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Mesangial hypercellularity predicts antiproteinuric response to dual blockade of RAS in primary glomerulonephritis

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The greater antiproteinuric efficacy of converting enzyme inhibitor and angiotensin II receptor blocker combination (CEI + ARB), versus monotherapy with either drug, is not a consistent finding. We evaluated the clinicopathologic predictors of response to CEI + ARB in 43 patients with primary glomerulonephritis (GN), never treated with immunosuppressive drugs, and with persistent proteinuria after CEI alone. Main histological lesions were analyzed by obtaining on 557 glomeruli and 165 arteries formal score of mesangial cellularity, glomerulosclerosis, tubulointerstitial damage, mononuclear cell infiltration, arteriosclerosis, and arteriolar hyalinosis. Duration of CEI and CEI + ARB therapy was similar (4.7 ± 2.4 and 5.0 ± 1.5 months). Proteinuria (g/day) decreased from 3.5 ± 2.9 to 2.4 ± 2.3 after CEI, and to 1.5 ± 1.3 after CEI + ARB ($P < 0.0001$). Reduction of proteinuria after CEI + ARB was greater in proliferative versus non-proliferative GN (−63.3 ± 23.4 versus 42.4 ± 23.7%, respectively; $P = 0.006$). When patients were categorized in responders and non-responders to CEI + ARB, no difference between the two groups was detected in any demographic or clinical variable, whereas histology showed in responders a greater prevalence of proliferative GN (71.4 versus 31.8%, $P = 0.009$) and higher score of mesangial cellularity (1.76 ± 0.53 versus 1.20 ± 0.22, $P < 0.0001$). At multiple regression analysis ($r^2 = 0.476$, $P = 0.001$), response to CEI + ARB resulted independently related only to mesangial cellularity ($P < 0.0001$). In conclusion, the best independent predictor of antiproteinuric efficacy of CEI + ARB in patients with primary GN is the degree of mesangial cellularity. This finding supports the experimental evidence that high angiotensin II contributes to proliferation of mesangial cells.

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The worldwide expansion of population with chronic kidney disease (CKD), the high cost of renal replacement therapies, and their scarce availability in underdeveloped countries as well, have made imperative a major change in the clinical approach to CKD from treatment of advanced stages to much more aggressive primary and secondary prevention.[1] According to this goal, reduction of proteinuria has become the cornerstone of conservative treatment of CKD;[2,3] proteinuria, in fact, is now recognized as the main independent determinant of onset and progression of renal insufficiency.[4,5] As angiotensin II (AII), proteinuria, and worsening of renal function are intimately connected,[4–6] it has been proposed to maximize inhibition of renin–angiotensin system (RAS) to the antiproteinuric effect and to start such a treatment early in the course of disease, that is, well before the development of overt renal insufficiency.[2–4,7]

Dual blockade of RAS, by combining converting enzyme inhibitors (CEI) with AII type 1 receptor blockers (ARB), has stimulated a great interest in the nephrology community as small-scale clinical studies have documented, on average, a greater antiproteinuric effect of combination treatment, as compared to monotherapy with either CEI or ARB, in patients with different chronic renal diseases.[8–10] The therapeutic advantage has been strengthened by the large combination treatment of angiotensin II receptor blocker and angiotensin-converting enzyme inhibitor in non-diabetic renal disease (COOPERATE) trial that demonstrated halving of risk of end-stage renal failure of dual blockade versus monotherapy with either CEI or ARB.[11]

The larger antiproteinuric response to combination therapy in patients with glomerulonephritis (GN), however, is not a consistent finding. In patients with either membranous nephropathy or focal-segmental glomerulosclerosis,[12] our group did not confirm the higher efficacy of this intervention observed in immunoglobulin A nephropathy (IgAN).[8,9] Heterogeneous response to combined

treatment was also hypothesized by the investigators of the COOPERATE trial.[11] Whether the different antiproteinuric effect is related to the type of renal lesions or clinical characteristics of patients, or changes of systemic or renal hemodynamics during treatment, remains undefined. This point is crucial because maximal suppression of RAS is costly and not totally exempt from relevant side effects, such as hyperkalemia and acute renal failure.[10] Knowledge of clinical and histological predictors of high antiproteinuric response to CEI and ARB combination is also critical to gain more insights into the mechanisms underlying the superiority of dual blockade of RAS. This information may encourage nephrologists at identifying and treating the patients who will potentially gain most benefit from combined therapy. Nowadays, in fact, less than 5% of CKD patients regularly followed by nephrologists are receiving co-administration of CEI and ARB in spite of significant proteinuria.[13,14]

