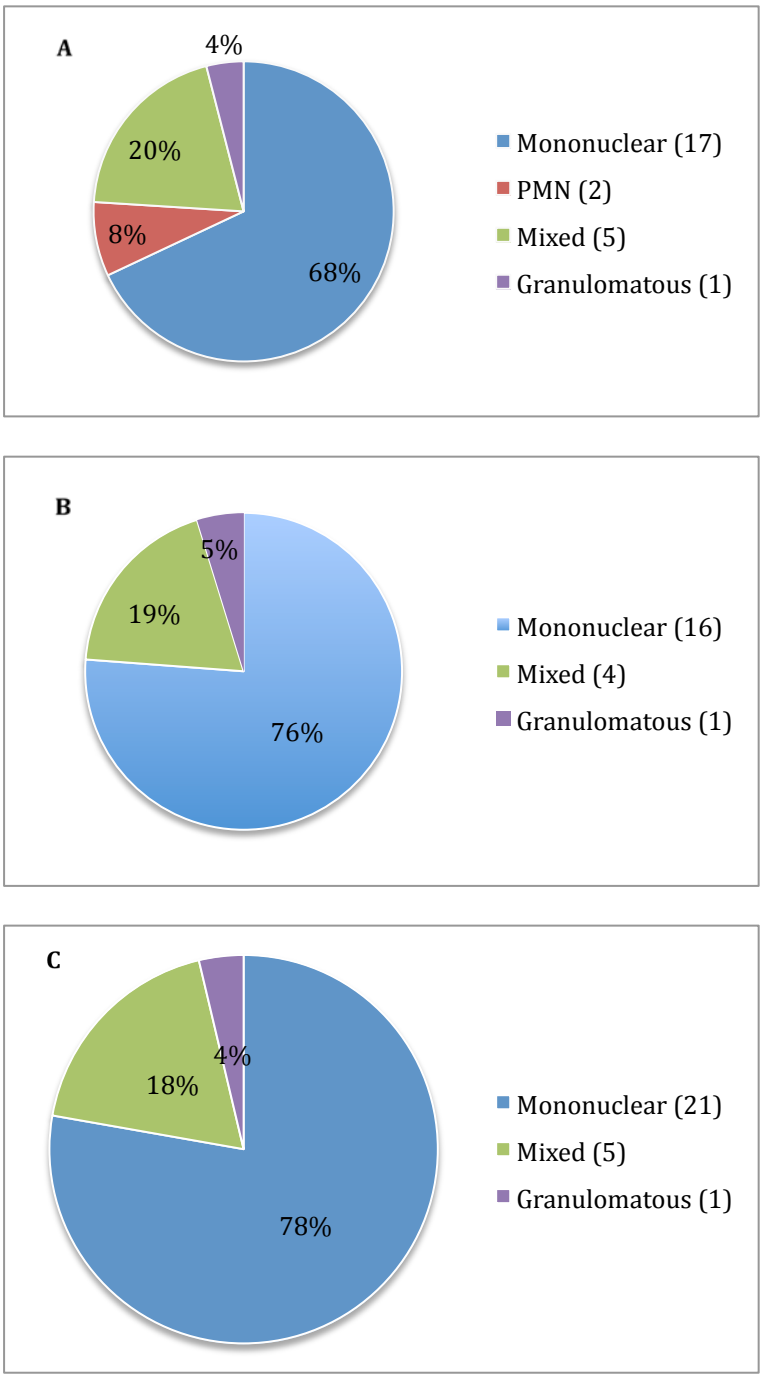


Figure 12A, B&C: The interstitial infiltrate in omeprazole-induced AIN (A), in omeprazole induced AIN after exclusion of patients with UTI's (B) and vasculitis (C) differentiated into main groups of the most presenting cell type: mononuclear, polymorphonuclear, mixed when there was a similar degree of mononuclear cells and polymorphnuclear infiltrate present) and

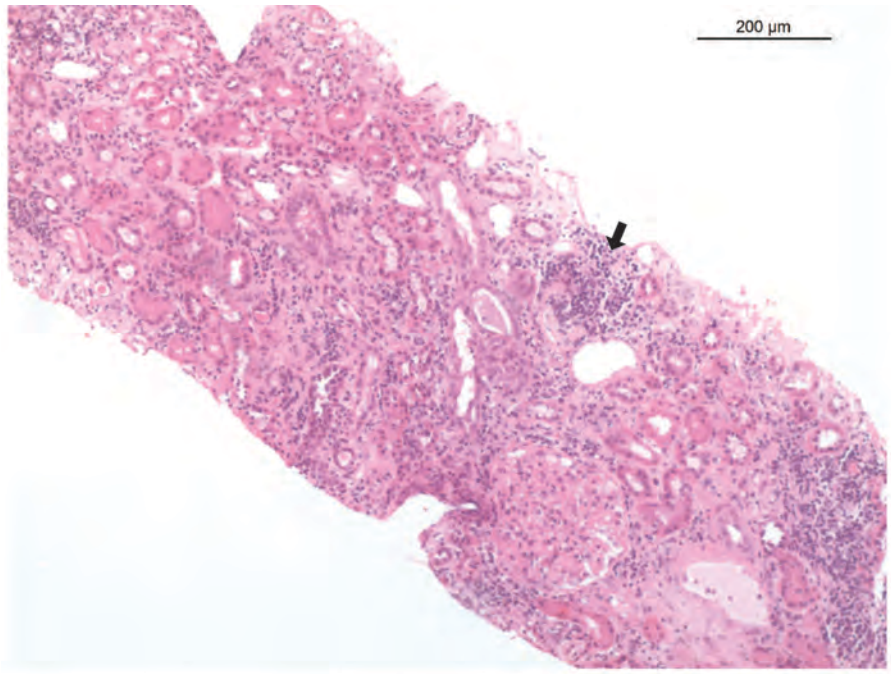


Figure 13: Low magnification of histological features in a renal biopsy of a patient with omeprazole-induced AIN. Tubulointerstitial compartment with lymphoplasmatic infiltrate, some scattered eosinophils and neutrophils (H&E, x100). Black arrow highlights a lymphocyte agreggate.

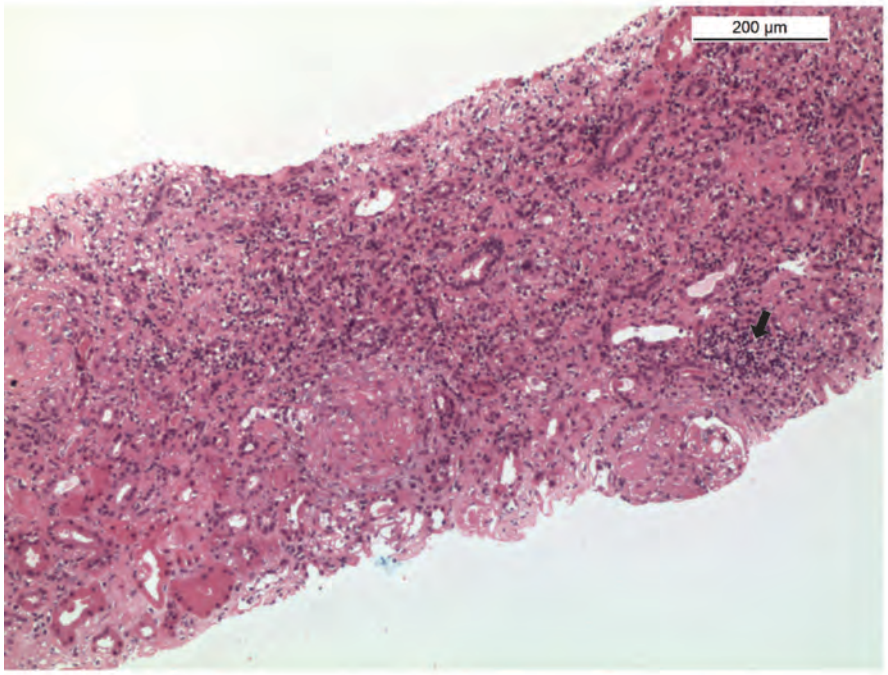


Figure 14: Low magnification of histological features in a renal biopsy of a vasculitis patient. Tubulointerstitial compartment with lymphoplasmatic infiltrate, some scattered eosinophils and neutrophils (H&E, x100). Black arrow highlights a lymphocyte agreggate.

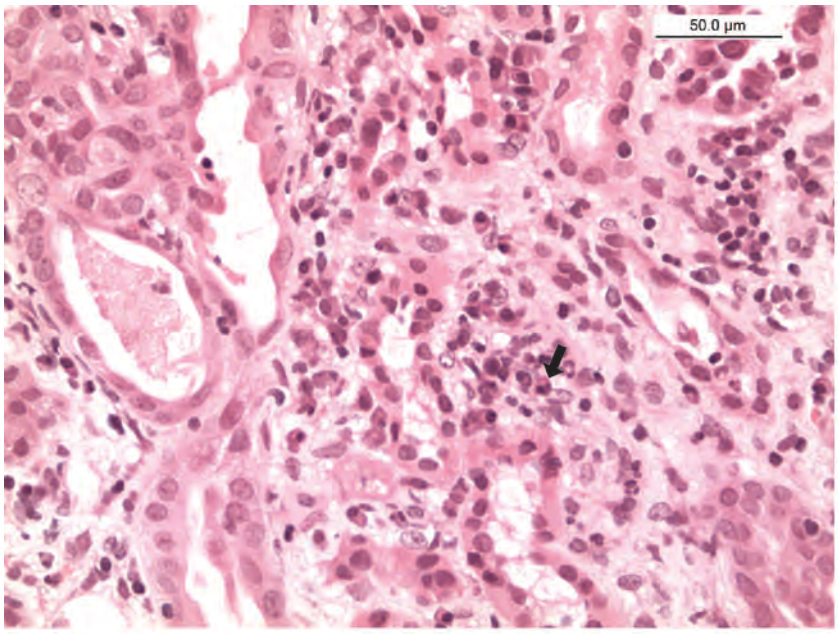


Figure 15: High magnification of histological features in a omeprazole-induced AIN case with tubulointerstitial compartment with lymphoplasmatic infiltrate, some scattered eosinophils and neutrophils. Black arrow highlights an eosinophil (H&E x400).

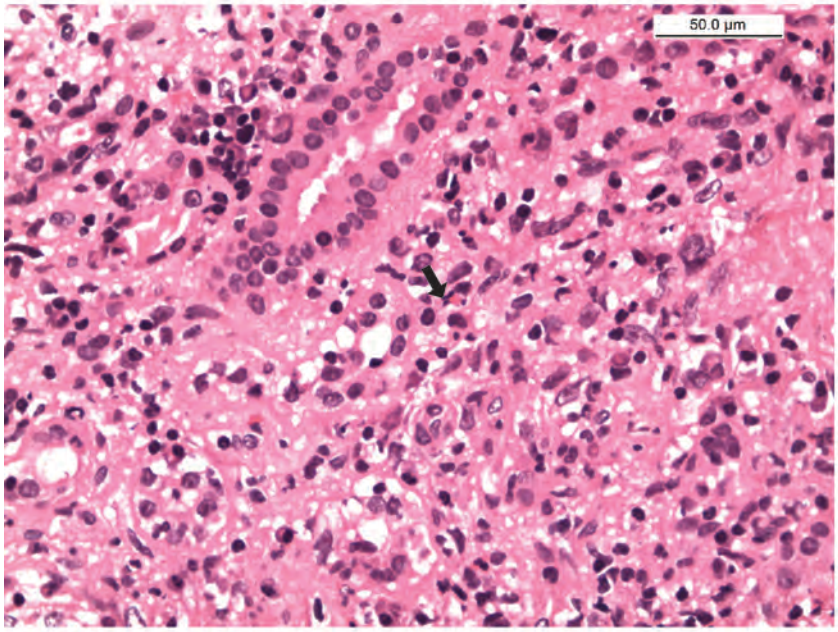


Figure 16: High magnification of histological features in a vasculitis case with tubulointerstitial compartment with lymphoplasmatic infiltrate, some scattered eosinophils and neutrophils. Black arrow highlights an eosinophil (H&E x400).

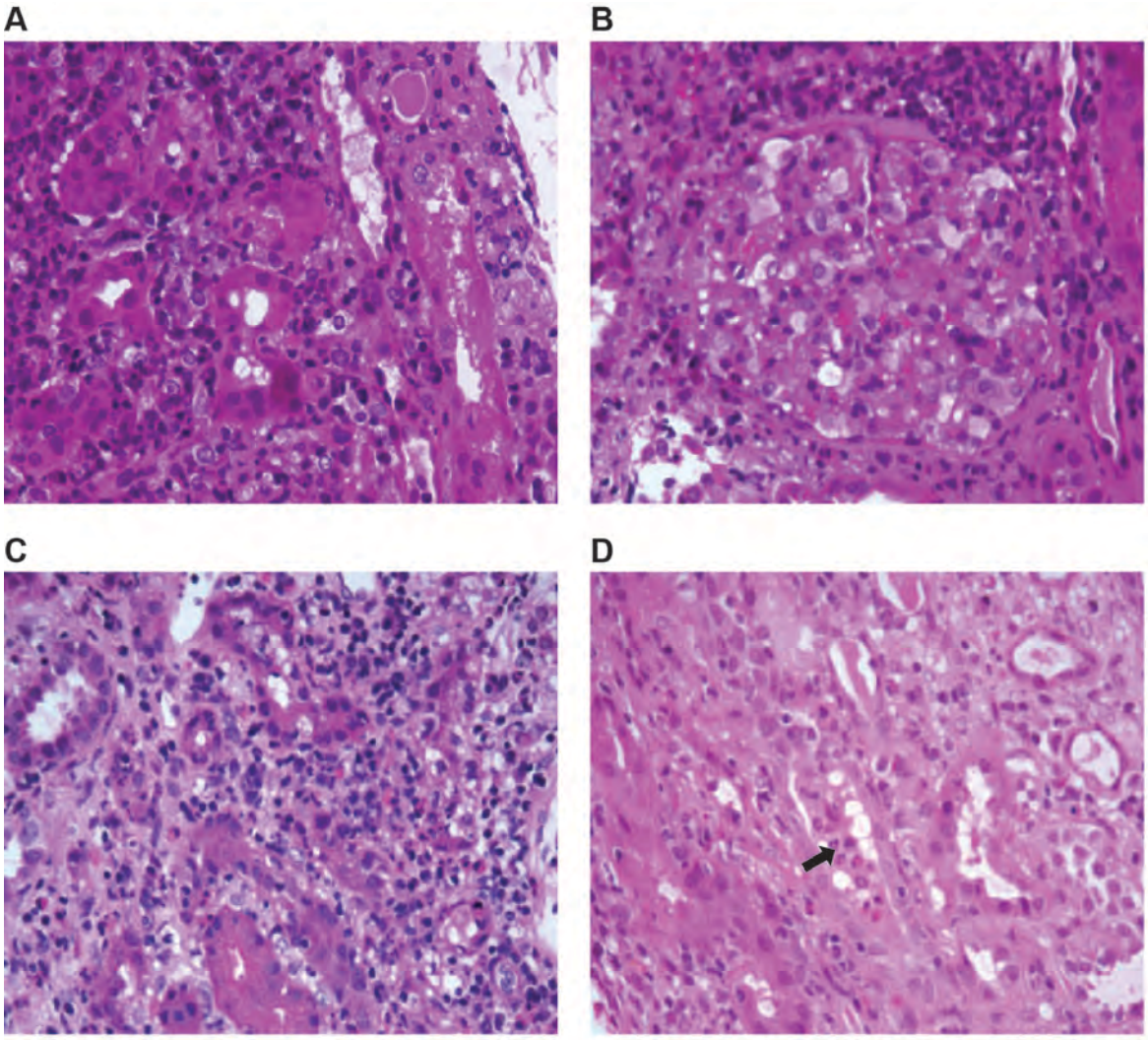


Figure 17: Histological features in omeprazole-induced AIN cases. A Polymorph, plasma and myelodysplatic cells in a patient with background history of myelodysplastic syndrome (H & E x400). **B** Mononuclear infiltrate in the glomerulus in the same patient with myelodysplatic syndrome. **C** Mixed interstitial nifiltrate with lymphocytes, plasma cells and polymorphs including a neutrophilic abscess and eosinophils in a patient with a concommitant UTI (H&E, x400). **D** Intratubular eosinophils almost forming a cast, the case with the most eosinophils (H&E x400). Black arrow highlights the intratubular eosinophils.

