

## Original article

### CXCR3 renal expression in glomerulonephritis in children: is there a connection with the course of the disease?

**Background:** Glomerulonephritis (GN) is a common childhood disease that may represent a significant cause of chronic kidney disease at one point of its course. The role of chemokines in glomerulonephritis, has been long anticipated and studied and the possible link between certain chemokines and different renal pathologies, if proved, can pave the road for future use of such markers for early prognosis and possible therapies for this common disease. **Objective:** in this study, we aimed at detecting CXCR3 in the renal biopsies done for children with glomerulonephritis and to correlate it to the nature of renal pathology and response to therapy. **Methods:** The glomerular and interstitial expression of CXCR3 in renal biopsies done for 22 patients with glomerulonephritis was studied using immunohistochemical staining. Pathologies already diagnosed in these biopsies were proliferative GN (mesangioproliferative GN, diffuse proliferative GN, focal proliferative GN, IgA nephropathy and crescentic GN) as well as non-proliferative GN (Minimal change disease, focal segmental glomerulosclerosis, membranous nephropathy, diffuse mesangial sclerosis and advanced hypertensive nephrosclerosis). History, clinical findings and laboratory investigations in the initial presentation and at the time of the study were obtained. **Results:** The degree of glomerular and interstitial CXCR3 expression did not vary with gender, age of presentation, response to steroids, or cumulative doses of steroids. Percentage of strong glomerular CXCR3 expression was much higher in proliferative GN compared to non-proliferative GN although the difference was not statistically significant, percentage of renal dysfunction was more among strong glomerular and mild/moderate interstitial CXCR3 expression with no statistically significant difference from the counterparts. **Conclusion:** Our study revealed that enhanced CXCR3 renal expression on glomerular and interstitial levels did not affect the response to steroids along the course of the disease and so can probably act as a therapeutic target rather than a prognostic marker.

**Keywords:** glom. CXCR3, int. CXCR3, glomerulonephritis, renal biopsy.

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## INTRODUCTION

Chemokine receptor CXCR3 is a G $\pm$ i protein-coupled receptor in the CXC chemokine receptor family. Other names for CXCR3 are G protein-coupled receptor 9 (GPR9) and CD183<sup>1</sup>. Increased expression of the CXCR3 receptor has been demonstrated in patients with inflammatory kidney disease like IgA nephropathy, membranoproliferative glomerulonephritis, rapidly progressive glomerulonephritis, or nephrotoxic nephritis<sup>2</sup>.

Activated Th1 cells and macrophages are able to produce IFN- $\gamma$  and TNF- $\alpha$ , which in turn can amplify both CXCL10 and CXCL9 production and up-regulation of CXCR3, the interaction of CXCL10 and CXCL9 with their receptor expressed

by mesangial cells can account for chronic stimulation of their proliferation and allows the inflammatory reaction characteristic of proliferative GN<sup>3</sup>. Glomerular macrophage infiltration is associated with fibrin deposition. Fibrin deposition is a potent mediator of glomerular injury in crescentic GN<sup>4</sup>.

Chemokines produced in glomeruli seem not only to induce recruitment of inflammatory cells, but also activation of chemokine receptors in podocytes and may therefore contribute to the pathogenesis of proteinuria via activation of NADPH-oxidases, leading to a release of superoxide anions. Podocyte damage leads to the retraction of their foot processes, resulting in proteinuria, especially in diabetic nephropathy, minimal change glomerulonephritis (MCGN),

membranous nephropathy (MGN), and focal segmental glomerulonephritis (FSGS)<sup>5</sup>.

These findings are very much suggestive that CXCR3 probably plays a significant role in the pathogenesis of GN whether the nature of the pathology is proliferative or non-proliferative. Our aim was to check the presence and distribution of CXCR3 in renal tissue of patients with GN and to correlate it with the nature of renal pathology and response to steroid therapy.

