Successful Treatment of Membranous Glomerulonephritis with Rituximab in Calcineurin Inhibitor-Dependent Patients

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Background and objectives: Calcineurin inhibitors (CNIs) induce remission of proteinuria in most nephrotic patients with membranous glomerulonephropathy (MGN). However, 60% of patients become treatment dependent and are at risk of chronic nephrotoxicity. The aim of this study was to evaluate the efficacy of rituximab in patients with long-term dependence on CNIs.

Design, setting, participants, and measurements: Thirteen patients with MGN, normal renal function, and proven dependence on CNIs, despite previous treatment with other immunosuppressant drugs, received a single trial of four weekly doses of rituximab (375 mg/m^2). Outcome measures were the percentage of patients with CNI withdrawal and no evidence of relapse and the percentage of patients with complete or partial remission 30 mo after CNI withdrawal.

Results: After rituximab, proteinuria decreased significantly (2.5 ± 0,76 basal *versus* 0.85 ± 0.17 at 6 mo; $P = .0003$). CNIs and other immunosuppressant drugs could be withdrawn in all patients with no evidence of relapse. After CNI withdrawal, GFR increased significantly (90.3 ± 15 basal to 106.4 ± 20 at 3 mo with a mean increase of 15.3% [range 0–20]). Three patients suffered a relapse of nephrotic proteinuria 19, 23, and 28 mo after rituximab treatment; all were successfully treated with a second course of rituximab. At 30 mo, all patients were in remission.

Conclusions: In patients with MGN with long-term CNI dependence, rituximab can be an effective tool to overcome dependence on CNI, thus avoiding the risk of nephrotoxicity related to the chronic exposure to these drugs.

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Membranous glomerulonephropathy (MGN) is the most frequent cause of nephrotic syndrome in adults. There is general agreement that patients with persistent nephrotic syndrome are at risk of developing progressive renal insufficiency (1–4). In these patients, prospective randomized clinical trials have demonstrated that the calcineurin inhibitors (CNIs) cyclosporine (5,6) and tacrolimus (7) induce complete or partial remission of proteinuria in more than 70% of patients. However, more than 60% of patients treated with CNI suffer subsequent relapses or become treatment dependent (5–8) and need prolonged therapy to maintain remission, which exposes them to the nephrotoxic effects of this drugs. Consequently, for these patients, there is a need for the development of new treatment strategies aimed at reducing the risk of chronic nephrotoxicity. MGN is an antibody-mediated disease induced by deposits of immunoglobulins and complement components on the subepithelial layer of the glomerular capillary wall (9). This immune deposition promotes injury to the glomerular filtering barrier, proteinuria, and eventual renal failure (10). Infiltration of CD-20+ cells has also been demonstrated in renal biopsies of patients with MGN (11). Results in experimental MGN have shown that the inhibition of B cell function is associated with beneficial effects on proteinuria, (12) and human studies clearly demonstrated that the inhibition of B cells with alkylating agents induces remission of the nephrotic syndrome (13). The availability of monoclonal antibodies targeted to the cell surface antigen CD-20 of B cells permits an analysis of the effect of more selective and specific B cell inhibition in the outcome of several antibody-mediated diseases in clinical studies (14). In recent years, observational studies have shown that the administration of the anti-CD20 monoclonal antibody rituximab can reduce urinary protein excretion and preserve renal function in patients with MGN and persistent nephrotic syndrome (15–19).

This pilot observational study was conducted in patients with MGN with normal renal function, who experienced long-term dependence on CNI despite previous treatment with high-dose immunoglobulins and mycophenolate mofetil. The study aim was to evaluate whether a single course of rituximab could allow either dose reduction or withdrawal of CNI.

Patients and Methods

We recruited 13 patients with IMN who were being treated in two nephrology departments in Spain and showed evidence of long-term dependence on treatment with CNI (either cyclosporine or tacrolimus) and GFR higher than 60 ml/min calculated by endogenous creatinine clearance. CNI dependence was defined as the occurrence of at least

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four CNI-responsive relapses of nephrotic proteinuria while being weaned off these drugs. The last relapse after CNI dose reduction had to occur within the 2 mo before study entry. Exclusion criteria were pregnancy, infections (including hepatitis C and B and HIV), diabetes mellitus, malignancy, glomerulopathies other than GMN, or any systemic disease associated with GMN.