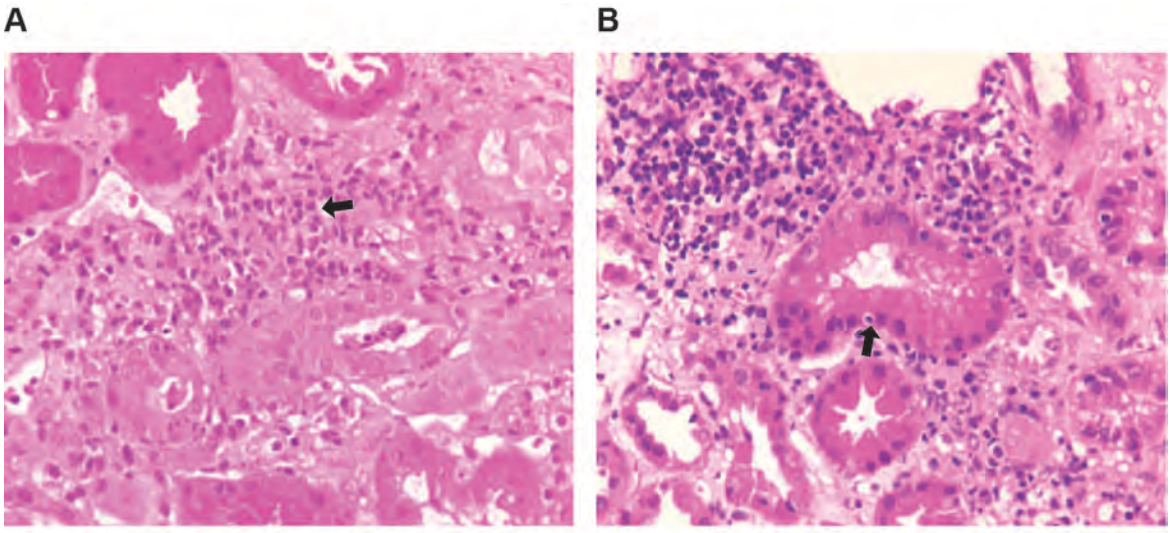


Figure 18: Histological features in two vasculitis cases. A Second example with tubulointerstitial compartment with lymphoplasmatic infiltrate (highlighted by the black arrow), some scattered eosinophils and neutrophils at higher magnification (H&E, x400oil). **B** Lymphoplasmatic infiltrate with tubulitis (H&E, x400 oil). Black arrow highlights the tubulitis.

In order to semi-quantify the extent of inflammation, tubular injury (tubulitis), tubular damage and fibrosis histologically, we utilized the Banff criteria, for the reasons stated in the methods section, and found a very similar spectrum of severity of infiltrate in both groups (Figure 19). At least 60% and more of both patient groups had evidence of severe inflammation. There were no patients without tubulitis (t0) in omeprazole-induced AIN group versus 7% in the vasculitis group, minimal tubulitis (t1) in 56% of the omeprazole-induced AIN group versus 63% of the vasculitis group, intermediate tubulitis (t2) with 24% of the omeprazole-induced AIN group versus 15% of the vasculitis group and severe tubulitis (t3) in 20% of the omeprazole-induced AIN group versus 15% of the vasculitis group. Likewise the extent of fibrosis and cortical tubular atrophy was similar in both groups (Figures 20 -22). Vessel involvement was not a feature of any of the omeprazole-induced AIN cases and therefore this aspect of the Banff criteria was not analysed.

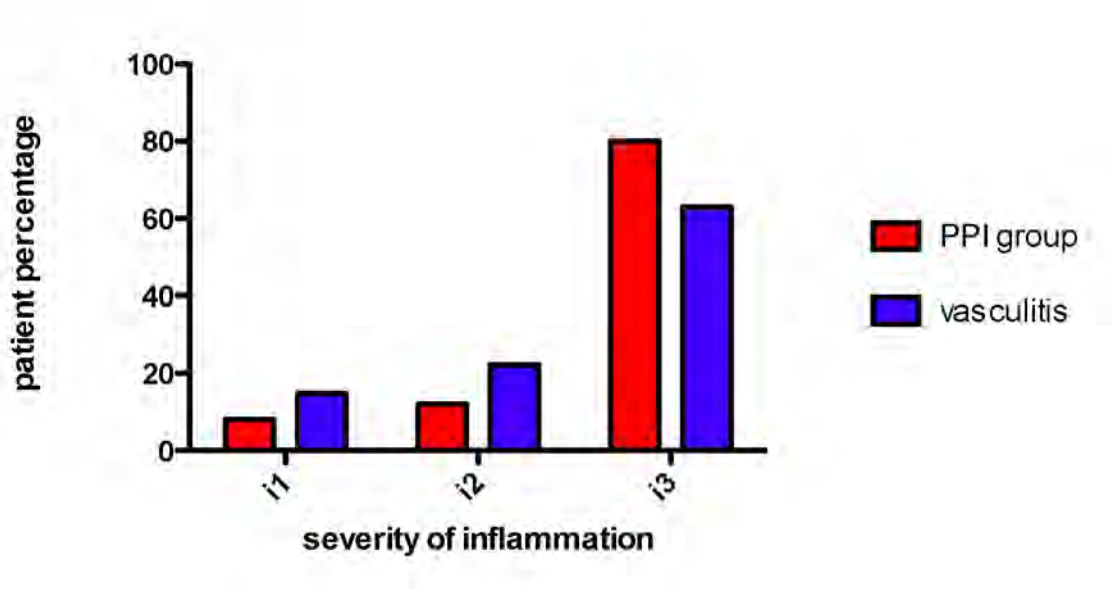


Figure 19: The degree of severity of inflammation (i) is similar in both groups with minimal inflammation of 8% in the omeprazole-induced AIN group versus 15% in the vasculitis group, intermediate inflammation of 12% in the omeprazole-induced AIN group versus 22% in the vasculitis group, severe inflammation of 80% in the PPI-IN group versus 63% in the vasculitis group. Most of the patients had severe inflammation in both groups.

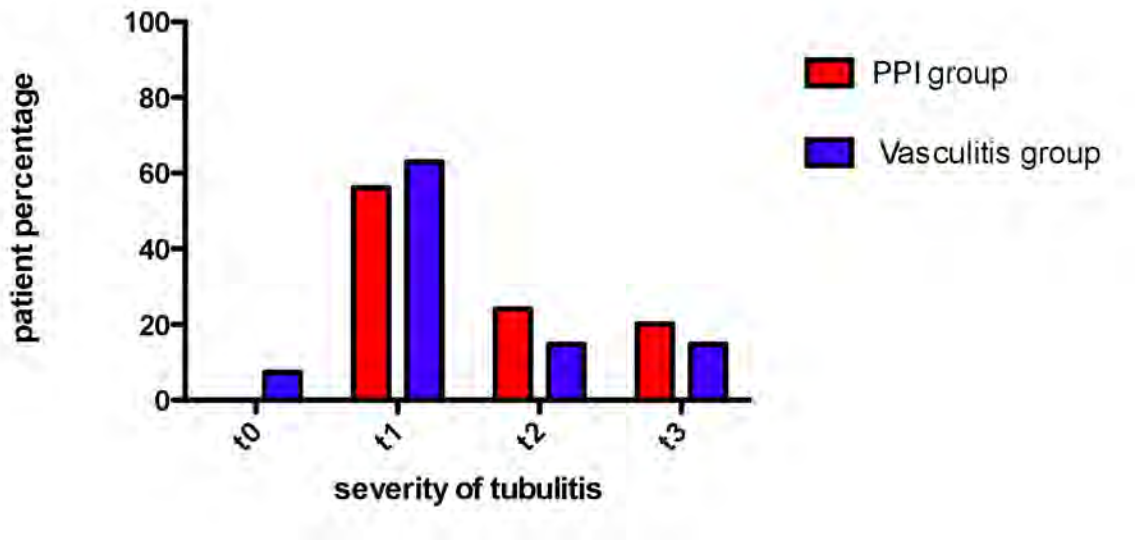


Figure 20: **Severity of tubulitis (t) on H&E grading**. There were no patients without tubulitis in the omeprazole-induced AIN group verus 7% with no tubulitis (t0), minimal tubulitis (t1) in 56% of the omeprazole-induced AIN group versus 63%of the vasculitis gropu, intermediate tubulitis (t2) with 24% of the PPI-IN group versus 15% of the vasculitis group and severe tubulitis (t3) in 20% of the PPI-IN group versus 15% of the vasculitis group.

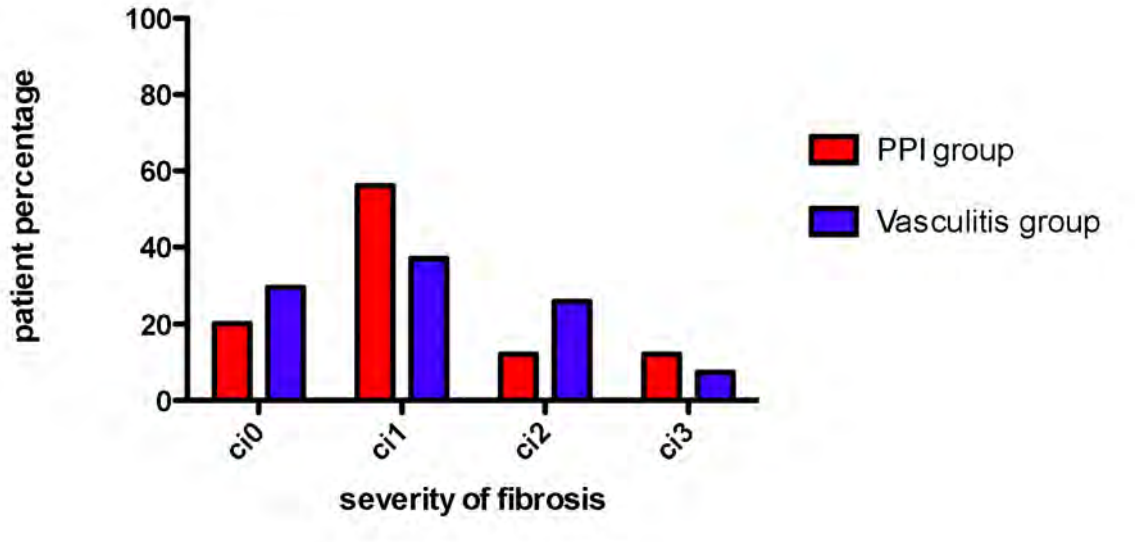


Figure 21: Severity of chronic inflammation (ci) on H&E grading. No fibrosis (ci0) was seen in 20% of the omeprazole-induced AIN group versus 29% of the vasculitis group, minimal fibrosis (ci1) in 55% of the omeprazole-induced AIN group versus 37% in the vasculitis group, intermediate fibrosis (ci2) in 12% of the omeprazole-induced AIN versus 26% and severe fibrosis (ci3) in 12% omeprazole-induced AIN versus 7% of the vasculitis group.

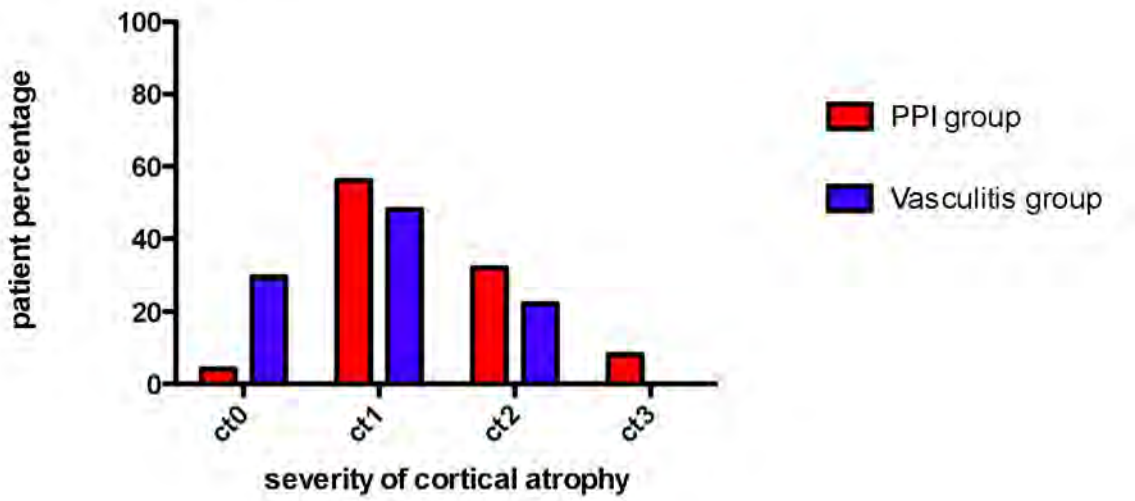


Figure 22: Severity of cortical tubular atrophy (ct) on H&E grading. No cortical atrophy (ct0) was seen in 4% of the PPI-IN group versus 30% of the vasculitis group, minimal cortical atrophy (ct1) was seen in 56% of the PPI-IN group versus 48% of the vasculitis group, intermediate cortical tubular atrophy (ct2) was seen in 32% of the PPI-IN group versus 22% in the vasculitis group and severe cortical tubular atrophy (ct3) was seen in 8% in the PPI-IN group and none in the vasculitis group.

ANCA associated Glomerulonephritis Classification

As expected there was no significant inflammatory involvement of the glomeruli observed in the omeprazole-induced AIN group whereas in the vasculitis group all patients had evidence of glomerular injury (by definition) (Figure 23). Grading the glomerulitis according to the ANCA associated glomerulonephritis classification (Berden *et al.*, 2010) showed 54% of patients with crescents, 21% of patients with focal glomerulonephritis, 14% with sclerosed glomerulonephritis and 11% with mixed glomerulonephritis (Figure 24).

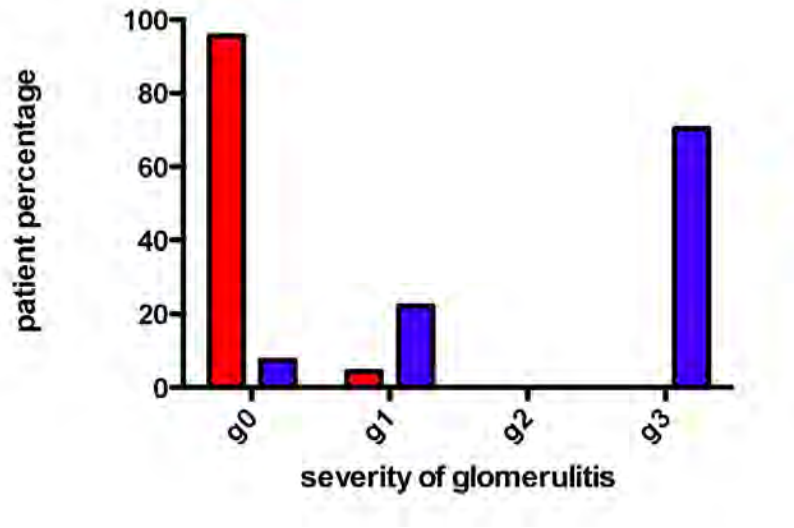


Figure 23: **Severity of glomerulitis (g) on H&E grading.** More severe degree of glomerulitis in the vasculitis group as expected.