## METHODS

Twenty two patients suffering from GN were included in this study. Only patients with clinical course necessitating, at one point, the performance of a renal biopsy were enrolled. Patients with diabetes mellitus, Hashimoto's thyroiditis, or auto-inflammatory diseases were excluded. Paraffin embedded sections of renal biopsy tissues previously obtained, clinical examination, and cumulative doses of steroids were obtained from patients' records. Careful clinical examination was done at the time of the study. Levels of urinary proteins (24 hours urinary proteins quantitation), serum creatinine, blood urea nitrogen, serum albumin, serum triglycerides (TGs) and total serum cholesterol were determined.

### *Immunohistochemical staining*

Paraffin sections were fixed on poly-L-lysine coated slides, dried overnight in a 60°C oven. Then they were deparaffinized and dehydrated. The slides were treated in microwave oven in ready to use antigen retrieval citrate buffer for 10 minutes, then sections were left to cool at room temperature for 20 minutes. Slides were stuck to cover plates using PBS pH 7.6 and placed in sequenza center for immunostaining. Four Endogenous peroxidase activity was blocked by adding 2-3 drops of hydrogen peroxide blocking serum for 5 minutes at room temperature, then sections were rinsed well with PBS for 5 minutes. Two drops of protein blocking serum were added for 10 minutes. The primary antibody was applied by adding 3 drops to each section CXCR3 polyclonal antibody PA5-23679 and incubated for 2 hours at room temperature, followed by rinsing in PBS pH 7.6. The secondary antibody was applied by adding 2 drops of biotinylated secondary antibody to each section a supersensitive immunodetection system (Bigenex, catalog No. AD 000-SL) for 30 minutes at room temperature. Slides were then rinsed in PBS pH 7.6. Two drops of peroxidase labeled streptavidin were added for 20 minutes at room temperature, then rinsing with PBS pH 7.6. Slides

were incubated for 10 minutes with substrate chromogen (DAB) mixture. Slides were then rinsed with distilled water. Slides were immersed in Harris Haematoxylin for 3 seconds, rinsed in tap water, dehydrated in absolute alcohol. Lastly, slides were cleared in xylene and mounted by Canada Balsam then covered by glass cover.

Sections of gut for Crohn's disease were regarded as positive controls for CXCR3. They were stained in each run to judge the effectiveness of the technique. Negative control slides, were processed as the previous immunostaining procedure, but the primary antibody was omitted from the steps, and PBS was used instead. The extent of positive staining of CXCR3 was examined in glomerular cells (glom. CXCR3) and interstitial (int. CXCR3) and extent of CXCR3 staining was graded using a scale of 0-3, where 0=no staining, 1=mild staining, 2=moderate staining, 3=strong staining<sup>6</sup>.

### *Statistical Methods*

Statistical analysis was performed using Statistical Package for Social Sciences, Version 17.0 (SPSS, Inc., Chicago, III., USA) for Windows. Continuous variables were analyzed as mean values  $\pm$  standard deviation (SD) or median and range as appropriate. Rates and proportions were calculated for categorical data.

*Kolmogorov-Smirnov test* of normality was done to assess normality of continues variables before starting the analysis.

$\chi^2$  (chi square) test and Fisher's exact test when appropriate were used to examine the relation between qualitative variables

Comparison between two continues variables was done using t student test for normally distributed variables and its non-parametric analogue Mann Whitney test was used for not normally distributed ones.

Correlations among different parameters were determined by using spearman rho test. P value of  $\leq 0.05$  was considered statistically significant.

## RESULTS

Patients were classified into 2 groups: proliferative GN and non-proliferative GN.

**Group I:** Proliferative group included 5 (38.5%) females and 8 (61.5%) males. The age of the patients ranged between 3.5 and 13 years with a mean  $\pm$  SD of  $7.5 \pm 3.2$  years. Duration of illness ranged from 2 months to 2 years with a mean  $\pm$  SD of  $1.4 \pm 0.9$  years.