This study was aimed at evaluating the potential demographic, clinical, and morphological (type and severity of renal lesions) predictors of antiproteinuric response to CEI + ARB in patients with a variety of primary GNs and persistent proteinuria after CEI alone.

RESULTS

Seventy-two patients were selected on the basis of inclusion criteria. We excluded 15 patients treated with steroids or immunosuppressive agents, two patients who started CEI more than 6 months after biopsy, three patients treated for less than 2 months with either CEI or CEI + ARB, three patients with inadequate biopsy specimen, and six patients with incomplete data collection and/or inaccurate 24-h urine collection. Forty-three patients were therefore included in the final analysis. Histological scores were obtained on 557 glomeruli and 165 arteries. A good agreement between the two observers in scoring histological lesions was testified by kappa index >0.75 for all the examined scores.

Demographic and clinical characteristics of enrolled patients, which were not different from those of excluded patients (data not shown), are reported in Table 1. All patients had proteinuria >1 g/day at the time of renal biopsy. When patients were categorized on the basis of type of glomerular lesion traditionally prominent (proliferative or non-proliferative GN), patients with proliferative GN were characterized by younger age, lower proteinuria, and higher serum albumin level, as well as by higher score of mesangial cellularity (Table 2).

In the whole group of patients, combination therapy was more effective than CEI in decreasing proteinuria (-53.1 ± 25.5 versus -28.3 ± 28.7%, $P<0.0001$). In particular, as depicted in Figure 1, the percentage decrement of proteinuria after CEI + ARB was significantly greater in proliferative than in non-proliferative GNs. As expected, the difference disappeared when considering the absolute reduction (-1.55 ± 1.53 and -2.47 ± 2.78 g/day, respectively; $P=0.191$) because of the different basal levels of proteinuria. The antiproteinuric response to combination did not correlate with basal proteinuria ($r=-0.151$, $P=0.335$); the same held true when examining separately proliferative and non-proliferative GNs. Similarly, it did not differ between patients with and without nephrotic proteinuria ($\geqslant 3.5$ g/day) at baseline (-56.4 ± 24.3 and -51.3 ± 26.4%, respectively; $P=0.438$). Finally, the efficacy of combination treatment was not influenced by the specific diagnosis of IgAN; in this subclass of GN, in fact, entity of proteinuria reduction after CEI + ARB was analogous to that found in the other GNs (-59.3 ± 20.2 and -49.4 ± 27.9%, respectively; $P=0.227$).

As reported in Table 3, the progressive decrement of proteinuria was associated with a slight but significant improvement of serum albumin levels. Both systolic and diastolic blood pressure (BP) were diminished by CEI and by CEI + ARB; however, the entity of systolic/diastolic BP reduction did not significantly differ between monotherapy and combined therapy (CEI: $-6.5 \pm 12.2/-3.2 \pm 13.5$%; CEI + ARB: $-9.1 \pm 10.0/-6.1 \pm 9.3$%). Estimated glomerular filtration rate (GFR) also diminished after combination therapy, but, again, the changes were similar to those observed after CEI alone. Neither hemoglobin nor serum potassium was affected by the magnitude of RAS inhibition. Daily sodium excretion did not differ from baseline to CEI + ARB, testifying a constant salt intake in the three periods (10.5 ± 4.2, 10.6 ± 3.6, and 10.5 ± 5.4 g NaCl/day).