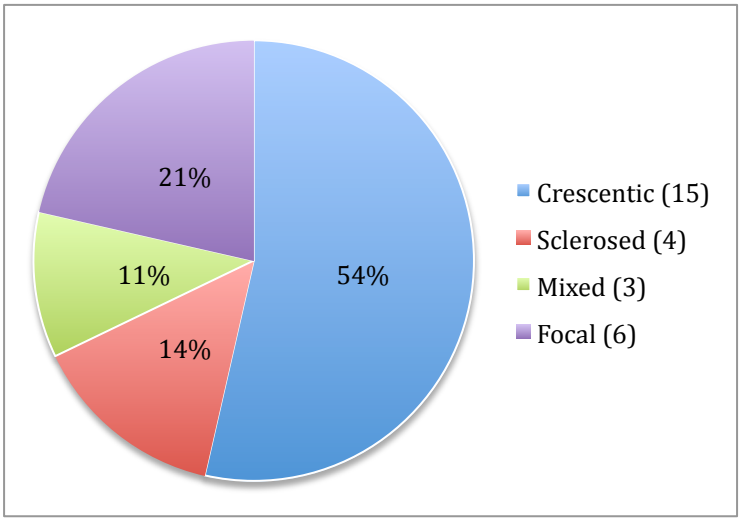


Figure 24: Classification of the vasculitis group according to the ANCA-associated glomerulonephritis classification schema (Berden et al., 2010). 54% with cresentic, 21% with focal, 11% with mixed and 14% with sclerosed glomerulonephritis.

3.3 Immunohistochemistry

CD8 and CD20 staining

Representative renal biopsies stained for CD8 (brown) and CD20 (red) show examples of CD8 and CD20 cells in clusters (Figure 25A,B 26A (omeprazole AIN) and 26B (vasculitis)) and invading the tubules as tubulitis (Figure 27A vasculitis, 27B omeprazole AIN).

A

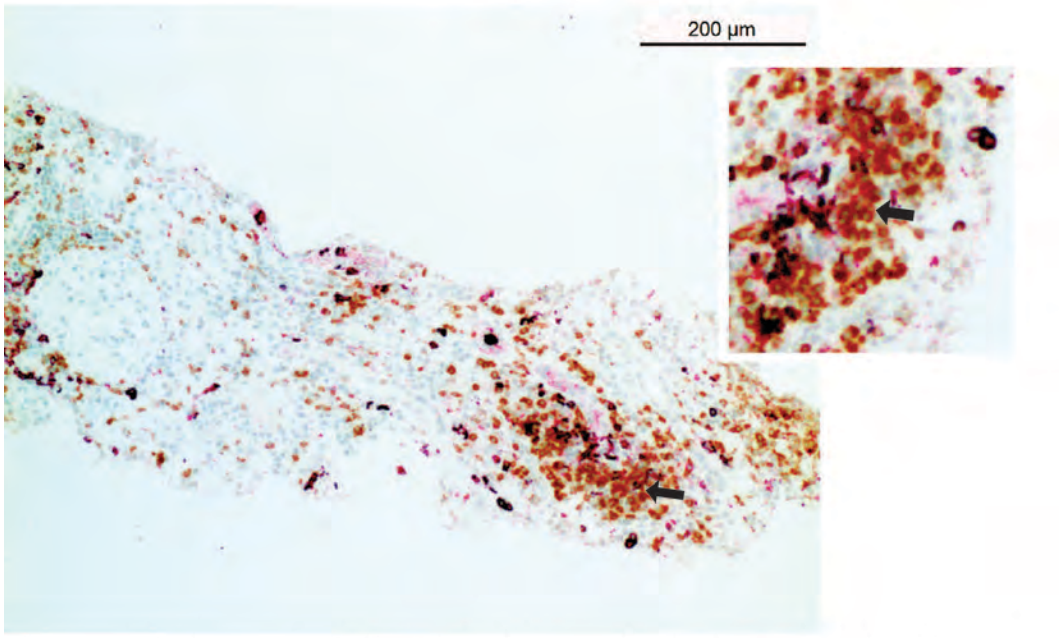


Figure 25A: Low resolution CD8 (brown) clusters and CD20 (red) staining in a renal biopsy of a patient with omeprazole-induced AIN x100. Black arrow highlights the CD8 clusters.

B

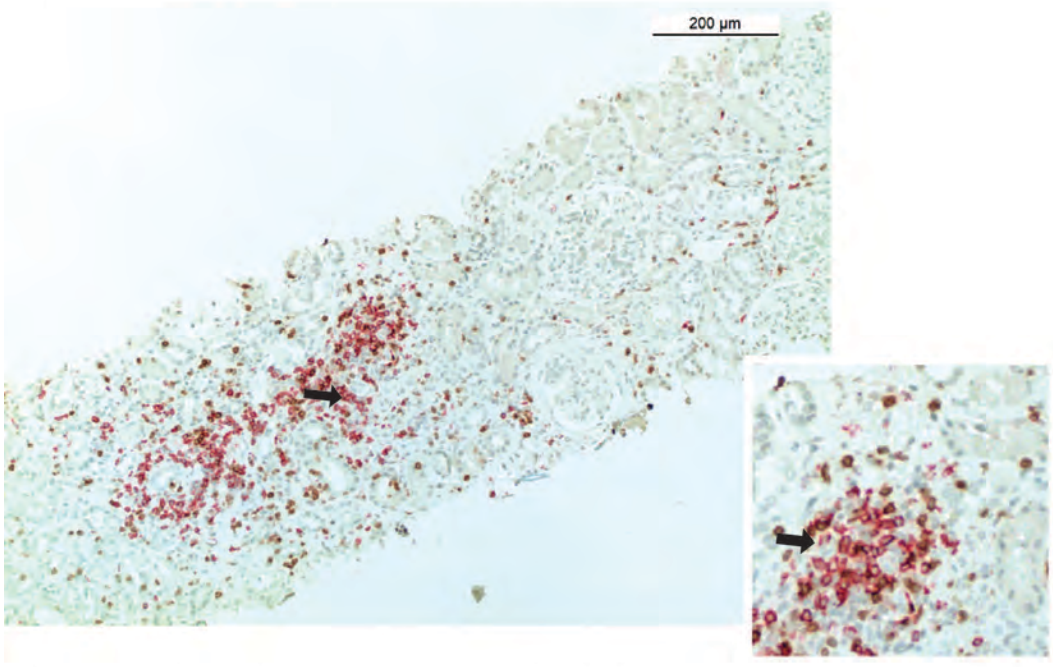


Figure 25B: Low resolution CD8 (brown) staining and CD20 (red) clusters in a renal biopsy of a patient with omeprazole -induced AIN x100. Black arrow highlights the CD20 clusters.

A

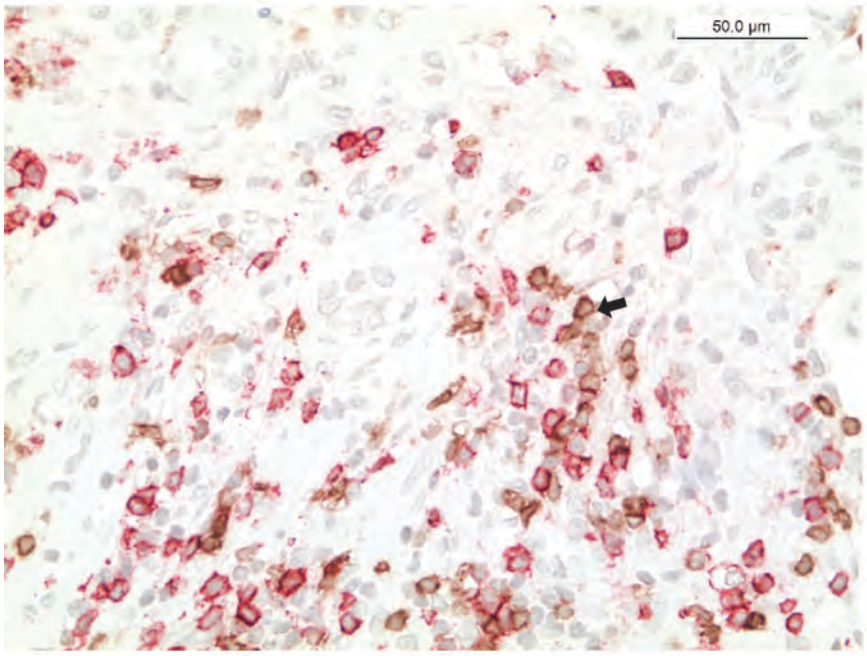


Figure 26A: High resolution CD8 (brown) in clusters and CD20 (red) in a renal biopsy of a patient with omeprazole-induced AIN x400. Black arrow highlights the CD8 clusters.

B

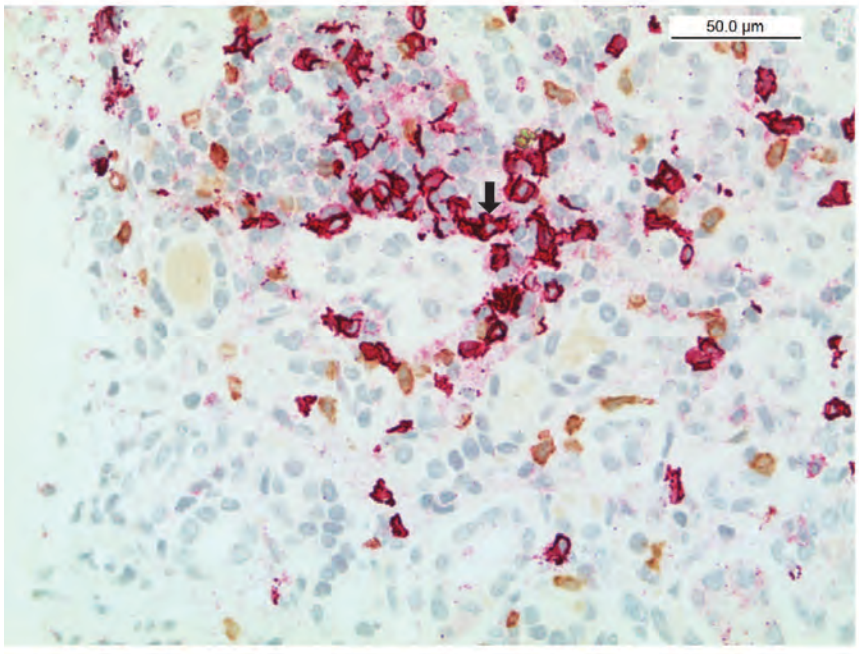


Figure 26B: High resolution CD20 (red) in clusters and CD8 (brown) staining in a renal biopsy of a vasculitis patient x400. Black arrow highlights the CD20 clusters.

A

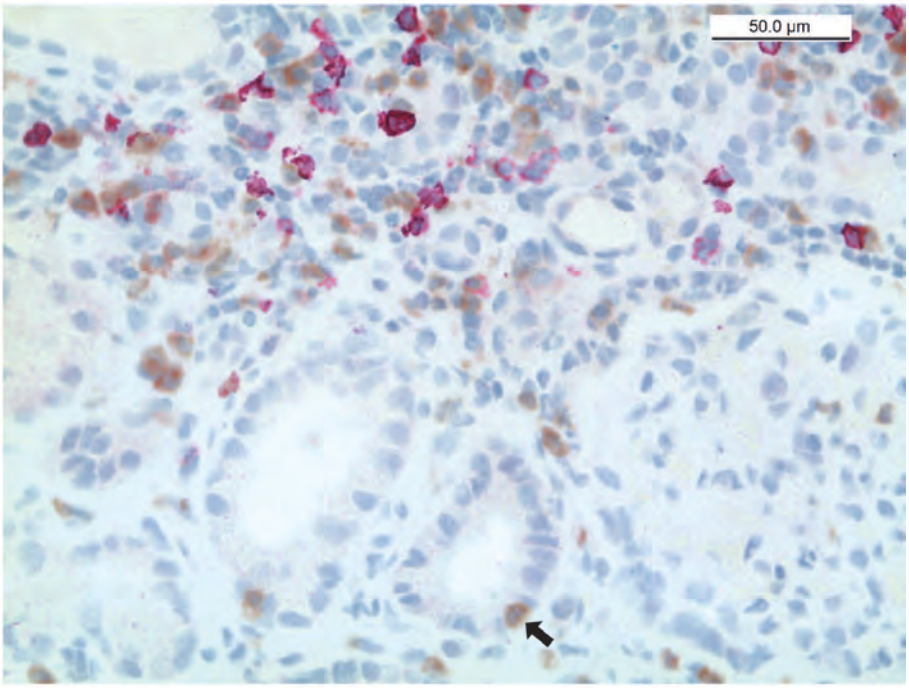


Figure 27A: CD8 (brown) tubulitis and CD20 (red) staining in a vasculitis patient x400. Black arrow highlights the CD8 tubulitis.

B

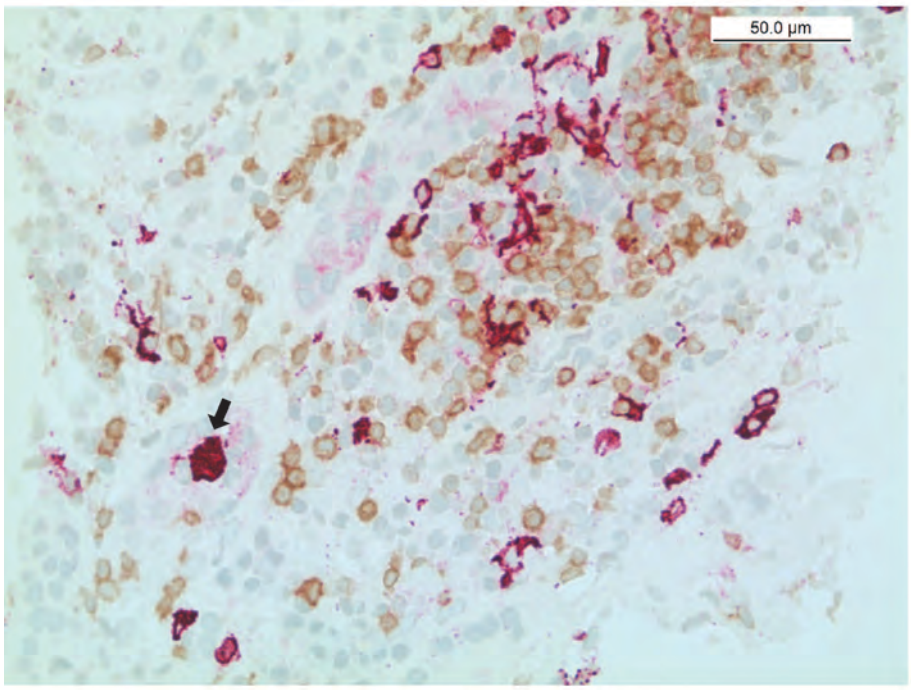


Figure 27B: CD20 (red) tubulitis and CD8 (brown) staining in a omeprazole induced AIN patient x400. Black arrow highlits the CD20 tubulitis.