**Group II:** Non-proliferative group included 3 (33.3%) females and 6 (66.7%) males. The age of the patients ranged between 7 months and 11 years

with a mean  $\pm$  SD of  $4.6 \pm 3.7$  years. Duration of illness ranged from 2 months to 3.8 years with a mean  $\pm$  SD of  $1.1 \pm 1.2$  years. No significant difference in duration of illness was found between both groups. Other demographic and laboratory data were assessed in both groups (table 1).

Nine out of 13 (69.2%) of the proliferative GN group had moderate/ strong CXCR3 glomerular (glom. CXCR3) staining compared to 4 out of 9 (44.4%) in the non-proliferative group, yet with no statistically significant difference between both groups (table 2). Interstitial CXCR3 expression (int. CXCR3) was either absent or mild in proliferative GN (5 and 8 patients respectively) while in non-proliferative GN, 3 patients showed absence of interstitial CXCR3 expression, 2 patients with mild and 3 with moderate expression. A significant difference was found in the distribution of int. CXCR3 expression between proliferative and non-proliferative GN groups (table 3).

The studied patients with mild/moderate expression of glom. CXCR3 had comparable age at presentation and gender to those with strong marker expression. The same was found between those with absent expression of int. CXCR3 and those with mild/moderate expression.

Response to steroid treatment and cumulative doses of steroids showed no significant difference between the patients with mild/moderate glom. CXCR3 renal expression and those with strong

marker expression and also between patients with mild/moderate interstitial CXCR3 expression and those with absent expression of the marker although resistance to steroid therapy was significantly higher among non-proliferative GN group compared to proliferative GN group.

Clinical presentation (hypertension, oliguria and hematuria) did not vary significantly between patients with mild/moderate glom. CXCR3 renal expression and those with strong marker expression. Hematuria was significantly more frequent (71.4%) among the patients showing mild/moderate int. CXCR3 expression compared to those who did not where only 1 patient (12.5%) presented by hematuria ( $p=0.024$ ). other clinical presentations did not significantly vary between both groups.

Renal functions, s. albumin, degree of proteinuria or s. lipids were comparable between mild/moderate glom. CXCR3 expression and strong marker expression on the one hand and between mild/moderate int. CXCR3 expression and absent marker expression on the other hand.

The extent of both glom. and int. CXCR3 expression was not significantly different between steroid resistant and steroid sensitive patients. Two of the patients with proliferative GN presented with pure nephritis and so did not receive steroid therapy (tables 4, 5).

**Table 1.** Some demographic and laboratory data in patients with proliferative and non- proliferative GN.

	<b>Proliferative GN N (%): 13 (59%)</b>	<b>Non proliferative GN N (%): 9 (41%)</b>	<b>P value (sig. d 0.05)</b>
<b>Cumulative corticosteroid dose (grams/kg)</b>			
Median	0.44	0.26	0.22
Range	(0.02-2)	(0.06-0.9)	
<b>Response to steroids treatment</b>			
Resistant	3 (27.3)	8(88.9)	0.006
Sensitive	8(72.7)	1(11.1)	
<b>Initial lab. Investigations</b>			
<b>24 hrs urinary proteins (g/24h)</b>			
Median	3.3	3.2	0.48
Range	(1.1-7)	(1.3-6.5)	
<b>S. Creatinine (mg/dl)</b>			
Median	0.9	0.7	0.27
Range	(0.2-4.1)	(0.2-2,4)	
<b>S. albumin (gm/dl)</b>			
Median	2.5	1.9	0.07
Range	(1.2-4.2)	(1.1-3.1)	
<b>S. cholesterol (mg/dl)</b>			
Median	243.2	306.4	0.23
Range	(124-500)	(208-429)	

**Table 2.** Extent of glomerular CXCR3 staining in proliferative and non-proliferative GN groups.

Extent of glomerular CXCR3 expression	Proliferative GN gp. No (%) 13 (100%)	Non-proliferative GN gp. No (%) 9 (100%)	P value (sig. d 0.05)
0	1 (7.7%)	0(0%)	0.295
1	3 (23.1%)	5 (55.6%)	
2	4 (30.7%)	3 (33.3%)	
3	5 (38.5%)	1 (11.1%)	

Extent of CXR3 was graded using a scale of 0-3, where 0=no staining, 1=mild staining, 2=moderate staining, 3=strong staining<sup>6</sup>.