The administered CEIs were ramipril ($n=25$, mean dose: 5.6 ± 1.7 mg/day, range: 5–10), enalapril ($n=10$, mean dose: 19.0 ± 3.2 mg/day, range: 10–20), and lisinopril ($n=8$, mean dose: 18.8 ± 3.5 mg/day, range: 10–20). The three CEIs used had the same antiproteinuric effect ($P=0.521$). In the combination therapy, two different ARBs were added, while maintaining unchanged type and dose of CEI; specifically, irbesartan was used in 29 patients (mean dose: 295 ± 28 mg/day, range: 150–300) and losartan in 14 patients (mean dose: 89 ± 21 mg/day, range: 50–100). Similarly to CEI, add-on therapy with either ARB led to analogous antiproteinuric response ($P=0.209$). Type and dose of other antihypertensive drugs, prescribed in six patients (furosemide $n=1$, amlodipine $n=3$, doxazosin $n=2$), did not change throughout the study.

When patients were divided in responders and non-responders to CEI + ARB (Table 4), no major clinical difference, except for the extent of proteinuria reduction, became manifest. Also, the duration of combined therapy was

Table 1 | Demographic and clinical characteristics of patients

Gender (M:F)	28:15
Age (years)	38.2 ± 16.1
Body mass index (kg/m^2)	26.1 ± 4.0
Proteinuria (g/day)	3.51 ± 2.85
Serum creatinine (mg/dl)	1.03 ± 0.27
Duration of CEI alone (months)	4.7 ± 2.4
Duration of CEI+ARB (months)	5.0 ± 1.5

ARB, angiotensin II receptor blocker; CEI, converting enzyme inhibitor; F, female; M, male.

Table 2 | Comparison of main basal clinical and pathological features in patients with proliferative (*n*=22) and non-proliferative GN (*n*=21)

	Proliferative GN	Non-proliferative GN	*P*-value
Diagnosis	IgAN (*n*=16)	MN (*n*=13)	—
	MesP (*n*=5)	FSGS (*n*=7)	—
	MP (*n*=1)	MC (*n*=1)	—
Age (years)	31.8±12.6	44.8±17.1	0.007
Male gender (%)	59.1	71.4	0.396
Proteinuria (g/day)	2.27±1.49	4.80±3.35	0.004
Serum creatinine (mg/dl)	1.01±0.31	1.05±0.24	0.637
eGFR (ml/min/1.73 m^2)	105±33	94±24	0.216
Serum albumin (g/dl)	3.92±0.61	3.46±0.68	0.023
Systolic BP (mmHg)	125±19	132±18	0.202
Mesangial cellularity	1.69±0.56	1.25±0.26	0.002
Cronicity index	6.84±3.53	7.05±3.04	0.838

BP, blood pressure; eGFR, GFR estimated by Cockroft-Gault equation; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; IgAN, immunoglobulin A nephropathy; MC, minimal change glomerulonephritis; MesP, mesangioproliferative glomerulonephritis; MN, membranous nephropathy; MP, membranoproliferative glomerulonephritis.

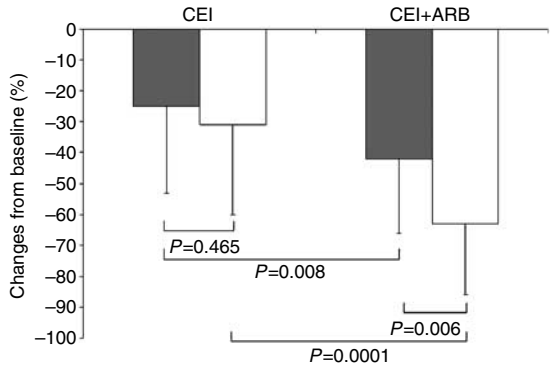

Figure 1 | Changes of proteinuria from baseline after monotherapy and combination therapy in 21 patients with (gray bars) non-proliferative and in 22 patients with (white bars) proliferative GN.