Semiquantitative analysis CD8

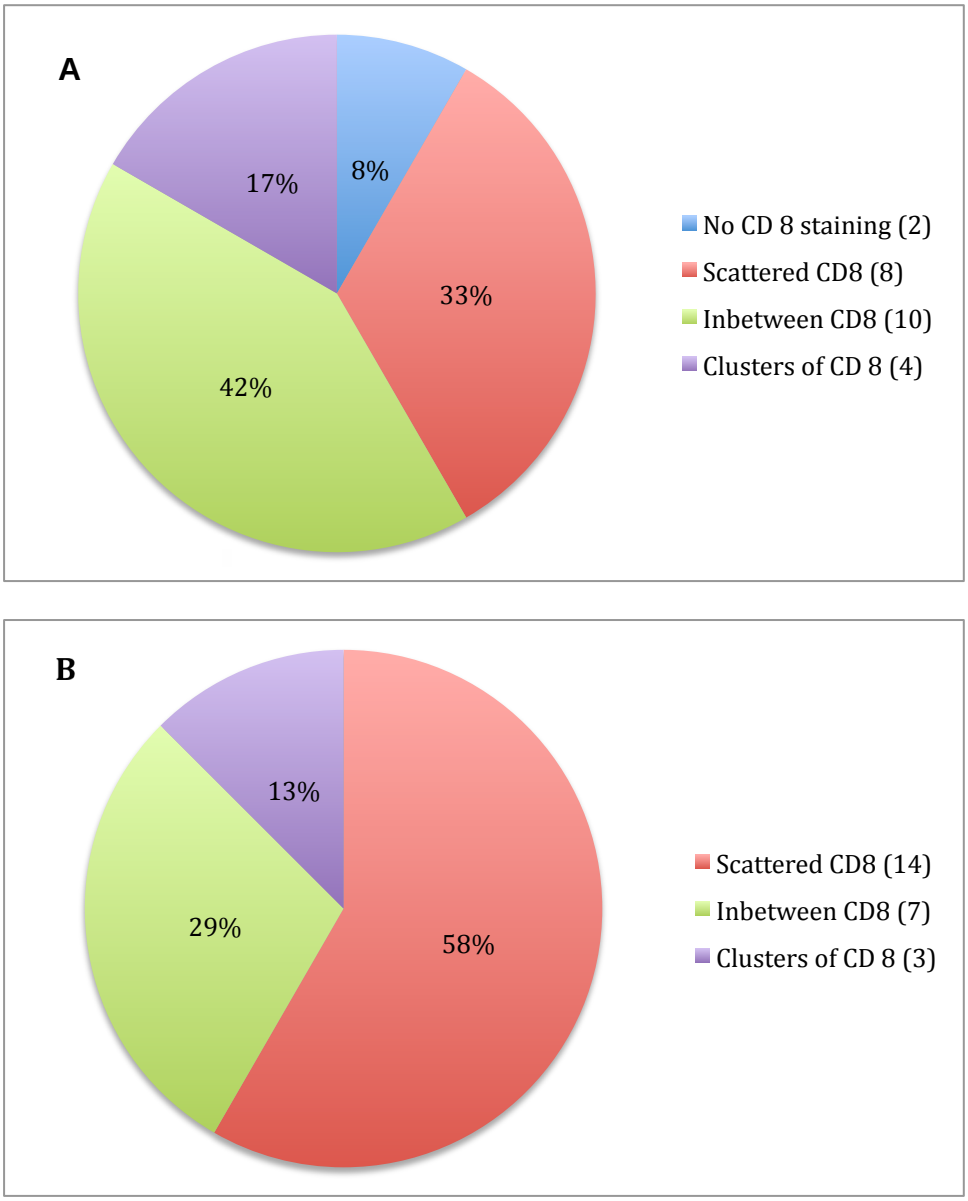
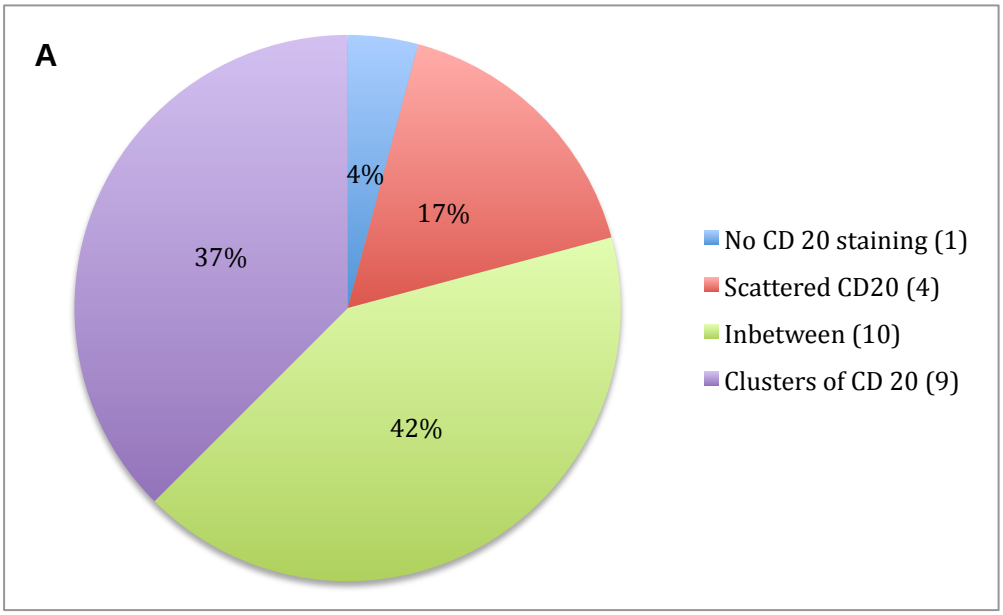


Figure 28A&B: CD8 staining in the omeprazole-induced AIN and the vasculitis group. 2 patients were excluded in the omeprazole-induced AIN and 3 patients in the vasculitis group due to limited availability of tissue.

In conclusion 17% versus 13% had CD8 positive staining in clusters in the omeprazole-induced AIN group and vasculitis group respectively. CD8 is involved but probably not the main driver of the disease.

Semiquantitive analysis CD20



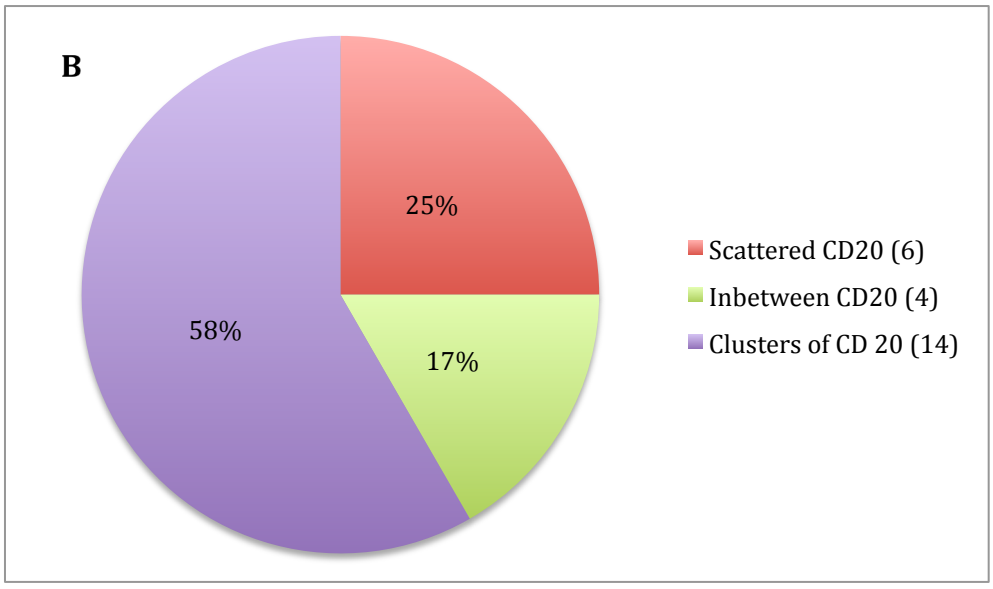


Figure 29A&B: CD20 staining in the omeprazole-induced AIN and the vasculitis group. 1 patient was excluded in the omeprazole-induced AIN and 3 patients were excluded in the vasculitis group due to limited availability of tissue.

There is a considerable amount of CD20 staining present in both groups with 58% versus 37% in clusters in the omeprazole-induced acute interstitial nephritis and vasculitis group respectively. This would support a role for B cells in the inflammatory process.

3.4 Immunofluorescence

CD4 and IL17A staining

To determine the type of T-cell mediated response we undertook double-staining immunofluorescence for CD4 (green) and IL17A (red) to demonstrate examples of a potential Th1-Th17 response. Figure 30 demonstrates a moderate tubulitis (t2 tubulitis according to the Banff-criteria) double-staining for CD4 and IL17A cells in an omeprazole-induced acute interstitial nephritis case. A similar picture is demonstrated in figure 31 where inflammatory cell clusters demonstrate co-staining for CD4 and Th17 in an other omeprazole-induced acute interstitial nephritis case. Of interest, an omeprazole-induced interstitial nephritis case with a concurrent UTI showing a neutrophilic abscess also has clusters of Th17 positive cells (Figure 32). The difficulty however of this stain was to reliably grade the IL17A staining due to problems with high background in the kidney.

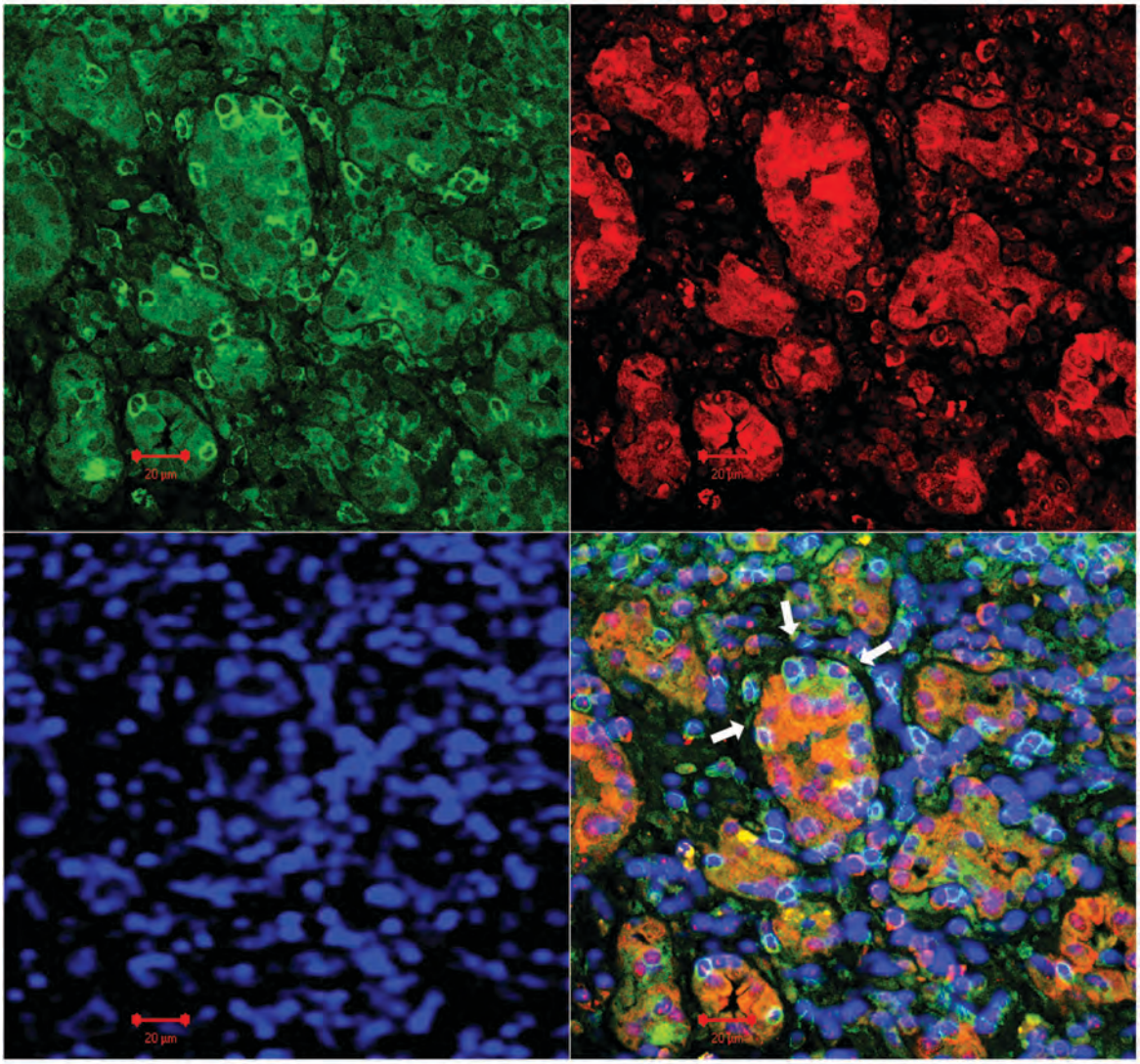


Figure 30: CD4 and IL17A in omeprazole-induced AIN with tubulitis (t2). CD4 (green), IL17A (red), DAPI (blue) and merged image bottom right. The white arrow highlights the tubulitis.

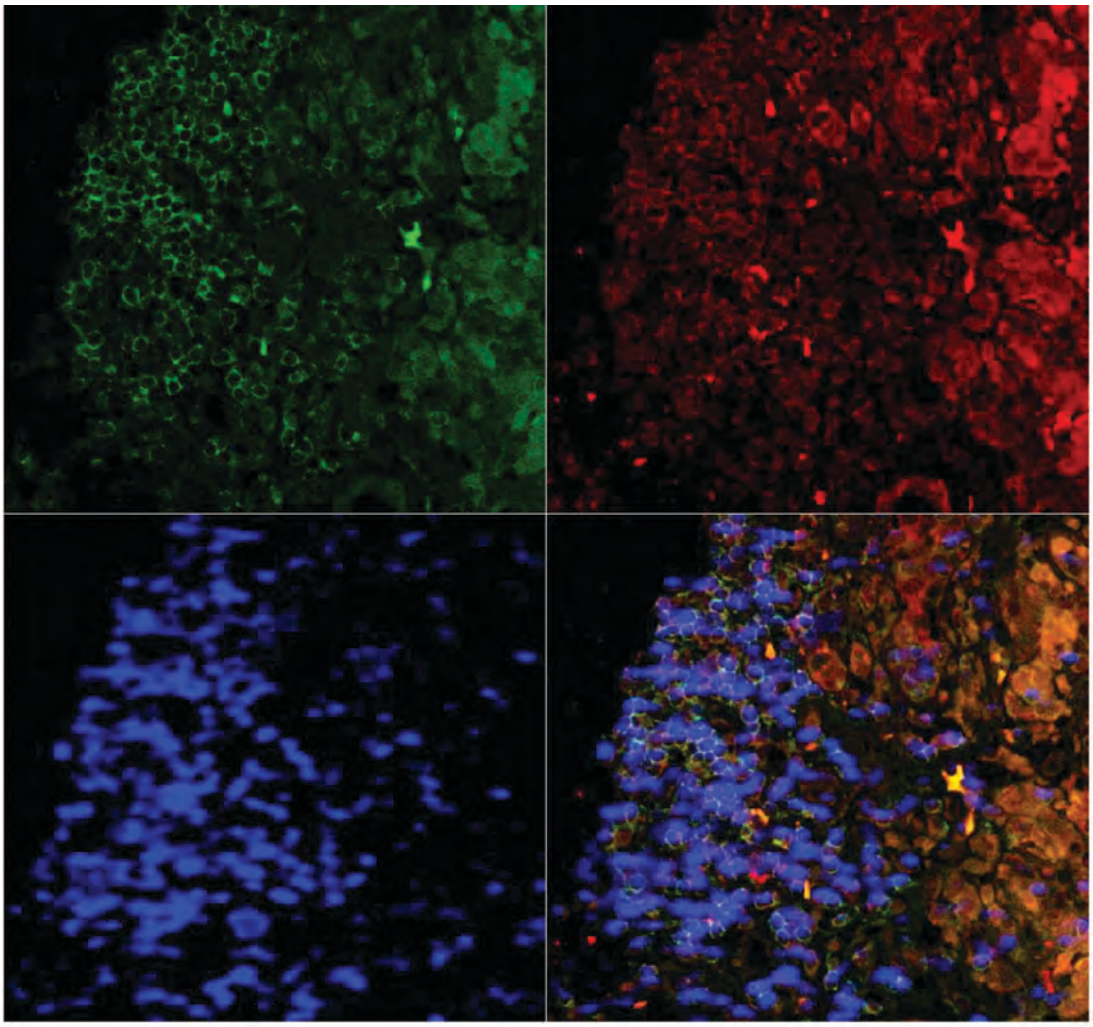


Figure 31: CD4 and IL17A in clusters in an omeprazole-induced AIN case. CD4 (green), IL17A (red), DAPI (blue) and merged image bottom right.