**Table 3.** Extent of interstitial CXCR3 staining in proliferative and non-proliferative GN groups.

Extent of interstitial CXCR3 expression	Proliferative GN gp. No (%) 13 (100%)	Non-proliferative GN gp. No (%) 9 (100%)	P value (sig. d 0.05)
0	5 (38.5%)	3 (33.3%)	0.022
1	8 (61.5%)	2 (22.2%)	
2	0 (0%)	4 (44.4%)	
3	0 (0%)	0 (0%)	

Extent of CXR3 was graded using a scale of 0-3, where 0=no staining, 1=mild staining, 2=moderate staining, 3=strong staining<sup>6</sup>.

**Table 4.** Extent of glomerular CXCR3 staining in steroid resistant and steroid sensitive patients.

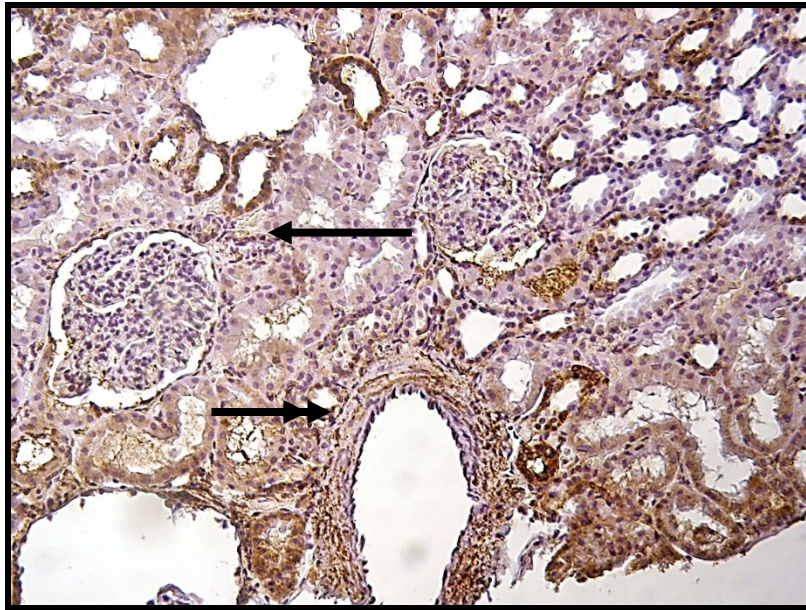
Extent of glomerular CXCR3 expression	Steroid resistant No (%) 11	Steroid sensitive No (%) 9	P value (sig. d 0.05)
0	0 (0%)	1 (11.1%)	0.26
1	6 (54.5%)	2 (22.2%)	
2	4 (36.4%)	3 (33.3%)	
3	1 (9.1%)	3 (33.3%)	

Extent of CXR3 was graded using a scale of 0-3, where 0=no staining, 1=mild staining, 2=moderate staining, 3=strong staining<sup>6</sup>.

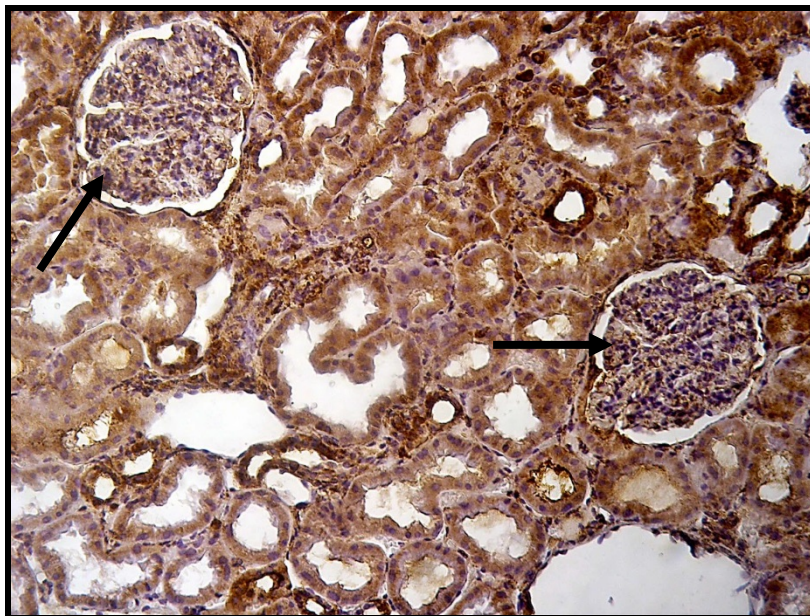
**Table 5.** Extent of interstitial CXCR3 staining in steroid resistant and steroid sensitive patients