similar (5.2±1.6 and 4.8±1.4 months, $P=0.480$). On the other hand, when examining the histological patterns of biopsies (Table 5), the responder group was characterized by greater prevalence of proliferative GNs and higher score of mesangial proliferation than non-responder group, whereas severity of other renal lesions did not differ. In agreement with the observation of a similar entity of Uprot reduction after CEI + ARB in patients with and without IgAN, the prevalence of IgAN did not differ between responders and non-responders ($P=0.287$). Correlation analysis demonstrated that the extent of antiproteinuric response to CEI + ARB and mesangial score were strictly associated ($r=0.573$, $P<0.0001$); this association similarly occurred in proliferative ($r=0.501$) and non-proliferative ($r=0.512$) glomerulonephritides (Figure 2). On the basis of these results, we used multiple regression analysis to determine the independent role of the main potential predictors of antiproteinuric response to CEI + ARB (Table 6). This analysis, which explained almost 50% of variance of percent change of proteinuria, identified mesangial score as the sole independent predictor of antiproteinuric response to combination therapy.

Table 3 | Changes of main clinical and laboratory parameters during follow-up

	Baseline	CEI	CEI+ARB
Proteinuria (g/day)	3.51±2.85	2.42±2.32[a]	1.51±1.33[b,c]
Body weight (kg)	75.4±15.3	74.0±13.0	74.6±14.0
Serum albumin (g/dl)	3.69±0.68	3.97±0.62[a]	3.98±0.65[a]
Systolic BP (mmHg)	128±18	120±22[a]	116±18[b]
Diastolic BP (mmHg)	80±12	76±10	74±12[a]
Serum creatinine (mg/dl)	1.03±0.27	1.06±0.33	1.13±0.34[d]
eGFR (ml/min/1.73 m^2)	100±29	100±32	94±32[d]
Hemoglobin (g/dl)	13.9±1.6	14.0±1.5	13.8±1.3
Serum potassium (mEq/l)	4.5±0.3	4.5±0.4	4.6±0.5
Sodium excretion (mEq/day)	178±71	180±61	176±91

ARB, angiotensin II receptor blocker; BP, blood pressure; CEI, converting enzyme inhibitor; eGFR, GFR estimated by Cockroft–Gault equation.
[a]$P<0.01$ versus baseline.
[b]$P<0.001$ versus baseline.
[c]$P<0.01$ versus CEI.
[d]$P<0.05$ versus others.

DISCUSSION

Suppression of AII activity is the main intervention indicated to reduce proteinuria and retard progression of renal disease in CKD patients.[2,3,15] However, CEI alone allows only a modest reduction of proteinuria and does not satisfactorily reduce progression toward end-stage renal disease.[6–12,16] A likely explanation derives from experimental evidence that concentration of AII is much higher in the kidney than in plasma,[17] and that CEI administration, while completely inhibiting systemic AII production, does not significantly affect intrarenal AII because most of it is formed via non-angiotensin-converting enzyme-dependent pathways, such as that of chymase.[17,18] This hypothesis has been confirmed by clinical studies that evidenced in the human kidney the predominant role of angiotensin-converting enzyme-independent generation of AII in states of elevated RAS activity.[10,19–21] Noteworthy, a similarly moderate nephroprotective efficacy has been found for ARBs,[8–12] likely because in the course of ARB monotherapy, the high AII concentrations reached can overcome inhibition of type 1 AII receptor.[10] On the contrary, in the presence of activated RAS, maximal reduction of intrarenal AII has been obtained by combining low doses of CEI + ARB, with the decrement being greater than that observed after administration of higher doses of either agent alone.[22] Overall, these data support the use of combined treatment in conditions characterized by high intrarenal AII levels. This study verifies, for the first time in patients with various biopsy-proven primary GNs, the validity of this rationale behind the administration of CEI + ARB.