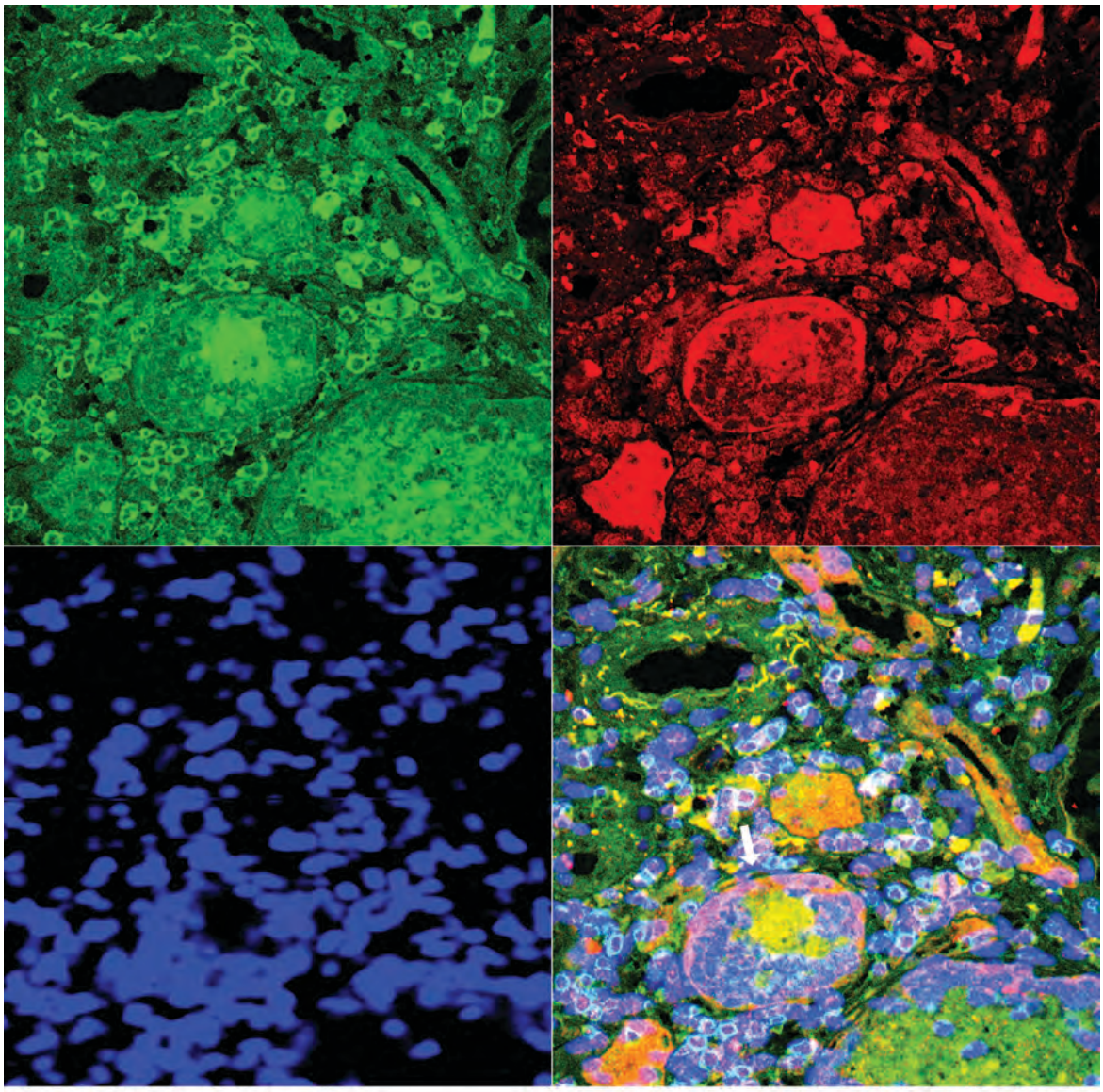
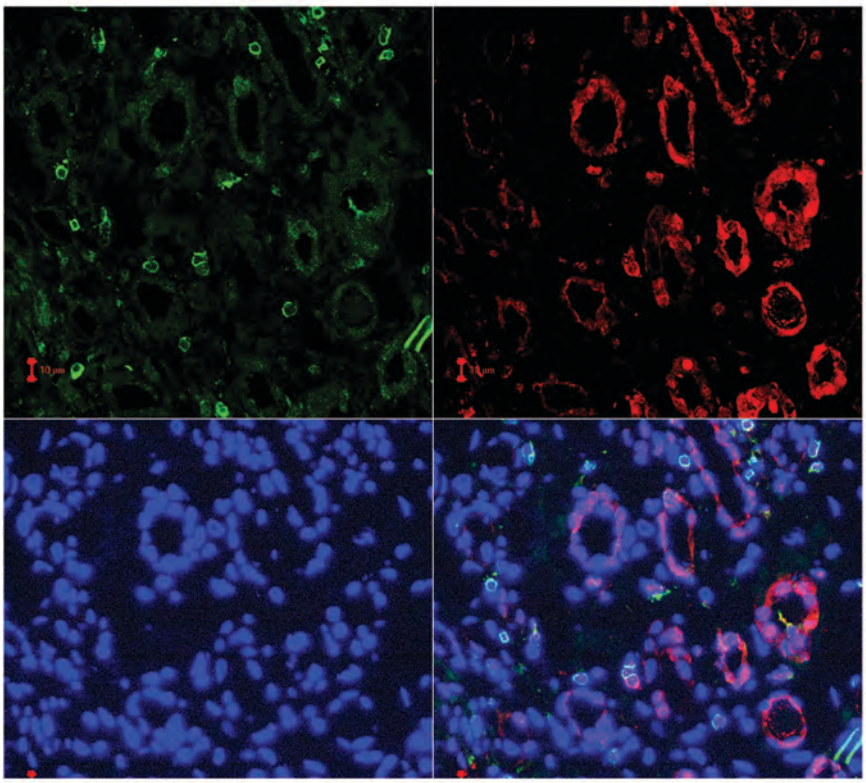


Figure 32: Omeprazole-induced AIN with asymptomatic E.coli UTI. CD4 (green), IL17A (red), DAPI (blue) and merged image bottom right with a tubular abscess. The white arrow highlights the tubular absess

IL17A and CD4 with sudan black

In order to try and reduce autofluorescence and therefore enhance the different immunostaining, sudan black was applied to the slides. Although CD4 still had the characteristic green glow, IL17A lost its red glow, making the grading of the slides even more difficult and unreliable (Figure 33A&B).

A



B

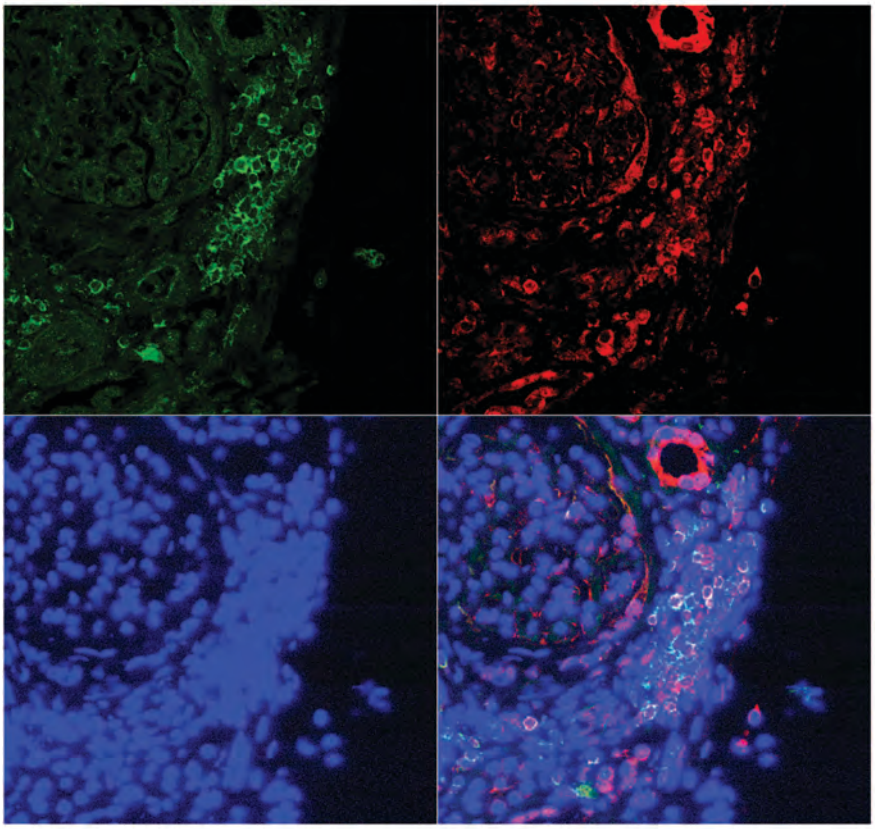
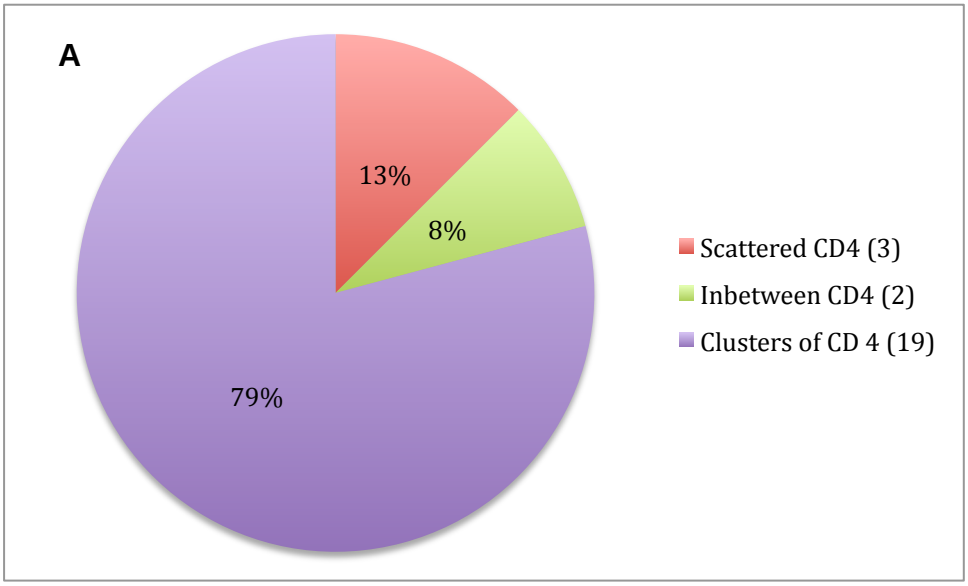


Figure 33A&B: IL17A and CD4 with sudan black, CD4 cells (green) with typical glow, IL17A (red) loosing the glow after administration of sudan black.

Semiquantitive analysis of CD4



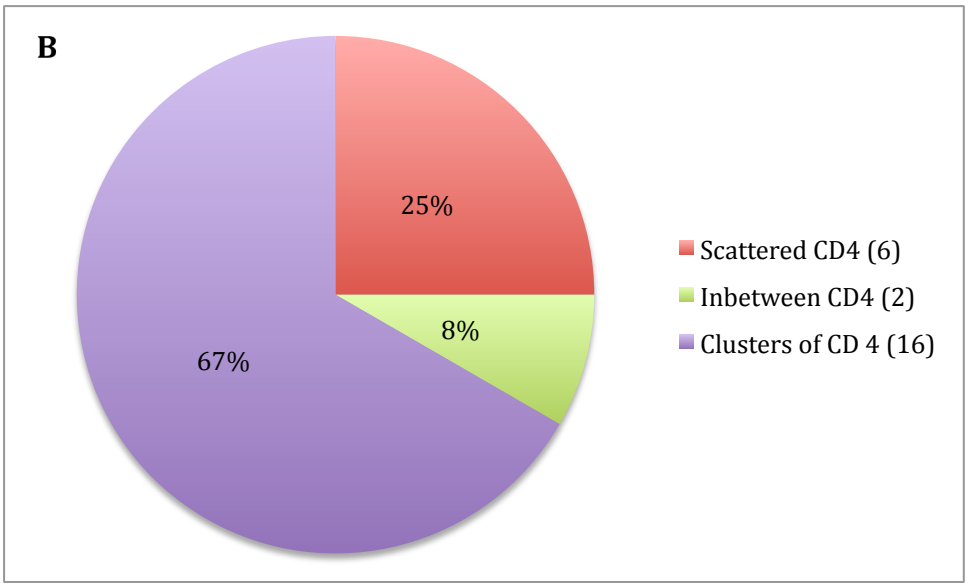
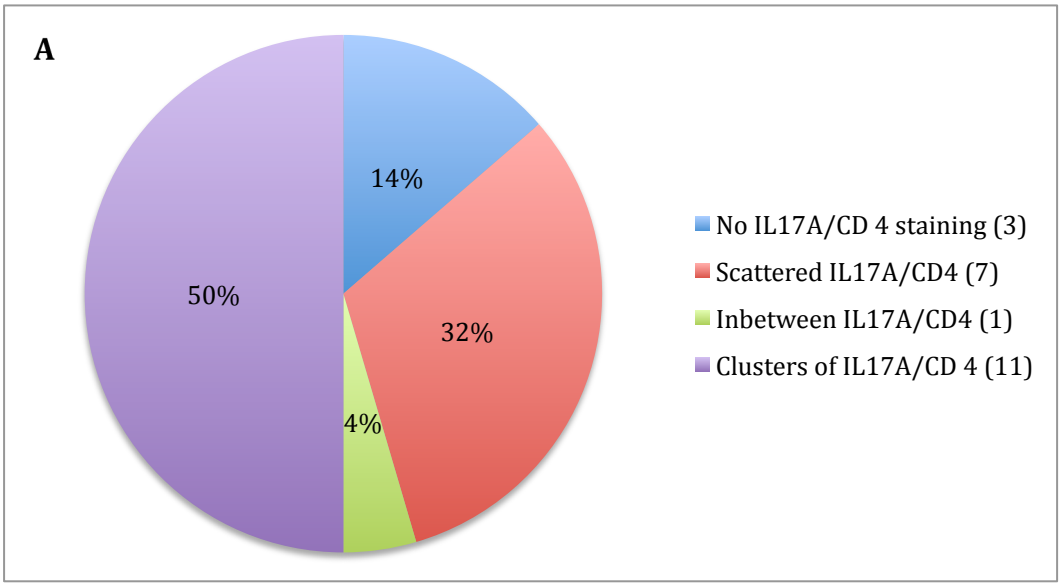


Figure 34A&B: CD4 staining in the omeprazole-induced AIN and the vasculitis group. 1 patient was excluded in the omeprazole-induced AIN and 3 patients were excluded in the vasculitis group due to limited availability of tissue.

CD4 positive cells were present in clusters in 79% of the omeprazole-induced AIN patients and 67% of the vasculitis patients. This supports a cell-mediated inflammatory process.

Semiquantitive analysis of IL17A and CD4



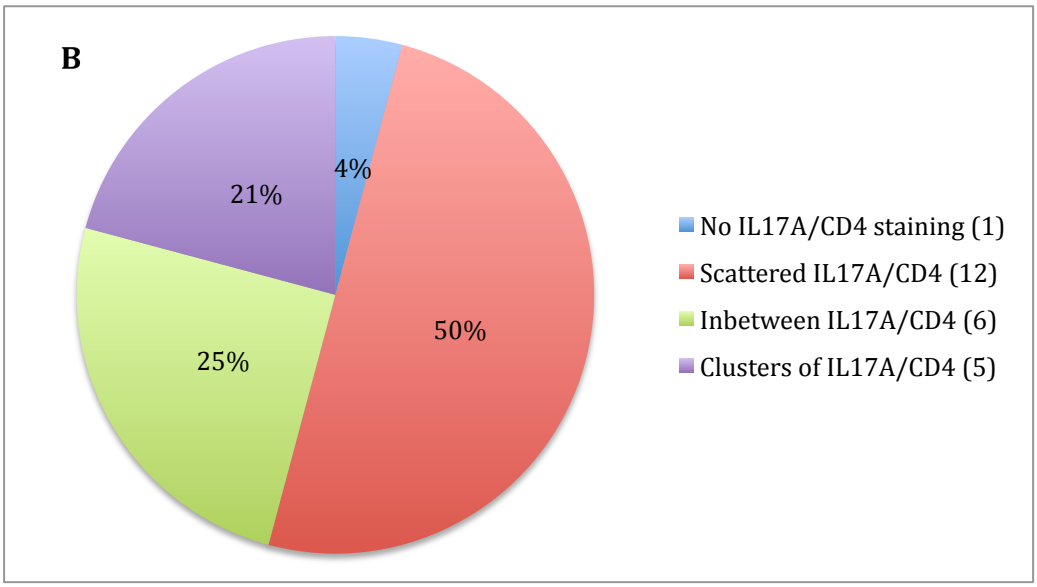


Figure 35A&B: **IL17A and CD4 staining in the omeprazole-induced AIN and the vasculitis group.** 3 patients were excluded in the omeprazole-induced AIN group and 3 patients in the vasculitis group due to limited availability of tissue.