Extent of interstitial CXCR3 expression	Steroid resistant No (%) 11	Steroid sensitive No (%) 9	P value (sig. d 0.05)
0	4 (36.4%)	4 (44.4%)	0.11
1	3 (27.3%)	5 (55.6%)	
2	4 (36.4%)	0 (0%)	
3	0 (0%)	0 (0%)	

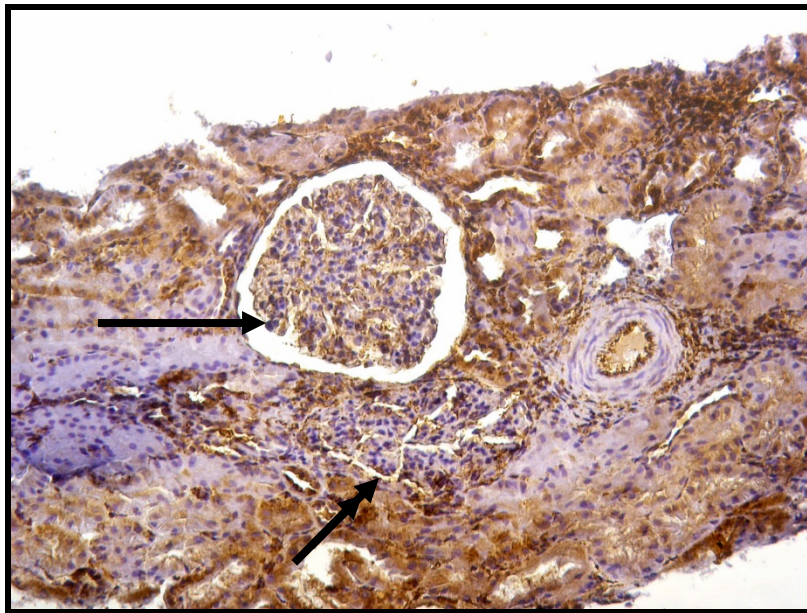
Extent of CXR3 was graded using a scale of 0-3, where 0=no staining, 1=mild staining, 2=moderate staining, 3=strong staining<sup>6</sup>.



**Figure 1.** Mild CXCR3 expression in the glomerulus with positive immunostaining of the inflammatory cells (arrow) and vascular smooth muscle cells (double arrows) (X200, CXCR3 immunohistochemical staining).



**Figure 2.** Moderate CXCR3 expression by mesangial cells of the glomerulus (arrow) (X200, CXCR3 immunohistochemical staining).



**Figure 3.** Strong CXCR3 expression by mesangial cells of the glomerulus (arrow). The periglomerular inflammatory cells (double arrows) also immunostained (X200, CXCR3 immunohistochemical staining).

## DISCUSSION

We compared glomerular and interstitial expression of CXCR3 among patients with proliferative and those with non-proliferative GN. We found that the glom. and int. marker expression was comparable among both groups although strong glom. marker expression was found in 41.7% (5 patients) among proliferative group in comparison to 11.2% (1 patient) among non-proliferative group.

This can be explained by the involvement of many inflammatory cells and chemokines in proliferative GN and this can be the cause of a stronger CXCR3 glomerular staining, the disease being primarily of glomerular origin. A significant difference in the distribution of int. CXCR3 expression was found between proliferative and non-proliferative GN groups, the non-proliferative showing higher percentage of moderate expression.