The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved the Ethics Committee, and written informed consent was provided by all participants. The Spanish Ministry of Health authorized the treatment with rituximab.

Treatment Protocol and Follow-Up

Follow-Up Before Study Admission. Before study entry, all patients were advised to ingest a diet containing 5 g salt/d and angiotensin converting-enzyme inhibitors (ACEIs) or AIIRA treatment for at least 9 mo. ACEIs or AII RA were titrated at their maximal tolerated doses. Amlodipine and other antihypertensive drugs were added when necessary to achieve a BP of $< 130/80$. Before CNI treatment, eight patients had been unsuccessfully treated with a 6-month trial of cyclophosphamide and prednisone and two patients were treated with chlorambucil and prednisone. The remaining three patients received CNI monotherapy as a first-line treatment.

During follow-up, after demonstration of CNI dependence, patients received treatment with other drugs in an effort to reduce the dose of CNI. Ten patients were treated with high-dose immunoglobulins (Igs; 0.4 g/Kg/d given for 4 consecutive days) for 6 mo, and 11 patients were treated with mycophenolate mofetil (MMF; (2 g/d) for at least 6 mo. Ig therapy did not allow either CNI dose reduction or withdrawal in any patient, whereas MMF allowed a CNI dose reduction in three patients, being ineffective in the rest. These three patients were maintained on combined treatment with CNI and MMF at the minimum dose required to maintain remission of proteinuria.

At study entry, four patients were receiving tacrolimus monotherapy, three were receiving tacrolimus with low-dose steroids (7.5 mg/d), three were receiving combined tacrolimus and MMF (1000 mg/d), and three were receiving monotherapy with cyclosporine. The doses of these drugs are shown in Table 1 and were maintained unaltered during the 4-wk period of treatment with rituximab.

Treatment Protocol. All patients received one intravenous infusion of rituximab (375 mg/m^2) per week for 4 consecutive weeks. Before each rituximab infusion, patients were pretreated with a single dose of 125 mg i.v. 6-methylprednisolone, 50 mg diphenhydramine, and 1 g i.v. propacethamol. One month after the last infusion of rituximab, both steroids and MMF were discontinued and the dose of CNI was tapered down at a rate of 30% every month until either discontinuation was achieved or evidence of relapse of nephrotic proteinuria (proteinuria > 3.5g/d) was noted. Relapses of proteinuria taking place within the period of CD-19 depletion were treated by either reintroducing or increasing the dose of CNI. Relapses occurring after CD-19 cell recovery were retreated with a second trial of rituximab at the minimum dose necessary to achieve the depletion of CD-19 cells. The doses of ACEIs were maintained unchanged during the whole follow-up. No patients received concurrent treatment with both ACEI and AIIRA agents or with aldosterone receptor antagonists. In addition to ACEIs, four patients were taking diuretics, six were taking statins, and five were taking amlodipine.

Patients were monitored weekly during rituximab treatment, every month during the 3 mo of CNI withdrawal, every 2 mo during the first year, and every 4 mo thereafter. Mean follow-up after rituximab treatment was 35 mo (range, 31 to 54 mo). In each control, we measured BP, proteinuria, serum creatinine, urea, endogenous creatinine clearance, immunoglobulins, albumin, and blood cell count including CD-19+ cells by flow cytometry. Measurements were performed by staining 50 μl of EDTA anticoagulated whole blood using MultiTEST CD3FITC, CD16+CD56PE, CD45PerCP, and CD19 APC reagent (BD) and lysing red blood cells (FACS Lysing solution), following manufacturer's instructions. Acquisition and analysis of stained samples was done on a FACSCalibur (BD) cytometer using MultiSET software. To evaluate whether the urine was properly collected we measured 24-h urinary

Table 1. Baseline clinical and biochemical characteristics of individual patients