As mentioned above, our IL17A staining was difficult to interpret. Therefore, we chose to stain for CD4 and IL17F to support our findings of role for Th17 cells in omeprazole-induced AIN.

IL17F and CD4 staining

The following renal biopsies stained for CD4 (green) and IL17F (red) demonstrate

examples of CD4 and IL17F cells in clusters in a patient with omeprazole-induced AIN (Figure 36), CD4 and IL17F double staining in an omeprazole-induced AIN case with tubulitis (Figure 37) and a case which demonstrates single staining for CD4 as well double staining for CD4 and IL17F (Figure 38).

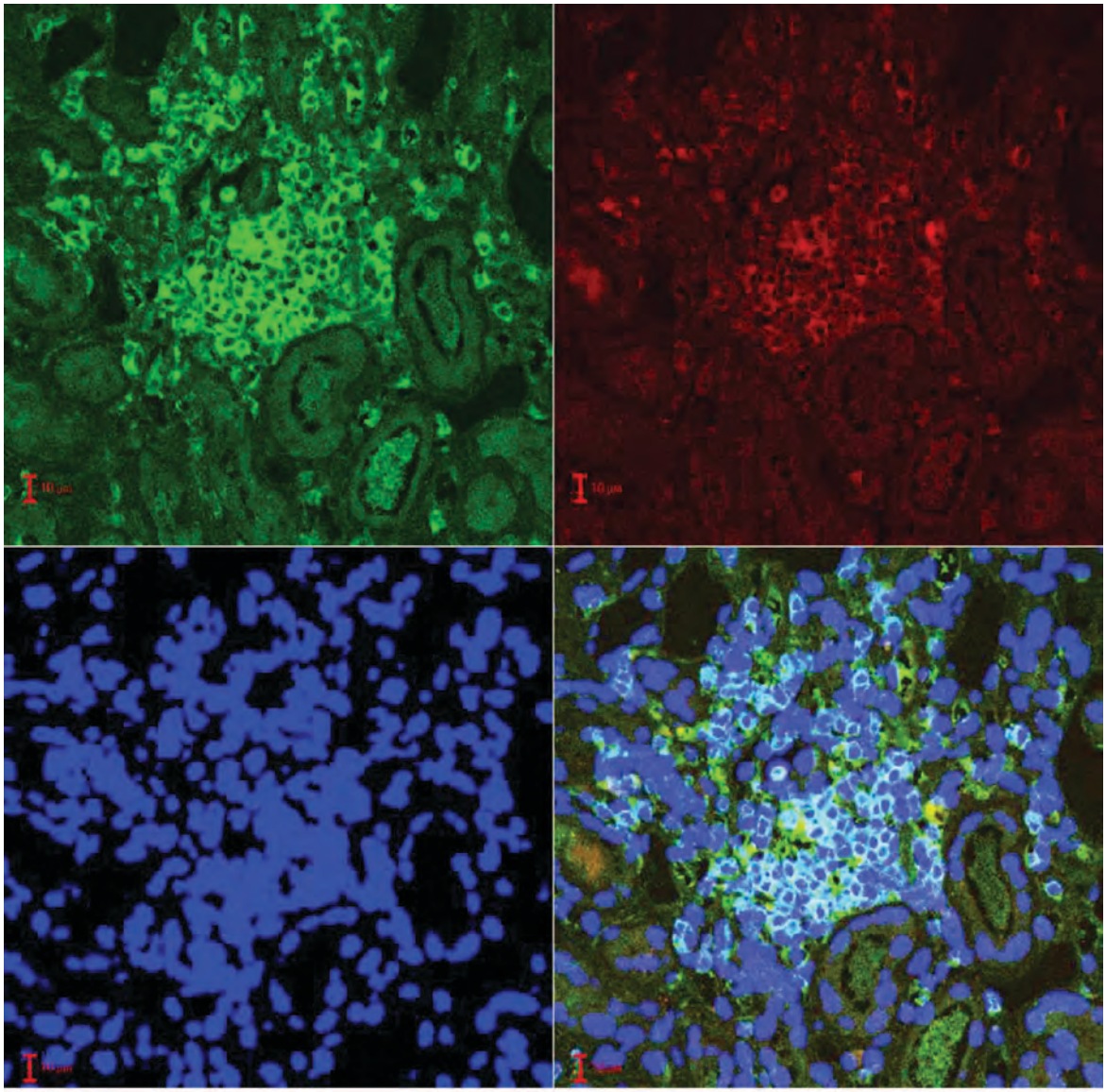


Figure 36: Cluster of IL17F and CD4 double staining in an omeprazole-induced AIN patient. CD4 (green), Il17F (red), DAPI (blue) and merged image bottom right.

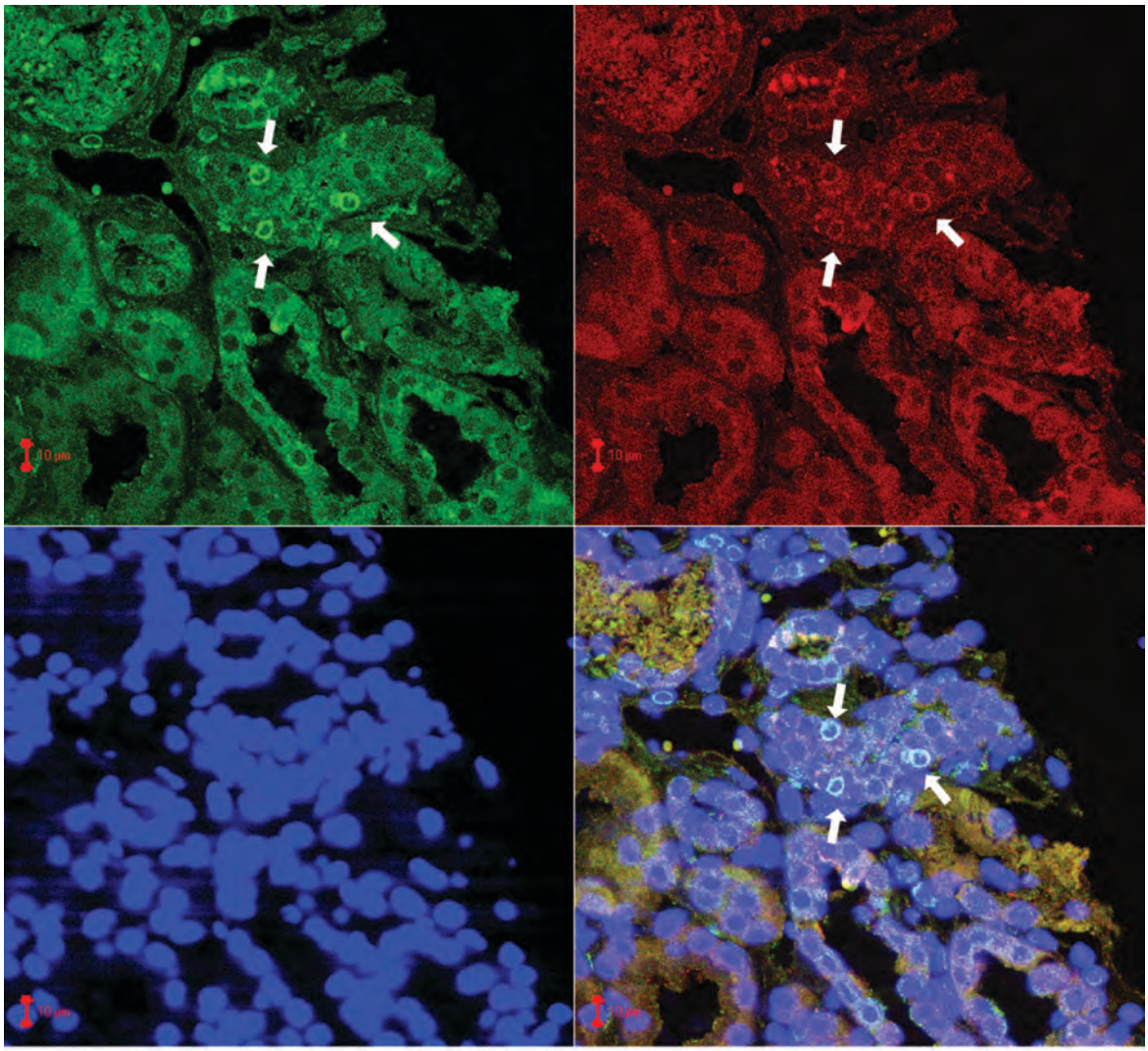


Figure 37: IL17F and CD4 double stained cell tubulitis in a vasculitis case. CD4 (green), IL17F (red), DAPI (blue) and merged image bottom right. Double staining for CD4 and IL17F is highlighted with white arrows.

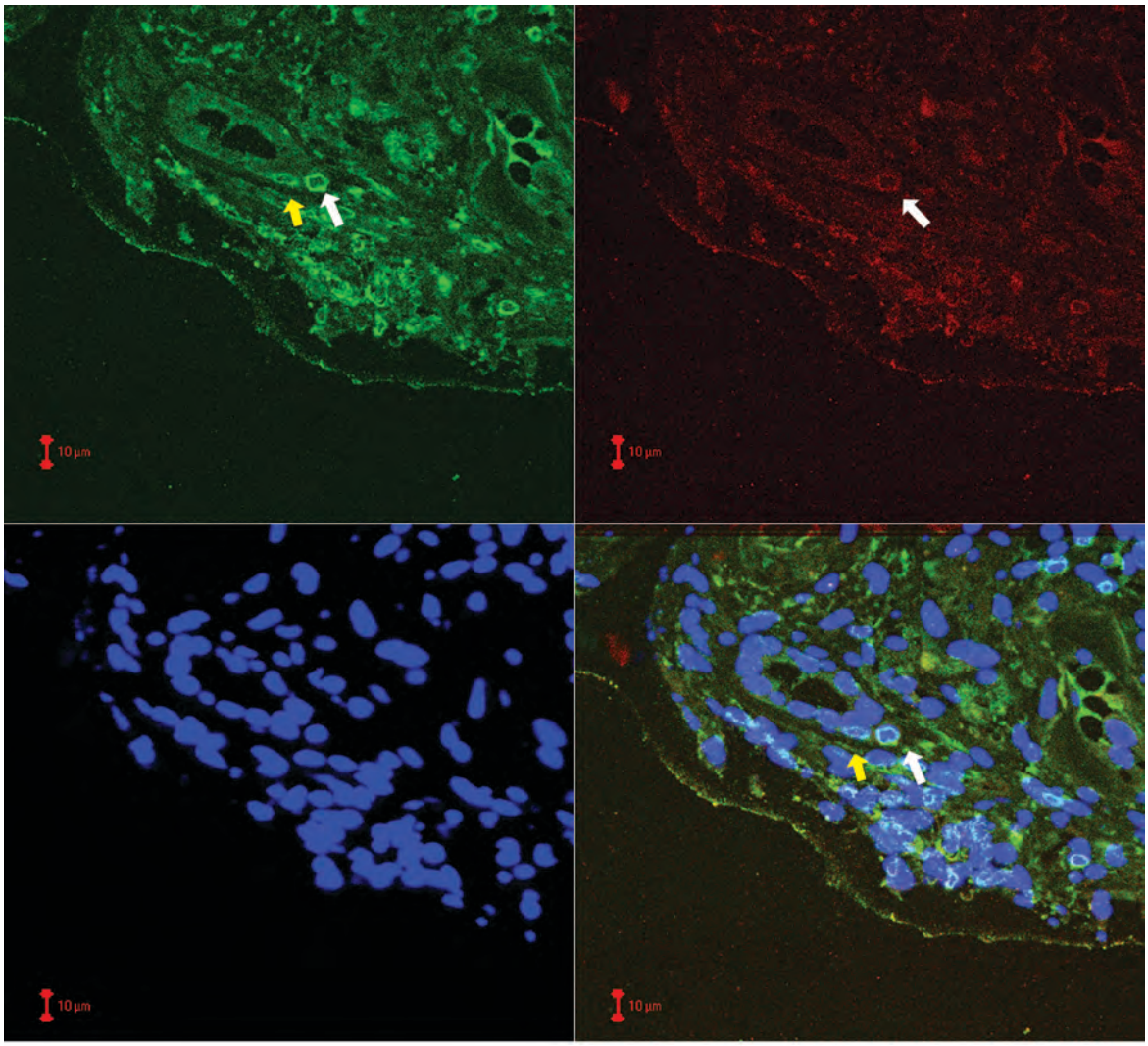


Figure 38: IL17F and CD4 double and single stained tubulitis in an omeprazole-induced interstitial nephritis patient. CD4 (green), IL17F (red), DAPI (blue) and emerged image bottom right. The yellow arrow highlights the single staining, the white arrow the double staining.

Semiquantitive analysis of IL17F and CD4

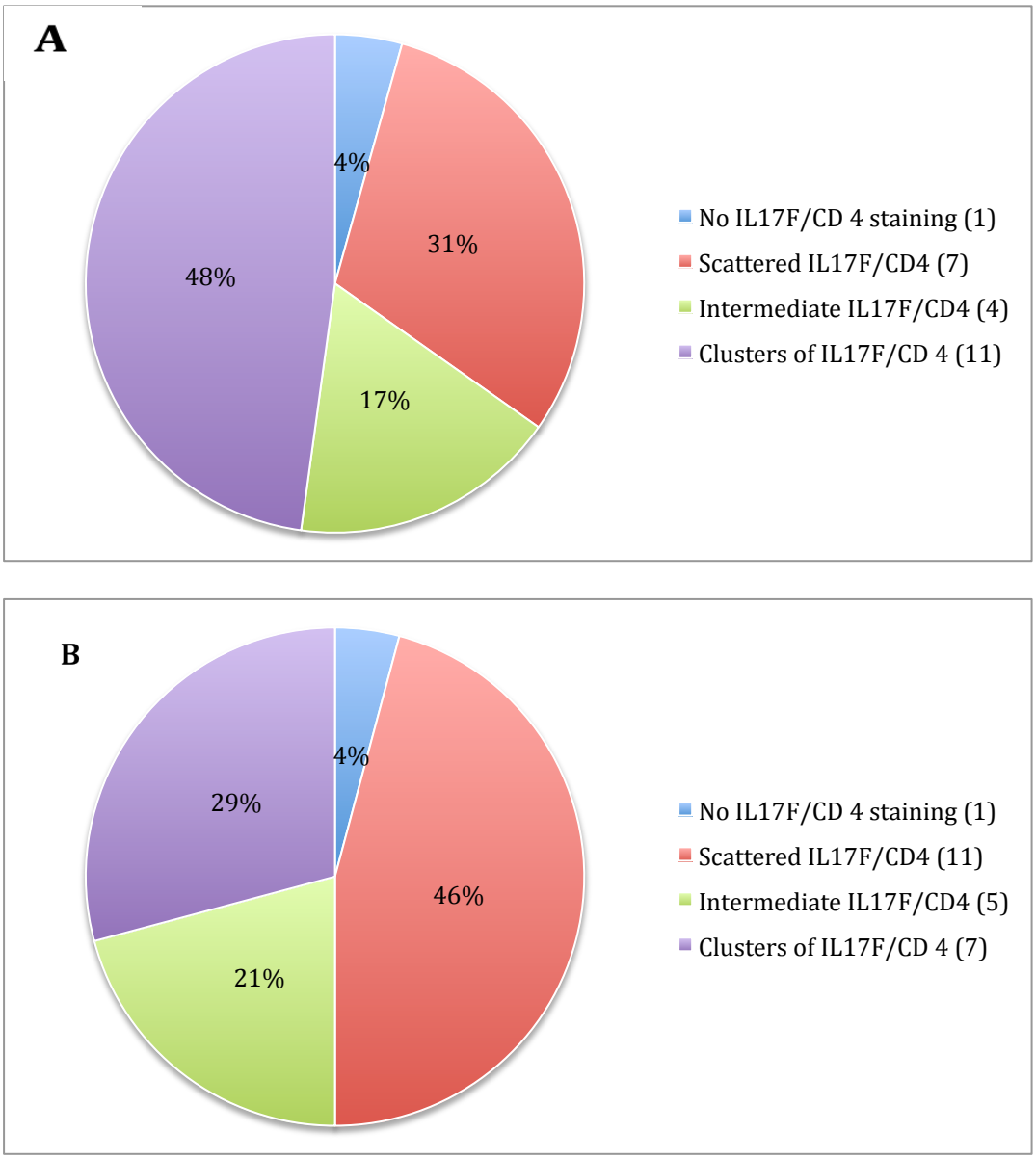


Figure 39A&B: IL17F and CD4 staining in the omeprazole-induced acute interstitial nephritis and vasculitis group. 2 patients had to be excluded from the omeprazole-induced acute interstitial nephritis and 3 patients from the vasculitis group due to limited tissue availability.