This comes in agreement with several studies; one, performed on renal specimens from 23 subjects between 35 and 65 years of age with proliferative and non-proliferative GN, found that in non-proliferative GN, CXCR3 expression in glomerular cells was rarely found whereas in proliferative GN, very strong CXCR3 immunoreactivity was distributed all over glomerular structures<sup>7</sup>. Similarly, another report of high expression of CXCL9 and CXCL10 by resident glomerular cells and CXCR3, in mesangial cells of patients with proliferative GN was made in a total of 45 human renal biopsies<sup>8</sup>. Another study reported that CXCR3 have been detected in mesangial cells of patients with IgA nephropathy, membranoproliferative glomerulonephritis, and

rapidly progressive glomerulonephritis, indicating that CXCR3 might contribute to mesangial cell proliferation in these diseases and suggesting that CXCR3 expression is up regulated during glomerular inflammation<sup>5</sup>. A third study compared CXCR3-deficient mice (CXCR3<sup>-/-</sup>) to normal mice. Nephrotoxic nephritis was induced leading to an increased renal mRNA expression of IP-10/CXCL10, Mig/CXCL9, and I-TAC/CXCL11. This increased chemokine expression was paralleled by the renal infiltration of T cells, followed by renal tissue injury, severe glomerulosclerosis with crescent formation and tubulointerstitial damage in wild-type mice, whereas CXCR3-deficient nephritic mice showed significantly reduced renal T cell infiltrates, less severe nephritis, less glomerulosclerosis and infrequent glomerular crescent formation. The data indicated that the decreased susceptibility of CXCR3<sup>-/-</sup> mice to develop nephrotoxic nephritis is due to impaired T cell trafficking into the inflamed kidney<sup>9</sup>. On the contrary, in a study population that consisted of biopsies from patients with IgA nephropathy, lupus nephritis, membranoproliferative glomerulonephritis, the number of CD3-positive T cells, CXCR3-positive cells, were very low within glomerular tufts in contrast to tubulointerstitial infiltrates which were the main site of CXCR3<sup>10</sup>. The inclusion of lupus nephritis patients in the study may be the cause of this difference

Comparing glom. and int. marker expression as regards the response to steroids, no statistically significant variation was found between steroid

sensitive and steroid resistant groups in the extent of glom. and int. CXCR3 expression yet the percentage of patients with mod, /severe glom. CXCR3 expression was higher in steroid sensitive compared to steroid resistant patients. Steroid resistance was significantly higher among non -proliferative GN compared to proliferative GN.

The cause of these findings may be a better response to steroid therapy among the proliferative GN group and a tendency of higher glom. CXCR3 expression in the group of patients with better steroid response as shown in our study. Similarly, a study working on 148 renal allograft biopsies reported that, in patients with acute vascular rejection, the area of CXCR3-positive cells decreased significantly after treatment with high dose steroids (prednisolone)<sup>11</sup>.

Our study showed that glom. and int. renal expression of CXCR3 did not bear any significant effect on the cumulative dose of corticosteroids used by our patients. However, we found that cumulative steroids doses were higher among those with strong glom marker expression. This may be due to strong inflammation with enhanced expression of CXCR3 that needs more anti-inflammatory treatment. Fufuichi et al., (2000) studied the biopsies of 38 patients with several renal diseases, including 13 crescentic glomerulonephritis patients. The results suggested that chemokine receptor signaling may be pivotal for human renal diseases through the recruitment and activation of T cells; which may participate in glomerular and interstitial lesions and a resolving phase occurred after glucocorticoid therapy<sup>12</sup>. Also a genome-wide mRNA expression analysis of glomeruli microdissected from lupus mice was performed. Renal histology revealed mesangial proliferative glomerulonephritis with cellular infiltration of macrophages, T cells and neutrophils. Of note is the finding that chemokines and chemokine receptors CXCR3 that are related to T helper 1 (Th1) cells accumulation were up-regulated concomitantly with increased mRNA expression of many genes that were inducible by Th1 cytokine interferon-gamma. Prednisolone (10 mg/kg per day) for 4 weeks was orally given. Markedly attenuated glomerular lesion and leukocyte influx parallel with the reduction of enhanced gene expression have occurred after prednisolone administration<sup>13</sup>.