When considering the whole group of subjects, reduction of proteinuria after CEI + ARB was almost the double than

Table 4 | Distribution of demographic and clinical characteristics according to responders and non-responders to CEI+ARB

	Responders (n=21)	Non-responders (n=22)	P-value
At baseline			
Age (years)	37.3 ± 18.1	39.0 ± 14.5	0.743
Male gender (%)	66.7	63.6	0.835
Body mass index (kg/m^2)	26.0 ± 3.8	26.2 ± 4.3	0.894
Basal proteinuria (g/day)	3.83 ± 3.29	3.20 ± 2.39	0.472
Serum creatinine (mg/dl)	0.96 ± 0.27	1.09 ± 0.27	0.123
eGFR (ml/min/1.73 m^2)	106 ± 33	95 ± 24	0.229
Serum albumin (g/dl)	3.68 ± 0.67	3.71 ± 0.71	0.890
Systolic BP (mmHg)	129 ± 21	128 ± 16	0.907
Diastolic BP (mmHg)	80 ± 14	80 ± 10	0.943
After treatment			
Δ proteinuria (g/day)	−2.94 ± 2.75	−1.09 ± 1.07	0.008
Δ proteinuria (%)	−75.6 ± 11.7	−31.7 ± 13.7	<0.0001
Δ eGFR (%)	−6.2 ± 17.5	−7.6 ± 16.2	0.785
Δ systolic BP (%)	−10.6 ± 10.3	−7.7 ± 9.7	0.352
Δ diastolic BP (%)	−5.8 ± 12.4	−6.4 ± 12.5	0.874
Sodium excretion (mEq/day)	184 ± 79	173 ± 64	0.666

ARB, angiotensin II receptor blocker; BP, blood pressure; CEI, converting enzyme inhibitor; eGFR, GFR estimated by Cockroft-Gault equation; Δ, change versus baseline.

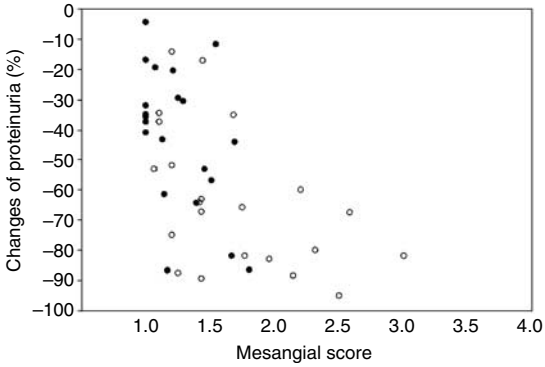

Figure 2 | Association between score of mesangial cellularity and percentage changes of proteinuria after CEI + ARB therapy (r = 0.573; P < 0.0001). Black circles identify patients with non-proliferative GN, whereas open circles identify patients with proliferative GN.

that detected after CEI alone (Table 2). Although BP values declined in parallel with the enhancement of RAS inhibition, no difference in the entity of BP control emerged between the two phases of the study; this finding, which this study shares with other works,[8–12,23–26] suggests that the major antiproteinuric effect of combination therapy is largely independent from its antihypertensive action. Similarly, the additive effect of a lower salt intake on proteinuria reduction can be excluded in this study because sodium excretion did not change throughout the period of observation. Hence, the present study confirms in patients with a variety of GNs that combination therapy leads, on average, to a greater antiproteinuric effect, which appears specifically related to a more profound inhibition of RAS.

More important, a novel question here addressed is whether the antiproteinuric response to CEI + ARB is unpredictable or depends on some particular characteristics of patients. The additional value of combination treatment, in fact, is not a consistent finding. Specifically, in non-diabetic CKD, as in the case of our patients, a greater antiproteinuric effect has been reported by studies that exclusively, or for the most part, enrolled patients with IgAN,[8,9,11,23,24] while conflicting results have been obtained in patients with non-proliferative GN.[12,25,26] That combination

Table 6 | Multiple linear regression analysis of antiproteinuric response to CEI+ARB

	β Coefficient	P-value
Constant	18.427	0.233
Age (years)	−0.413	0.110
Gender (female as reference)	4.931	0.483
Δ systolic blood pressure (%)	0.514	0.130
Δ eGFR (%)	−0.325	0.144
Mesangial score	−27.603	0.001
Cronicity index	−1.272	0.252
Type of GN (NP as reference)	−11.251	0.155

ARB, angiotensin II receptor blocker; CEI, converting enzyme inhibitor; eGFR, GFR estimated by Cockroft-Gault equation; GN, glomerulonephritis; NP, non-proliferative; Δ, change versus baseline.
Model summary: r^2=0.476, F=4.540, P=0.001.