Comparing the IL17A and CD4 staining (Figure 35A&B) with the IL17F and CD4 staining (Figure 39A&B) the grading of the staining looks very similar. There is a lot of positive staining with 50% of patients showing clusters, 4% intermediate, 32% of scattered and 14 % without any staining for IL17A and CD4 versus 48% in clusters, 17% intermediate, 31% scattered and 4% with no staining for IL17F and

CD4 double staining in the omeprazole-induced AIN and vasculitis group respectively.

This gives us stronger evidence that what we see with both stains are cells of a Th17 lineage. This is especially important since it is a novel marker and no one has stained omeprazole-induced acute interstitial nephritis kidneys in the past. We found Th17 cells within the interstitium and occasionally within the tubular epithelium. 7 of 23 omeprazole-induced AIN cases versus 9 of 24 vasculitis cases had a Th17 tubulitis. This is the first report of TH17 cells in omeprazole-induced acute interstitial nephritis.

Foxp3 staining

Following two examples of Foxp3 staining (green) show Foxp3-tubulitis (Figure 40) and Foxp3 in the interstitium (Figure 41) in a patient with omeprazole-induced AIN.

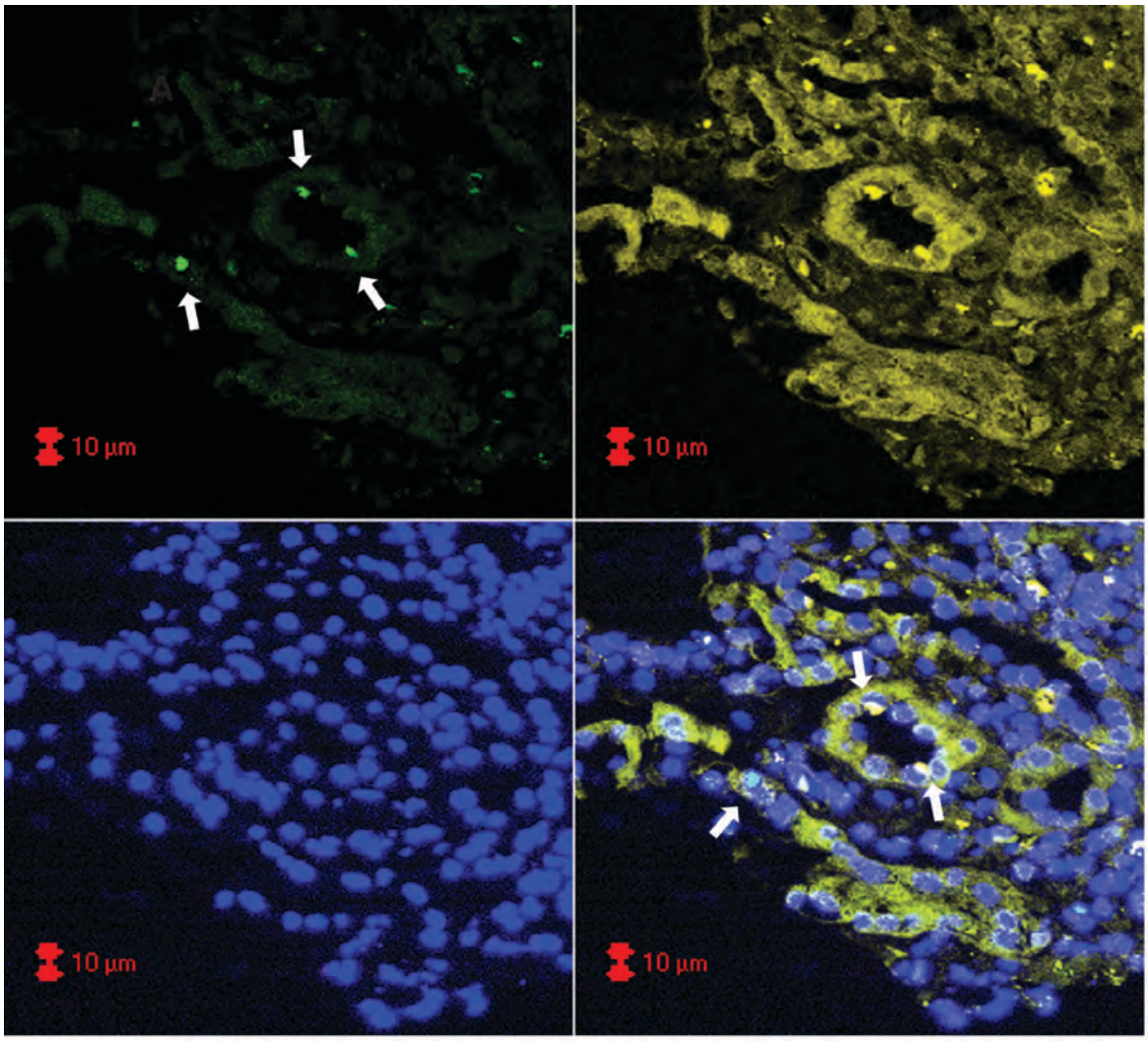


Figure 40: Foxp3 tubulitis in an omeprazole-induced acute interstitial nephritis case. Foxp3 (green, low setting for 488), kidney structure (yellow, high setting for 488), DAPI (blue) and merged image bottom right. The white arrow highlights the Foxp3 positive cells.

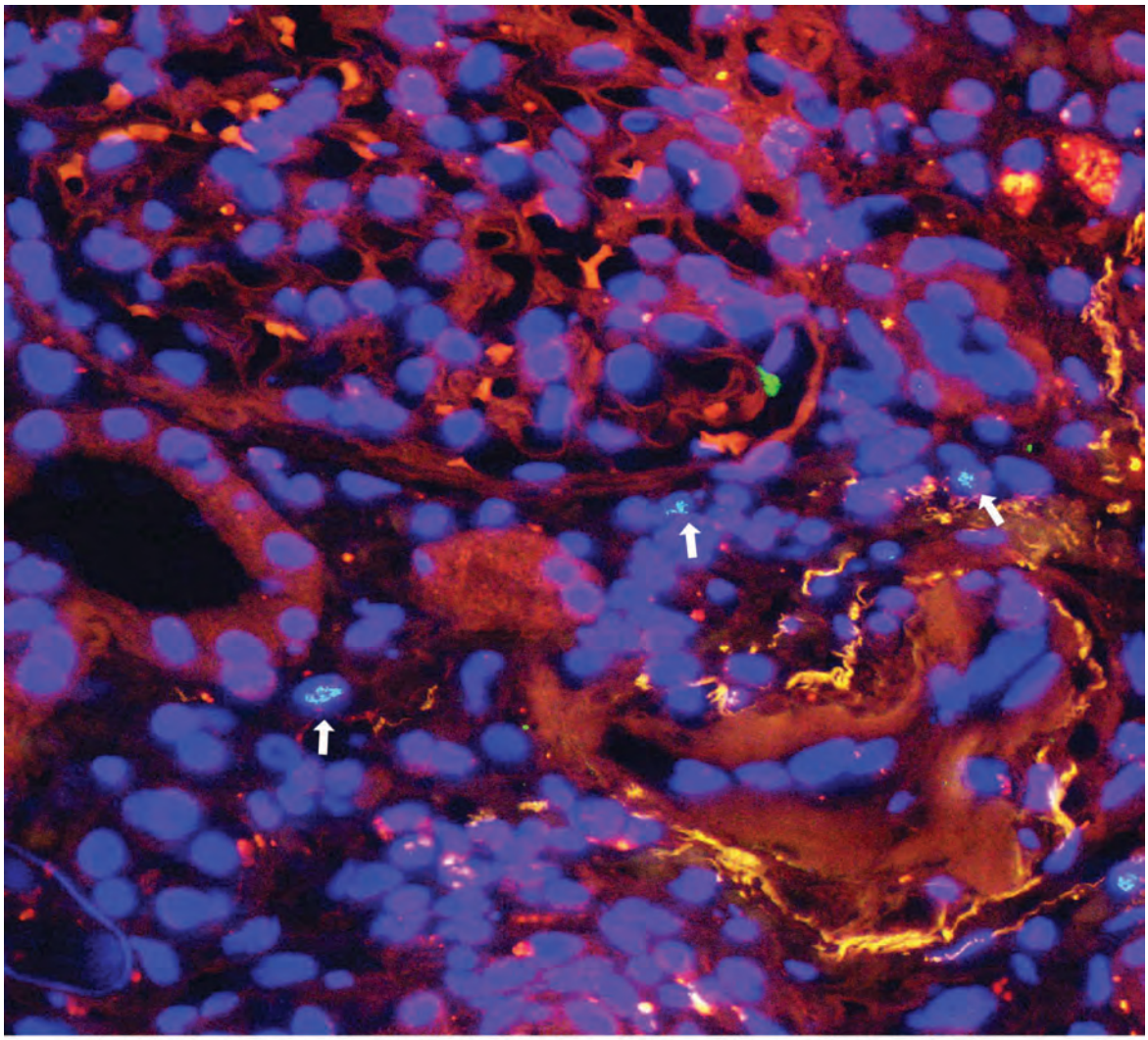
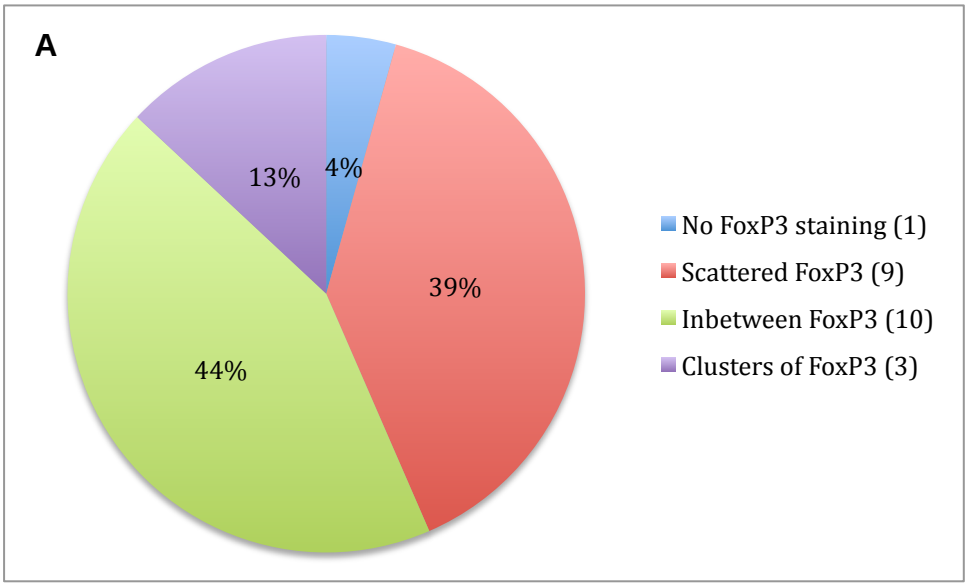


Figure 41: Foxp3 in the interstitium of an omeprazole-induced interstitial nephritis case. Foxp3 (green), DAPI (blue), kidney tissue (red). The white arrow highlights the positive Foxp3 cells.

Semiquantitive analysis of Foxp3



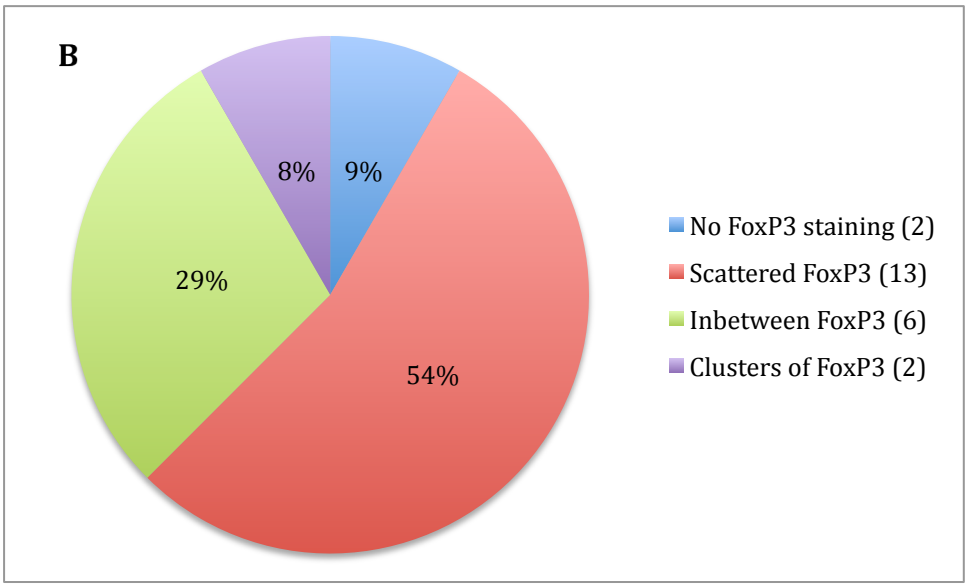


Figure 42A&B: Foxp3 staining in the omeprazole-induced AIN and vasculitis group. 2 patients were excluded in the omeprazole-induced AIN and 3 patients in the vasculitis group due to minimal availability of tissue.

Foxp3 regulatory cells were found within the interstitium and tubular epithelium of both vasculitis and omeprazole-induced AIN cases. However, we demonstrated fewer Foxp3 cells overall in the vasculitis cases, suggesting a dampened regulatory response.

Macrophages

Optimization was done for INOS (M1) and MMR (M2) (Figure 11). Further optimization and double staining was planned with CD163. Because of time constraints this was not done.

Summary of Immunostaining

In summary our cases showed numerous CD4 positive cells, and fewer numbers of CD20 and CD8. CD4 positive cells were present in clusters in 79% of the omeprazole-induced AIN patients and 67% of the vasculitis patients (Figure 34A&B). The majority of which were also Th-17 positive on double-staining. CD20 positive cells were present in clusters in 58% of all omeprazole-induced AIN patients and 37% of vasculitis patients (Figure 29 A&B). However, CD8 positive cells were present in clusters only in 17% of omeprazole-induced AIN patients and 13% of vasculitis patients (Figure 28A&B). Foxp3 regulatory cells were found in the interstitium and the tubular epithelium in both cohort groups, although there were fewer FoxP3 cells overall in the vasculitis group. For the first time we report the presence of Th17 cells in omeprazole-induced acute interstitial nephritis. Because of the difficulty of autofluorescence of kidney tissue we confirmed the presence of Th17 cells using double-staining for IL17A and CD4 as well as IL17F and CD4. This strengthens our important result in a novel marker such as IL17F showing Th17 cells are present within kidneys with omeprazole induced acute interstitial nephritis.

In this retrospective case series of omeprazole-induced AIN with 25 patients we have demonstrated, using immunohistochemistry, that the predominant cellular infiltrate is a Th1-Th17 cell-mediated process. In addition, lesser numbers of inflammatory B-cells (CD20), cytotoxic T-cells (CD8), and FOXP3+ Treg are also present within the interstitium.

3.5 References

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CHAPTER FOUR DISCUSSION

4.1 Discussion

In our study, which is the biggest biopsy proven case series for omeprazole-induced AIN with 25 cases to date, we provide data about the nature of the cellular infiltrate with predominantly Th1 –Th-17 cells, which would suggest that this is the pre-eminent inflammatory pathway mediating omeprazole - induced AIN. Associated inflammatory B-cells (CD20), T-cells with subtypes (cytotoxic (CD8), Th17 cell (IL17F+/CD4+), Treg (FOXP3)) are present within the lesion too. The nature of the interstitial inflammatory infiltrate was compared to the interstitial infiltrate evident in small vessel vasculitis with renal involvement.