Patient	Age	Sex	T RB	Time Tac/CsA	Tac/CsA Dose	Tac/CsA Trough Level	SBP	DBP	SCr	GFR	Alb	Prot	IgG
1	26	M	81	44	4	5.6	124	65	1.26	98	4.1	2.90	1330
2	32	M	49	51	4	7.0	123	54	1.34	100	4.0	2.60	1234
3	54	F	38	22	3	8.1	132	57	1.40	97	3.8	1.90	987
4	31	M	47	28	4	7.1	125	68	.98	101	4.0	2.20	1545
5	42	M	53	36	4	6.9	118	79	.87	97	4.1	3.20	1100
6	54	M	35	29	4	7.1	119	65	1.44	95	4.2	2.60	657
7	62	F	64	51	3	8.2	124	67	.89	131	4.0	3.10	1342
8	71	M	83	54	4	5.7	125	72	1.45	80	4.4	3.18	986
9	55	M	79	47	3.5[a]	198[a]	125	78	1.25	84	3.97	2.29	1134
10	45	M	90	80	4	7.7	114	69	1	80	3.8	0.7	1348
11	49	M	96	83	4	9	115	75	0.98	94	4.2	2.7	NA
12	38	M	75	21	3.6[a]	150[a]	106	65	0.85	115	3.8	1.85	NA
13	42	M	39	24	3.7[a]	175[a]	115	57	1.03	113	4.1	1.77	NA

TRB, time from renal biopsy in months; Time Tac/CsA, time on tacrolimus or cyclosporine A treatment in months; Tac/CsA dose, dose of tacrolimus (mg/day) or CsA (mg/Kg/day) at study entry; Tac/CsA trough levels, 12-trough levels (ng/ml); SBP, systolic blood pressure (mmHg), DBP, diastolic blood pressure (mmHg); SCr, serum creatinine (mg/dl); GFR, glomerular filtration rate (ml/min/1.73 m^2; Alb, serum albumin (g/L); Prot, proteinuria (g/day); IgG, serum levels of immunoglobulin G (mg/dl)

[a]Patients treated with CsA and CsA trough levels.

creatinine excretion and included samples with creatinine excretions within the physiologic range.

Before rituximab treatment, a kidney biopsy was performed in nine of thirteen patients. All biopsies were stained with hematoxylin-eosin, PAS, and Masson trichromic for light microscopy evaluation, and with fluorescent antibodies against IgG, IgM, IgA, C3, and fibrinogen for immunofluorescence analysis. In each biopsy, we determined the percentage of glomeruli with total sclerosis and the percentage of glomeruli with focal sclerosis. The amount of interstitial fibrosis was quantified in 5-μm trichromic stained sections using an Olympus WCUE-2 image autoanalyzer.

Outcome Measures

The main outcome measures were the percentage of patients who achieved treatment withdrawal with no evidence of relapse and the percentage of patients who maintained complete or partial remission 30 mo after treatment withdrawal.

Statistical Analyses

Data are summarized as the mean ± SD. Within-group changes in proteinuria and GFR over time were assessed by the nonparametric Friedman test for repeated measures or Mann-Whitney rank sum test with Bonferroni correction for repeated measures.