Table 5 | Histological scores in responders and non-responders to CEI+ARB

	Responders (n=21)	Non-responders (n=22)	P-value
Proliferative GN (% patients)	71.4	31.8	0.009
Diagnosis of GN	10 IgAN, 4 MesP, 1 MP, 5 MN, 1 FSGS	6 IgAN, 1 MesP, 8 MN, 6 FSGS, 1 MC	—
Mesangial cellularity	1.76 ± 0.52	1.20 ± 0.22	<0.0001
Interstitial fibrosis	2.48 ± 0.80	2.09 ± 1.15	0.211
Tubular atrophy	2.24 ± 0.96	1.93 ± 1.20	0.361
Interstitial infiltrates	1.62 ± 0.95	1.27 ± 1.20	0.302
Glomerulosclerosis	1.90 ± 0.52	1.77 ± 0.77	0.513
Arteriosclerosis	0.57 ± 0.81	0.73 ± 1.07	0.594
Arteriolar hyalinosis	0.88 ± 1.22	0.80 ± 1.25	0.822
Cronicity index	7.32 ± 2.83	6.58 ± 3.65	0.462

ARB, angiotensin II receptor blocker; CEI, converting enzyme inhibitor; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; IgAN, immunoglobulin A nephropathy; MC, minimal change glomerulonephritis; MesP, mesangioproliferative glomerulonephritis; MN, membranous nephropathy; MP, membranoproliferative glomerulonephritis.

therapy has different efficacy in subtypes of renal diseases has also been hypothesized by the authors of the COOPERATE study.[11] Besides the published data, also in clinical practice, it is common to observe incongruity in response to the dual blockade of RAS.

We addressed this problem by evaluating for the first time several clinopathologic correlates to antiproteinuric response to combination therapy. Indeed, the efficacy of any antiproteinuric intervention depends on several factors, such as age, gender, length of treatment, and changes of BP or GFR. It can also be influenced by less known clinical factors, such as body mass index, serum albumin, and salt intake.[27–29] Similarly, type and severity of renal lesions have an effect on proteinuria independently from underlying GN.[30–33] When we categorized patients in responders and non-responders to CEI + ARB, no significant difference emerged from the several demographic and clinical variables examined (Table 3). In particular, a comparable pattern of changes of both BP and estimated GFR was detected between the two groups. Also, basal proteinuria level did not affect response. Similarly, the antiproteinuric efficacy of combination was not specifically related to diagnosis of IgAN.

On the other hand, the histological study provided important information. We found similarities in cell infiltrates and in the specific histological markers of cronicity of GN, and of abnormalities of intrarenal arterial vessels as well; these negative findings, therefore, exclude a role of acute inflammation or senescence of renal structures in the quality of response. In contrast, the subgroup of patients highly responsive to CEI + ARB was characterized by a significantly greater prevalence of proliferative GNs. Indeed, these patients showed a greater decrement of proteinuria after CEI + ARB with respect to those with non-proliferative GN (Figure 1). Similarly, a significantly higher mesangial score was detected in responders. Indeed, as depicted in Figure 2, entity of mesangial cellularity and magnitude of antiproteinuric effect of dual RAS suppression strictly correlated with the association being similar in proliferative and non-proliferative GNs. Hence, the degree of mesangial cellularity influences response independently from the type of glomerular disease. To gain more insights into the independent role of these two histological figures – type of GN and mesangial score – a multiple regression analysis was run. The results confirm that the entity of mesangial cellularity is the sole independent predictor of the antiproteinuric response to dual blockade of RAS.