The first reported case of omeprazole-induced AIN appeared in 1992 (Ruffenach et al., 1992). Since then many further cases of proton pump inhibitor-induced AIN associated with all 5 PPI's in clinical practice (omeprazole, pantoprazole, esomeprazole, rabeprazole) have been reported suggesting the injury is a class effect. In New Zealand omeprazole is the pre-eminent proton pump inhibitor, with pantoprazole the only other agent available. All our cases are limited to omeprazole exposure.

Clinical presentation of omeprazole-induced AIN

Patients commonly presented with non-specific symptoms of malaise, fatigue, nausea, lethargy and weight loss, and importantly no specific renal symptoms, with an insidious onset of symptoms over 3 weeks to 6 months (1 patient presented at 2 years) after starting omeprazole. In the literature, symptom onset ranges from 1 week to 9 months after introduction of the proton pump inhibitor (Ruffenach et al., 1992, Brewster and Perazella, 2007, Christensen et al., 1993, Singer et al., 1994, Yip et al., 1997, Myers et al., 2001, Chan et al., 2009, Geetha, 1999, Shuster, 2000, Post et al., 2000, Delve et al., 2003). Similar to other reports, in our group the elderly are most affected, with age typically in the 7th decade and female (Chan et al., 2009).

The classical triad of fever, rash and peripheral eosinophilia were previously described as the presenting features of drug-induced interstitial nephritis, with 79% of

patients presenting with at least one of these features (Yip et al., 1997). However the clinical presentation of omeprazole-induced AIN is atypical, with less than 5% of patients presenting with the classical triad (Myers et al., 2001, Ra and Tobe, 2004). In our study none of the patients had any features consistent with the classic allergic acute interstitial nephritis.

The atypical presentation of omeprazole-induced AIN implies underlying histopathological differences too. Since the sentinel case by Ruffenach in 1992 implicating omeprazole as a cause of AIN, at least 114 cases of proton pump inhibitor-induced AIN have been published in the literature (Chan et al., 2009), of which 94 cases were biopsy-proven and 19 cases were non-biopsy proven. To date our study is the largest case series with biopsy proven omeprazole-induced AIN.

Omeprazole-induced AIN lacks substantial eosinophils

The immunopathogenesis of omeprazole-induced AIN is unknown, hence the focus of this project was to identify the key immune processes mediating the acute interstitial nephritis. Given the previously strong association of eosinophils in classic acute allergic interstitial nephritis, we initially semi-quantitified the extent of eosinophil infiltration in our biopsies. In contrast to the previous largest case series to date (Geevasinga et al., 2006) who reported eosinophils (not otherwise quantified) within the tubulointerstitium of 83% of patients (15 of 18), the occurrence of any eosinophils in our patients was only 52% (13 of 25). Interestingly, only one patient has an eosinophilic infiltrate, the other cases (12 of 25) showed only small numbers (3 – 7 eosinophils per high powerfield in some but not all views) of scattered eosinophils in the infiltrate. Similar findings were evident in the vasculitis group, with only a number of scattered eosinophils present predominantly in the group with the most severe infiltrate. The lack of a substantial eosinophilic infiltrate argues against a Th-2 mediated mechanism for omeprazole-induced AIN.

On light microscopy observations demonstrated a predominantly interstitial mononuclear infiltrate of lymphocytes and macrophages (68%) or a mixed inflammatory cell infiltrate, i.e. neutrophils, lymphocytes, macrophages (20%). This was confounded by 4 cases in the omeprazole-induced AIN group, who were found to have an asymptomatic ascending urinary tract infection, which would explain the

neutrophil infiltrate. Excluding these 4 patients the results show a very similar pattern of interstitial infiltrate in both groups with 76% of cases predominantly a mononuclear infiltration and 19% of cases a mixed inflammatory cell infiltration comparable to that evident in the vasculitis group with 78% of cases predominantly a mononuclear infiltration and 18% a mixed inflammatory infiltrate (Figure 12A, B&C).

Immune response in omeprazole-induced AIN is Th1-Th17 mediated rather than Th2-mediated

The immunopathological features of the inflammatory infiltrate in omeprazole-induced AIN is unknown where as the major immunopathological features of renal small vessel vasculitis have been defined as predominantly Th1 – Th17 cell mediated injury (Summers et al., 2009, Gan et al., 2010, Ooi et al., 2010, Kitching and Holdsworth, 2011). Given the very similar light microscopic features of omeprazole-induced AIN and renal small vessel vasculitis, the study sought to compare and contrast the possible immunopathogenic mechanisms between the two groups.

A basic examination of an immune response involves the determination of a predominantly T cell mediated-response or humoral B cell mediated response. The latter produces plasma cells, and some of our cases showed a plasma cell infiltrate. Thus it was important to determine the infiltration by CD4 T lymphocytes (identified by the T cell marker CD4), CD8 T lymphocytes (identified by the T cell marker CD8) and the B lymphocytes (identified with the pan B cell marker CD20). Plasma cells were recognized morphologically by their eccentric nucleus, clock-face clumped chromatin, and perinuclear pattern of the Golgi apparatus.

One of our main findings was that our cases showed numerous CD4 positive cells with over 65% present in clusters in both groups (Figure 34A&B), fewer numbers of CD20 cells in clusters with 58% versus 37% in the omeprazole-induced AIN, and vasculitis group respectively (Figure 29A&B) and only 17% versus 13% of CD8 cells evident in clusters in the omeprazole-induced AIN group, and vasculitis group respectively (Figure 28A&B). Plasma cells were present in the mixed inflammation. Overall these results suggest a T cell mediated Th1 response, rather than a Th2 mediated response.

However, the traditional division of T cell mediated mechanisms into Th1 and Th2 has recently been expanded to include Th17 and Treg cells (Korn et al., 2009). Th1, Th2, and Th17 cells eradicate intracellular pathogens, helminthes, and extracellular bacteria or fungi. Th1 and Th17 cells are involved in autoimmune diseases, whereas Th2 cells are involved in allergic responses. IL-17–producing T (Th17) cells produce cytokines IL-17A, IL-17F, IL-21, IL-22, IL-23 and retinoic acid receptor–related orphan receptor C2 (RORC2). Regulatory T (Treg) cells produce IL-10, IL-35, transforming growth factor (TGF)-b and master regulators forkhead box P3 (FoxP3).

Th17 cells have been recognised as a distinct lineage of Th cells, and associations between IL-17 and human disease have been known, especially in psoriasis, rheumatoid arthritis, and inflammatory bowel disease. IL-17 T lymphocytes induce a neutrophilic response that is followed by chronic inflammation, thus linking innate and cell mediated immunity, through the production of IL-17. Several disorders previously classified as typical Th1 disease are now considered to be primarily Th17-mediated responses (Ziolkowska et al., 2000, Fujino et al., 2003, Arican et al., 2005), and will require different therapeutic regimes.

Thus we further examined our cohort for the presence of Th17 lymphocytes and Foxp3 positive regulatory cells. As IL-17 is also expressed by other cells including IL-17–producing gd T (gd T-17) cells, natural killer T-17 cells, and IL-17–producing lymphoid tissue–induced cells, it was important to perform double antibody immunofluorescence with IL-17 and CD4. Other innate IL-17 producers have been postulated such as NK cells, eosinophils and neutrophils (Korn et al., 2009), but we could exclude these on the basis of location and morphology in combination with negative CD4 immunohistochemistry.

We show for the first time that the Th17 mechanism is involved in omeprazole-induced AIN. Th-17 cells were present in 82% of the biopsies, with more cells associated with the more active cases. Where there was a florid tubulitis with inflammatory cells actually crossing the tubular basement membrane, the majority were Th-17 positive cells (7 of 23 omeprazole-induced AIN cases versus 9 of 24 vasculitis cases). Comparing the IL17A and CD4 double staining (Figure 35A&B) with the IL17F and CD4 double staining (Figure 39A&B) for the omeprazole-induced AIN group and vasculitis group, the grading of the staining looks very similar supporting a

role for CD4+TH17+ cells mediating the injury. This is especially important since it is a novel marker and no one has stained omeprazole-induced AIN kidneys in the past. We found Th17 cells within the interstitium and occasionally within the tubular epithelium, which is the first report of TH17 cells in omeprazole-induced AIN.

Foxp3 regulatory cells were found within the interstitium and tubular epithelium of both vasculitis and omeprazole-induced AIN cases. However, we demonstrated fewer Foxp3 cells overall in the vasculitis cases, suggesting a lesser regulatory response, and consistent with reported decreased peripheral blood counts of Foxp3 CD4+ CD25+ regulatory T cells in granulomatosis with polyangitis (Wegener granulomatosis) patients (Flint et al., 2010). The exact role of T regs in omeprazole-induced AIN needs to be elucidated.

Conclusions

In this series of 25 renal biopsies with omeprazole-induced acute interstitial nephritis, the predominant inflammatory cells were indicative of a Th1-Th17 lineage, confirming a cell mediated immunologic process as the key inflammatory pathway. Eosinophils were mostly absent in the biopsies, making this unlikely to be the classical allergic acute interstitial nephritis (Th2 mediated) that has previously been reported with other drugs such as methicillin. This is despite recent publications (Chan et al., 2009) hypothesising such an underlying hypersensitivity reaction as the most likely aetiology. What is unknown is how omeprazole might induce this Th1-Th17 mediated inflammatory pathway of injury within the kidney.

It has been suggested that IL17 induces macrophage proliferation, which contributes to the excessive fibrosis and persistent chronic kidney disease (Duffield, 2011). Numerous macrophages were identified in our biopsies. Further staining for INOS (M1) and MMR (M2) is planned to identify the differences between a M1 (pro-injurious function or pro-inflammatory status) and M2 (wound-healing function or anti-inflammatory status) macrophage infiltrate. However, macrophages show a very high degree of plasticity and renal biopsies display only a snapshot of a dynamic process of injury and repair, which makes elucidation of the inflammatory pathways at play difficult to confirm.

Proton pump inhibitors are one of the most prescribed drugs in the Western

world (Chan et al., 2009) and are also available over the counter since 2003. It is generally regarded as a safe drug with relatively free complications. The true number of patients that may have developed an omeprazole-induced AIN may well be underestimated. Although the incidence of omeprazole-induced AIN seems to be rare, currently estimated in NZ to be in the order of 1:12'500 (Simpson et al., 2006) (Savage, 2006), it is a potentially important iatrogenic cause of acute kidney injury, which can lead to chronic kidney disease. In our study only 12% of omeprazole-induced AIN cases had fully recovered renal function 6 to 12 months after the acute event. Of clinical importance is the fact that 88% had some degree of persistent CKD although no patient needed dialytic support or had progressed to end stage kidney failure yet.

By understanding the clinicopathological features of omeprazole-induced acute interstitial nephritis, it is hoped that this can be translated into better prognostic indicators as well effective therapies to avoid the development of chronic kidney disease with long term detrimental effects on health (Ray et al., 2010).

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4.3 Appendix

Appendix 1- DAB

The PicTureTM-DOUBLE STAINING KIT using a commercial kit from Invitrogen Polymer Detection System, Invitrogen Corporation, Camarillo, CA, USA, CAT. NO 87-9999.

- De-paraffinized sections in three changes of xylene of 3 min each.
- Re-hydrated sections through graded alcohols of 3 min each wash and placed in PBS for 2 - 5 minutes.
- Peroxidase Quenching solution for 10 min (add 1 part of 30% hydrogen peroxidase to 9 parts of absolute methanol) then washed in PBS 5 min, 3 times.
- Antigen retrieval in Tris-EDTA buffer pH 9 and microwaved for 20 minutes (800 Watt). After cooling for 15 to 30 minutes biopsies were washed in de-ionised water.
- Protein Block with Serum Blocking Solution for 10 minutes, then blotted of solution.
- The biopsies were incubated with monoclonal primary antibody 1 (anti-human CD20) and monoclonal primary antibody 2 (rabbit anti-human CD8) for two hours and a half at room temperature in 1 in 100 dilution. Biopsies were washed in PBS 3 - 5 minutes.
- Post primary block solution for 30 minutes ,washed in PBS 2 - 5.
- Incubated with a secondary antibody (Novolink TMpolymer mouse and rabbit immunoglobulins) for 30 minutes, washed in PBS 2 - 5 with gentle rocking.
- Developing of DAB stain, rinse off in distilled water.
- Developing the fast red stain, rinse off in distilled water.
- DAPI for 5 min., dry slides and coverslip.

Appendix 2- Human leukocyte staining for formalin fixed paraffin embedded tissue)- Immunofluorescence

1. Cut 2µm sections
2. Oven dry at 60 ºC for 1 hour
3. Xylene 3x 3minutes (different:Histosol 2x20 minutes)
4. Graded alcohols 3 minutes each
5. PBS 3 min.
6. Antigenretrieval: HIER- 10mmol/L Tris, 1mmol/lEDTA pH 9.0 20 minutes
7. 20 minutes cool down step in HIER solution
8. Serum blocking solution: 3% blocking agent (10ml plus 1 drop goat serum) – 30 minutes at RT, drain or blot off solution. Do not rinse.
9. Add first primary antibody (optimizing single antibodies)

   - mouse CD 4 1 in 40 overnight at 4°C

   - rabbit IL17A 1 in 100 overnight at 4°C

   - goat IL17F 1 in 20 overnight at 4°C

   - mouse FOXP3 1:50 overnight at 4°C
10. Wash with PBS containing 0.05% Tween (500ul/1l PBS) 3x 5 min
11. Secondary to the first primary antibody 488 (1 in 500, on all the slides) for 30 min
12. Wash with PBS containing 0.05% Tween (500ul/1l PBS) 3x 5 min

(13. Second primary antibody IL17 1hour (for double staining))

(14. Wash with PBS-T)

13. Counterstain with DAPI (200ml PBS/20ulDPAI) 5min
14. Dry at 37°
15. Mount gold/cover slip slide

Appendix 3- The Banff 97 criteria. Figure from Kidney International, permission is given by the Nature publishing group. Licence number 2891470996710. (Racusen et al., 1999**).**

Table 2. Quantitative criteria for tubulitis ("t") score[a]

t0 - No mononuclear cells in tubules
t1 - Foci with 1 to 4 cells/tubular cross section (or 10 tubular cells)
t2 - Foci with 5 to 10 cells/tubular cross section
t3 - Foci with >10 cells/tubular cross section, or the presence of at least two areas of tubular basement membrane destruction accompanied by i2/i3 inflammation and t2 tubulitis elsewhere in the biopsy

[a] Applies to tubules no more than mildly atrophic

Table 4. Quantitative criteria for mononuclear cell interstitial inflammation ("i") scores

i0 - No or trivial interstitial inflammation (<10% of unscarred parenchyma)
i1 - 10 to 25% of parenchyma inflamed
i2 - 26 to 50% of parenchyma inflamed
i3 - more than 50% of parenchyma inflamed

Indicate the presence of remarkable numbers of eosinophils, PMNL, or plasma cells (specify which) with an asterisk (*).

Table 5. Quantitative criteria for early allograft glomerulitis ("g") score

g0 - No glomerulitis
g1 - Glomerulitis in less than 25% of glomeruli
g2 - Segmental or global glomerulitis in 25 to 75% of glomeruli
g3 - Glomerulitis (mostly global) in more than 75% of glomeruli

Table 7. Quantitative criteria for interstitial fibrosis ("ci")

ci0 - Interstitial fibrosis in up to 5% of cortical area
ci1 - Mild - interstitial fibrosis in 6 to 25% of cortical area
ci2 - Moderate - interstitial fibrosis in 26 to 50% of cortical area
ci3 - Severe - interstitial fibrosis in >50% of cortical area

Table 8. Quantitative criteria for tubular atrophy ("ct")

ct0 - No tubular atrophy
ct1 - Tubular atrophy in up to 25% of the area of cortical tubules
ct2 - Tubular atrophy involving 26 to 50% of the area of cortical tubules
ct3 - Tubular atrophy in >50% of the area of cortical tubules