In our study, we compared 24 hours urinary protein among patients in both glom. and int. renal tissue. However, proteinuria failed to show a significant variation among both groups despite the

issue that 24 hours urinary protein was found to be higher among those with strong marker expression on the glom. level. This can be explained by the suggestion that podocytes express functional CXCR receptors which contribute to podocyte damage and proteinuria during glomerular diseases. The release of oxygen radicals that accompanies the activation of CXCRs may contribute to podocyte injury and the development of proteinuria<sup>5</sup>.

In the same series, comparison of kidney functions namely serum creatinine and BUN levels among patients with mild/mod. versus those with strong marker expression on the glomerular level and absent versus mild/ mod. on the interstitial levels was done. The mentioned investigations though statistically non -significant were found to be higher among those with strong glom. CXCR3 expression. This could be due to the fact that 4 patients with strong glom. CXCR3 staining presented with severe manifestations and renal impairment compared to 2 patients presented with normal kidney functions. A study found that there was no significant difference between CXCR3<sub>-/-</sub> and CXCR3<sub>+/+</sub> wild-type mice as regards serum creatinine, blood urea nitrogen level and serum albumin<sup>8</sup>. On the other hand, a study found a higher degree of correlation between CXCR3-positive leukocyte infiltration, global glomerulosclerosis, and renal dysfunction. The highly significant correlation of the CXCR3-positive cell infiltrate with various parameters of renal dysfunction points toward the CXCR3-positive T cells as important mediators in progression of renal disease<sup>10</sup>.

In a similar way, serum triglycerides (TGs) were found to be higher in our group of patients with strong glom. CXCR3 expression as compared to with those with mild /mod. marker expression though statistically non- significant. This is in agreement with a study mentioning that IFN- $\gamma$ -inducible chemokines CXCL9, CXCL10, and CXCL11 have been detected in endothelial cells, submucosal cells, and macrophages in atheromas, and CXCR3, has been observed on lesional T cells, suggesting that CXCL10 likely stimulates atherogenesis and CXCR3 may have a pathogenic role in early stages of atherosclerosis<sup>14</sup>. Similarly, another study showed that treatment with a CXCR3 antagonist results in attenuating atherosclerotic lesion formation by blocking direct migration of CXCR3<sup>+</sup> effector cells from the circulation into the atherosclerotic plaque and by beneficially modulating the inflammatory response in the lesion<sup>15</sup>.

This was in agreement with reported enhanced expression of CXC-chemokine (IP-10/CXCL10) within human atherosclerotic lesions and its corresponding receptor. The role of chemokines in atherosclerosis was further supported by several studies showing that modified LDL particles are potent inducers of chemokines in various cells such as macrophages and vascular smooth muscle cells suggesting that chemokines may represent a link between lipids and inflammation in atherogenesis. In addition several other leukocyte/macrophage responses such as cell proliferation enzyme secretion, induction of reactive oxygen species, and promotion of foam cell formation have been observed in vitro after chemokine stimulation<sup>16</sup>.

Among our patients, hypertension was more prevalent in those with mild/mod. glom. CXCR3 expression. A finding which was also statistically non-significant. A study found that, the absence of CXCR3 in anesthetized and conscious mice led to increased angiotensin II type 1 receptor (AT1R) expression in mesenteric arteries and whole kidney protein extracts and thus leading to increased vasoconstriction, which in turn results in hypertension<sup>17</sup>

In conclusion, glom. CXCR3 renal expression rather than int.CXCR3 seems to have a pathogenetic effect in proliferative GN. Our study revealed that enhanced CXCR3 renal expression on glomerular and interstitial levels did not affect the response to steroids along the course of the disease and so can probably act as a therapeutic target rather than a prognostic marker.

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