Results

Table 1 summarizes the main clinical and biochemical characteristics of the 13 patients included. Mean number of relapses during treatment with CNI was 7 (range, 4 to 8). Mean time on CNI treatment before study entry was 47.8 ± 20.8 mo (range, 22 to 83 mo). Table 2 summarizes the histopathological data of kidney biopsies performed at diagnosis and after long-term treatment with CNI in the nine patients who underwent biopsy again before rituximab treatment. A significant increase in both the percentage of glomerular ($P = .0028$) and interstitial ($P = .0026$) sclerosis was observed. At study entry, all patients showed adequate control of BP and were in partial remission. All patients completed the rituximab treatment period with no evidence of adverse effects. CD-19+ cells decreased to undetectable levels after the first dose of rituximab in all patients and remained undetectable for an average of 7 mo (range, 6 to 11 mo). Despite the depletion of CD-19+ cells, serum IgG levels remained unaltered during the whole follow-up. Figure 1 summarizes the CD-19+ cell counts over the total observation period. Table 3 summarizes the evolution of the main biochemical variables over the observation period. Figure 2 summarizes the evolution of urinary protein excretion in each patient during follow-up, and Table 4 summarizes the evolution of urinary protein excretion in the cohort across the 30 mo of follow-up. Six months after rituximab, tacrolimus, CsA, MMF, and steroids could all be withdrawn in all patients with no evidence of relapse. After CNI withdrawal, GFR increased significantly, from basal levels of 95.4 ± 11 to 109.4 ± 8 at 3 mo and 110 ± 13 at 6 mo ($P = .0002$) with a mean percent increase of 15.3% (range, 0% to 20%). At 12 mo, all patients remained in complete (4) or partial (9) remission. Three patients suffered a relapse of nephrotic proteinuria 19, 23, and 28 mo, respectively after their first course of rituximab (Figure 2) and were treated with a second course of rituximab. The total dose of rituximab administered in this second course was adjusted to achieve depletion of circulating CD-19+ cells. A single dose of 650 mg/m^2 (1115, 1200, and 1378 mg, respectively) caused depletion of CD-19+ cells that lasted for 5, 7, and 9 mo, respectively and was followed by remission in all cases. All patients have completed the whole follow-up of 30 mo considered in the protocol and at this time remain in partial remission (Table 4 and Figure 2). Seven of these patients have been followed for more than 60 mo (range, 61 to 68 mo) and at last follow-up remained in partial remission with a mean GFR of 107 ± 23 ml/min/1.73 m^2 and a mean proteinuria of 0.78 ± 0.34 g/d. From month 6 after rituximab, neither GFR nor systolic BP showed significant changes. Diastolic BP showed a statistically significant increase (67.1 ± 7.5 *versus* 79 ± 6.3, $P < 0.05$); however, values were

Table 2. Comparison between kidney biopsies performed at diagnosis and after long-term treatment with CNI in the nine patients who were rebiopsed before rituximab treatment

Patient No.	Time	Biopsy at Diagnosis			Biopsy After Long-Term Treatment with CNI		
		No. of Glomeruli	Glom Scler %	Int Scler %	No. of Glomeruli	Glom Scler %	Int Scler %
1	81	12	8	0	21	10	0
2	49	9	0	0	11	12	5
3	38	11	0	0	13	10	5
4	47	13	0	0	9	0	0
5	53	14	0	0	10	0	0
6	35	22	0	0	11	20	15
7	64	10	0	0	13	0	10
8	83	13	0	0	8	15	5
9	79	16	0	0	12	16	23
Mean	58.7 ± 18.6	13.3 ± 3.8	0.11 ± 0.33	0	12 ± 3.7	9.2 ± 7.5[a]	7 ± 7.8[b]

Time between the two kidney biopsies in months. Glom sclera, percentage of sclerosed glomeruli; Int sclera, percentage of interstitial fibrosis. [a]$P = 0.028$. [b]$P = 0.026$.

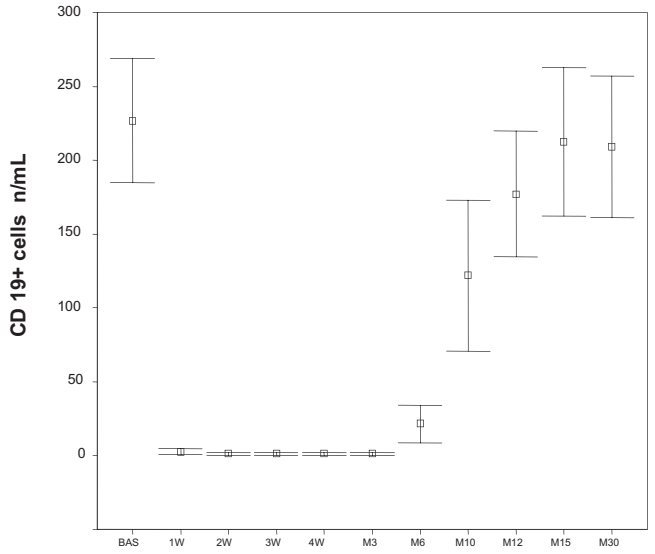

Figure 1. Evolution of CD-19+ cell count over the total observation period. Basal: CD-19+ cell counts before rituximab administration. Values within 15 and 30 mo exclude CD-19 counts of the three patients who suffered a relapse and needed retreatment.