These findings therefore disclose a linkage between mesangial cellularity and AII. The contribution of AII in the pathophysiology of proteinuria and progression of primary GNs has been amply described.[20,34–36] A recent study, moreover, has provided evidence that the glomerular expression of angiotensin-converting enzyme, chymase, and AII receptors correlates with the degree of mesangial hypercellularity in patients with IgAN, suggesting that locally synthesized AII is involved in this renal lesion.[37] Indeed, the mitogenic effect of AII on cultured mesangial cells is known from long time.[38,39] In addition, mesangial hypercellularity is associated with high sensitivity to the beneficial effects of CEI and ARB on glomerular structure and function,[35] whereas lysis of mesangial cells prevents any glomerular effect of AII.[40] Of note, mesangial hypercellularity is not limited to proliferative GNs, but it can also be observed in non-proliferative GNs, as in the case of this study. This occurs possibly because of the stimulation of intrarenal AII production secondary to the tubular overload of proteins.[41,42] Hence, the higher response to CEI + ARB in the presence of mesangial hypercellularity suggests that this lesion is, at least in part, determined by high intrarenal AII levels.

Limitations of the study are mainly inherent to the small sample size and the retrospective nature of analysis. Therefore, the results obtained, while reflecting clinical practice, should be confirmed by large prospective clinical trials. Furthermore, effectiveness of ARB alone was not investigated; nevertheless, several previous studies have shown analogous efficacy of ARB and CEI monotherapies.[8–12,23–26] Slightly different therapeutic regimens were also used; however, drug doses were therapeutically equivalent and analysis excluded a specific drug effect in agreement with the previous studies.[8–10] Finally, results may not be extrapolated to patients with advanced kidney disease or patients treated with immunosuppressive agents; on the other hand, this exclusion is compatible with the aim of this study that required adequate analysis of the morphological lesions.

Conclusion

This study provides evidence that in heterogeneous population of GN patients, the antiproteinuric effect of dual blockade of RAS is significantly greater in proliferative than in non-proliferative GNs. The best independent predictor of antiproteinuric response to CEI + ARB is the extent of mesangial cellularity. A reasonable explanation to this finding is that, as suggested in experimental studies, high intrarenal AII levels contribute to mesangial hypercellularity. Such a specific lesion therefore identifies patients who will gain most benefit from the combination therapy. In this subgroup of patients, the beneficial effect of intense RAS inhibition is probably not limited to the larger reduction of proteinuria; evidence has been in fact collected on the possibility to attain by means of RAS suppression the regression of renal disease, with the reversal of mesangial hypercellularity being a main initial event.[3,36,43–45]

MATERIALS AND METHODS

Patients

We conducted in three Nephrology Units (Second University of Naples, University Federico II of Naples, and University of Catanzaro) a retrospective analysis of all consecutive patients with primary biopsy-proven GN, which in the first semester of 2005 were treated with combination therapy (CEI + ARB) because of proteinuria >0.5 g/day, persistent after CEI alone.

Secondary GN forms were excluded according to traditional histological, clinical, and serological criteria. Additional exclusion criteria were: previous therapy, at any point of time of the clinical

history, with either steroids or immunosuppressive agents, treatment with RAS inhibitors before biopsy, delayed treatment with CEI (started more than 6 months after biopsy), treatment with either CEI or CEI + ARB lasting less than 2 months, inadequate biopsy specimen (less than seven glomeruli and one artery), incomplete collection of essential data, or inaccurate 24-h urine collection.

Clinical data

Data were extracted from clinical charts at three time points: last available evaluation after biopsy and before starting CEI (BASAL), last available evaluation of the effects of CEI monotherapy before prescription of combined therapy (CEI), and last evaluation of the effects of combination therapy performed in the first semester of 2005 (CEI + ARB). Data considered essential for the analysis at each time point of the study were: age, gender, body weight, BP, therapeutic regimen, serum levels of creatinine, albumin, and potassium, hemoglobin, 24-h urinary excretion of sodium, creatinine, and proteins.

The three participating centers shared the following routine features: presence of outpatient clinic dedicated to the conservative care of CKD; presence of clinical and laboratory standardized protocols, including three consecutive measurements of BP by a mercury sphygmomanometer (in the morning before drug administration) 5 min apart in sitting position after 10 min of rest, with Korotkoff phases I and V defining systolic and diastolic values, repeated after 5 min in standing position to detect orthostatic hypotension; measurement of creatinine in plasma and urine performed by means of the modified kinetic Jaffé reaction; and measurement of proteinuria by pyrogallol red-molibdate method.