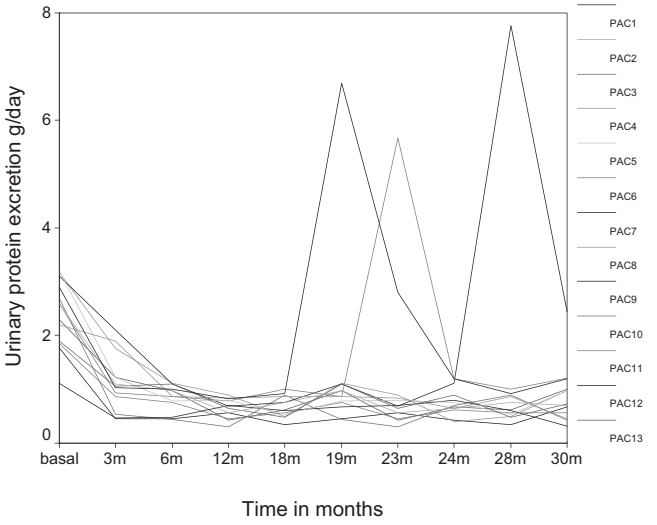

Figure 2. Time course of urinary protein excretion (g/24 h) in individual patients from entry into the study (basal) to end of follow-up (month 30) Values represent proteinuria in g/d

within the target BP levels. No side effects related to the rituximab administration were observed, and no opportunistic infection or other adverse events related to persistent immunosuppression were observed throughout the whole follow-up.

Discussion

This pilot study adds new information to what is currently available (15–19) concerning the potential utility of rituximab in the treatment of patients with MGN. In particular, our results show that in patients with MGN who suffered long-term dependence on CNI despite previous treatments with high-dose immunoglobulins and MMF, a single course of rituximab can allow withdrawal of CNI. The major limitations of our study are the small sample size and the lack of a control group. The latter makes it difficult to rule out definitively that the results observed could be attributed to the spontaneous evolution of MGN itself. However, the clinical course observed after rituximab treatment cannot be attributed to spontaneous remissions. All patients had been dependent of CNI for long and throughout this period of time, all attempts to treatment discontinuation, including treatment with other immunomodulating or immunosuppressant drugs were unsuccessful. All these patients met clear criteria for CNI dependence, and although CNI maintained remission of nephrotic proteinuria in all cases, analysis of kidney biopsies performed before study entry showed a significant increase in both glomerular and interstitial fibrosis scores. Taking into account the long period of time between the two kidney biopsies, these histopathological changes could be the result of the association of several variables such as the influence of age, repeated relapses of proteinuria, high BP, and/or mild chronic nephrotoxicity related to CNI treatment. The small but significant increase in GFR observed shortly after CNI withdrawal could be interpreted as the release of a vasoconstrictor effect induced by these drugs.

The second point of interest of our study is the evidence that patients who suffered relapses of the nephrotic syndrome after the first trial of rituximab could be successfully retreated. Although re-exposure to rituximab has the potential to induce the production of antichimeric antibodies which may limit the effect and/or increase the risk of hypersensitivity reactions in patients needing re-treatment, in our experience, relapses of nephrotic proteinuria were successfully treated with a second trial of rituximab with no adverse effects.

Table 3. Evolution of renal function , blood pressure, and urinary sodium excretion during the 30-mo follow-up

	Basal	3 mo	6 mo	12 mo	24 mo	30 mo
GFR	97 ± 14	113 ± 20[b]	111 ± 17	110 ± 24	114 ± 20	109 ± 19
SBP	121 ± 5.4	127 ± 5	129 ± 5.5	126 ± 7	129 ± 8.1	126 ± 10.1
DBP	67.1 ± 7.5	67 ± 10.2	69.6 ± 11	73 ± 8.3[a]	74 ± 7.9[a]	79 ± 6.3[a]
U Na	87 ± 15	86 ± 21	95 ± 35	118 ± 41	105 ± 32	113 ± 18

GFR, glomerular filtration rate (ml/min 1.73 m^2); Prot, proteinuria (g/24 h); SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); U Na, urinary sodium excretion (mEq/day). [a]$P < 0.05$, [b]$P < 0.01$ when compared to basal levels

Table 4. Evolution of urinary protein excretion in the whole group of patients during the 30-mo follow-up

	Basal	3 mo	6 mo	9 mo	12 mo	18 mo	24 mo	30 mo
N	13	13	13	13	13	13	13	13
Mean	2.41	0.99[a]	0.83[a]	0.65[a]	0.68[a]	1.29[b]	0.77[a]	0.89[a]
Median	2.60	0.97	0.86	0.69	0.62	0.86	0.70	0.73
SD	0.63	0.52	0.24	0.18	0.20	1.63	0.26	0.54
Minimum	1.12	0.45	0.44	0.30	0.34	0.44	0.40	0.32
Maximum	3.20	2.10	1.12	0.90	1.00	6.70	1.20	2.45
25%	1.87	0.70	0.62	0.51	0.52	0.72	0.63	0.50
75%	3.00	1.49	1.05	0.81	0.88	1.10	1.00	1.10

Values are proteinuria in g/day in biochemical controls carried out at fixed times. SD, standard deviation. [a]$P < 0.001$, [b]$P < 0.01$ when compared to basal levels.

Overall, our results concur with recent studies indicating that rituximab could play a relevant role in the treatment of nephrotic patients with MGN. However, their potential indications, the appropriate doses, and the treatment duration remain to be defined. In our patients, we prescribed a "lymphoma-based" protocol (20–22) consisting of four weekly doses of rituximab because this was the only treatment protocol available at the time the study was designed. A recent study by Cravedi *et al.* however, suggest that shorter treatments based on monitoring the number of circulating B cells could be as effective as the four-dose course of therapy, reducing the risk of re-exposure to rituximab as well as treatment-related costs. Moreover, according to the evidence of studies in patients with rheumatoid arthritis (23) indicating that two doses of 1000 mg rituximab induced long-term B cell depletion, we decided to treat late relapses of proteinuria using an strategy based on monitoring the number of circulating CD-19+ cells instead of giving a fixed four-dose protocol. Using this protocol, we observed that a single dose of rituximab was sufficient to achieve remission of the nephrotic syndrome in all cases.

The mechanisms accounting for the beneficial effects of rituximab in MGN remain poorly understood. Although there is no doubt that—as in other autoimmune diseases—the effects of rituximab in MGN are the consequence of its inhibitory actions on B cell differentiation and Ig secretion, certain data indicate that the clinical response to rituximab observed in patients with MGN does not run closely in parallel with the number of circulating CD-19+ cells. First, depletion of circulating B cells can be observed shortly after rituximab monotherapy in almost all patients, whereas the rate of clinical response reported in different studies averages 60% (15,16,19). Second, the data by Cravedi *et al.* (18) indicate that the number of circulating B cells increased gradually from the third month after rituximab administration, whereas the reduction of proteinuria was still evident during the entire 12-mo follow-up, even though a significant recovery of circulating B cells could be observed 6 mo after rituximab. Finally, in our study group, no patients suffered abrupt relapses of proteinuria after B cell recovery. After the first course of rituximab, the three patients who relapsed remained in remission for 7, 11, and 14 mo, respectively, with normal circulating B cell levels. After the second course of rituximab treatment, they maintained long-term remission of proteinuria despite the complete recovery of circulating CD-19 cells. Overall, these results suggest that the beneficial effects of rituximab in MGN cannot be attributed simply to the depletion of circulating B cells. In addition, rituximab could have other effects on B cell function (*i.e.*, interaction with T lymphocyte or antigen-presenting properties) that should be studied accurately.

It is worth mentioning that the administration of rituximab was safe and no patient suffered adverse effects. The overall tolerability of rituximab in immune-mediated diseases appears to be good (24,25) However, the Food and Drug Administration recently issued an alert concerning two fatal cases of progressive multifocal leukoencephalopathy caused by JC polyomavirus reactivation in two patients with systemic lupus erythematosus who had received rituximab therapy in combination with other immunosuppressant drugs (26). In our patients, no opportunistic infection or other adverse events related to persistent immunosuppression were observed throughout the whole follow-up. However, it should be taken into account that our study had a small sample size and a follow-up period restricted to 30 mo.

To summarize, the results of our pilot study provide new data regarding the potential usefulness of rituximab in MGN. Our data clearly indicate that rituximab is an effective tool to overcome long-term dependence of CNI, thus avoiding the risk of nephrotoxicity related to chronic exposure to these drugs. The efficacy of this approach, however, needs to be properly evaluated in a randomized clinical trial.

Disclosures

None.

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