Twenty-four hour urine collection was considered inaccurate if the value of measured creatinine excretion rate was outside the 60–140% range of the value calculated according to Dwyer and Kenler.[46] Daily salt intake (g/day) was calculated dividing 24-h urinary sodium excretion by 17. We estimated GFR by Cockcroft and Gault equation because our patients had normal or nearly normal renal function, and under these conditions, 24-h measured creatinine clearance overestimates GFR, whereas Modification of Diet in Renal Disease equation adequately predicts GFR in patients with more advanced CKD.[14,47] The GFR value was standardized to a body surface area of 1.73 m^2.

The median of percent reduction of Uprot after CEI + ARB (53%, range 4–95) was used to classify patients in responders (Uprot decrease $>53\%$) and non-responders (Uprot decrease $\leqslant 53\%$).

Kidney biopsy study

Two investigators independently reviewed kidney biopsies, and the means of individual scores were used for analysis. Diagnosis and classification of GN (proliferative and non-proliferative) were made according to traditional criteria.[48] Light microscopical changes were semiquantitatively estimated according to a scoring system previously used by our group,[49] which is partly based on the Banff schema.[50,51] The following variables were graded (0, 1, 2, and 3 that correspond to normal, mild, moderate, and severe, respectively): interstitial fibrosis/tubular atrophy, interstitial mononuclear cell infiltration glomerulosclerosis (GSC), arteriosclerosis, and arteriolar hyalinosis. In particular, we graded interstitial fibrosis/tubular atrophy and mononuclear cell infiltration on the basis of the extent of involved area, GSC on the basis of number of involved glomeruli and intraglomerular extent of sclerosis, arteriosclerosis on the basis of severity of the fibrointimal thickening of arteries, and arteriolar hyalinosis on the basis of the percentage of the circumference of arterioles affected by periodic acid Schiff-positive insudation or hyaline thickening. The cronicity index of each biopsy was defined by the sum of the scores of arteriosclerosis + arteriolar hyalinosis + GSC + mononuclear cell infiltration + interstitial fibrosis/tubular atrophy. We also graded mesangial proliferation on the basis of the number of mesangial cells per mesangial area (score 1 = 0–3 cells, score 2 = 4–5 cells, score 3 = 6–7 cells, and score 4 = $\geqslant$8 cells); each score was multiplied for the number of glomeruli with that score and then normalized for the total number of glomeruli according to the following formula:

$$\text{Mesangial score} = \text{NG1/TNG} + \text{NG2/TNG} + \text{NG3/TNG} + \text{NG4/TNG}$$

where NG1–4 is the number of glomeruli with that specific score (1–4) and TNG is the total number of glomeruli.

Statistical analysis

Values are reported as mean $\pm$ s.d. We used for analysis of continuous variables only parametric methods because Shapiro–Wilk test did not reject the hypothesis of normal distribution. Differences between responders and non-responders were evaluated by unpaired Student's *t*-test, whereas paired Student's *t*-test was used to detect intragroup differences. Analysis of variance for repeated measurements was used to compare data at baseline, after CEI, and after CEI + ARB. Bonferroni *post hoc* test was also used. Multiple linear regression analysis was used to identify the predictors of antiproteinuric response (percent changes of proteinuria from baseline) to CEI + ARB. The model was built by identifying *a priori* the main potential determinants of antiproteinuric response among demographic features (age and gender), laboratory and clinical variables (percent changes of estimated GFR and systolic BP), and histological patterns (mesangial score, cronicity index, and type of GN – proliferative, non-proliferative). Pearson's correlation coefficient was also used. Agreement between the two observers on histological scores was verified by kappa index.[52] Data were analyzed using SAS version 8.1 (SAS Inc., Cary, NC, USA). $P<0.05$ was considered statistically significant.

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