Supplemental Material

Human or chimeric monoclonal anti-CD20 antibodies for children with nephrotic syndrome.

A superiority randomized controlled trial

Table of Contents

Materials and methods

Dilution of rituximab. Children randomized to the active received rituximab following the same premedication described above. Rituximab was administered at a dose of 375 mg/m^2. For doses between 100 and 250 mg, rituximab was diluted in 100 ml of normal saline and administered at 2 ml/h for the first 30'; 3 ml/h for the second 30'; 6 ml/h for the third 30'; 15 ml/h until the end of the infusion. For doses between 260 and 500 mg rituximab was diluted in 250 ml of normal saline and administered at 6 ml/h for the first 30'; 9 ml/h for the second 30'; 18 ml/h for the third 30'; 36 ml/h until the end of the infusion. For doses between 510 and 1000 mg, rituximab was diluted in 500 ml of normal saline and administered at 9 ml/h for the first 30'; thereafter, the infusion rate was doubled every 30 minutes up to a maximum of 72 ml/h.

Relevant concomitant care and interventions that were permitted or prohibited during the trial.

Any medications not listed in the exclusion criteria, were permitted at the discretion of the investigator who recorded all concomitant medications in the appropriate section of the case report form. In view of a possible role in the reduction of proteinuria and worsening of glomerular filtration rate, inhibitors of the renin-angiotensin system were not allowed during the study and replaced with other antihypertensive agents.

Ancillary studies included biochemical or cell predictors of treatment failure. Anti-rituximab antibodies were determined in 64 patients who already received rituximab at the time of the study enrolment and at 6 months (Lisa-Tracker Theradiag LTR-005, Marne la Vallée, Fr). Circulating lymphocyte subpopulations were analyzed by multicolor flow cytometry in cryo-preserved peripheral blood mononuclear cells (PBMCs) isolated by Ficoll-Paque Plus (Amersham, Biosciences) density gradient centrifugation, at baseline and at different time points. Cells were stained with fluorochrome-conjugated antibodies directed against CD3, CD4, CD8, CD19, CD56 (Biolegend), CD24, CD25, CD27, CD38, CD127, IgD (BD Biosciences), and IgM (Jackson Immuno-Research Laboratories) and analysed by BD FACS CANTO II. **Figure S1** represents the gating strategy used to identify the different cell subsets, which were reported as the percentage of total circulating lymphocytes. Subsets of gated CD3$^+$ (total) T cells were identified as CD4$^+$ T helper cells, CD8$^+$ T cytotoxic cells and CD4$^+$CD25^{high}CD127^{low}Treg cells; total NK cells were identified as CD3$^-$ CD56$^+$ cells and total B cells were identified as CD19$^+$ cells. Transitional

($CD38^{high}CD24^{high}$), mature/naïve ($CD38^{intermediate}CD24^{low}$), total memory ($CD19^{+}CD27^{+}$), IgM memory ($CD19^{+}CD27^{+}IgM^{+}IgD^{intermediate}$), switched memory ($CD19^{+}CD27^{+}IgM^{-}IgD^{-}$) B cells and plasmablasts ($CD19^{+}CD27^{+}CD38^{high}$) were also identified in gated $CD19^{+}$ in 36 patients who received ofatumumab and 33 who received rituximab treatment matched for age and sex.

Blinding.

The following reasons justified the open-label design: (1) ofatumumab and rituximab require different methods for infusion; for example, the ofatumumab requires a filter that cannot be masked. According to the producer indications, the same filter may alter/reduce the availability/stability of rituximab; and (2) ofatumumab must be diluted in 1000 ml of normal saline and infused at a fixed rate; the producer does not guarantee rituximab stability using the same dilution/infusion strategy.

Data collection methods and monitoring.

Study visits occurred at baseline, after 1 month and every three months thereafter, unless complications or relapses occurred. Determination of 24 hours proteinuria at baseline and after 12 and 24 months was performed at a central laboratory (in order to assess the primary and secondary outcomes). Dipstick for proteinuria determination was evaluated daily. In case of dipstick positivity, the presence of proteinuria was confirmed with 24-hour urine collection. Complete blood count, kidney function, plasma proteins, immunoglobulins, lipid status (cholesterol and triglycerides), albumin and lymphocyte subpopulations were obtained at 1, 3, 6, 9, 12, 15, 18, 21 and 24 months during protocol visits. At discharge from the Nephrology Unit, each patient received a clinical diary form, to be filled with proteinuria levels at dipstick, body weight, current treatment and monthly send to our centre via fax or email. A study coordinator maintained on-going contact with the children, their families, and the family physician to collect clinical data including blood pressure and potential adverse events.

Statistical Methods

Continuous data were expressed as mean ± standard deviation or medians and interquartile-ranges otherwise. Variables were compared by unpaired t test or nonparametric

Mann–Whitney U test, as appropriate; a chi-squared test was used for categorical variables. Monitoring of levels of each circulating cell subpopulation at different time points was analyzed using a nonparametric Kruskal-Wallis test and a Dunn's multiple comparison test as appropriate. The association of previous rituximab administration with risk of relapse was evaluated by cox proportional hazards regression model. Statistical analyses were performed using R, version 4.0.3 (www.R-project.org/), SPSS 20.0 and Graphpad Prism 9.0.

Fig. Suppl. 1 Gating strategy for flow cytometry analysis

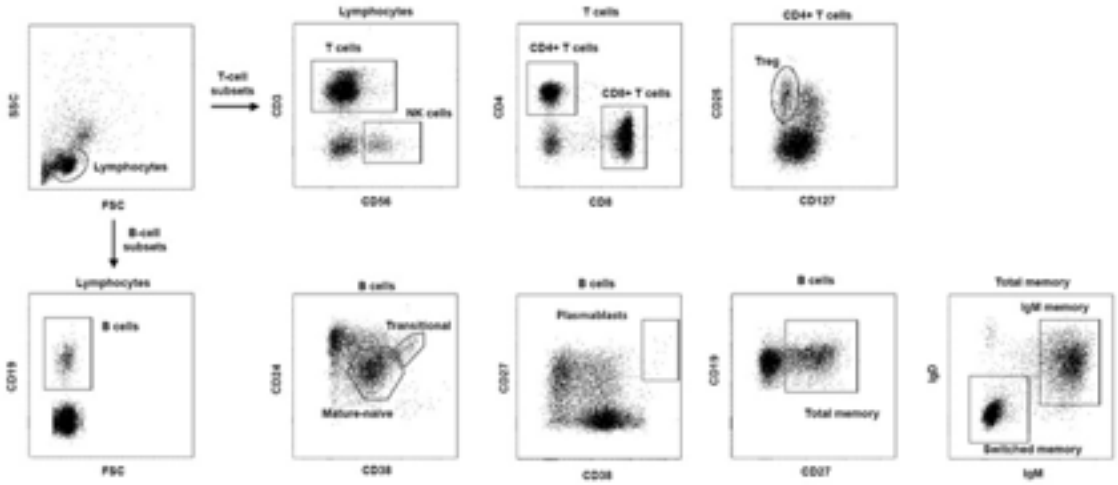

Fig. Suppl. 2 Time (months) to relpase in patients treated with ofatumumab (blue)and rituximab (red)

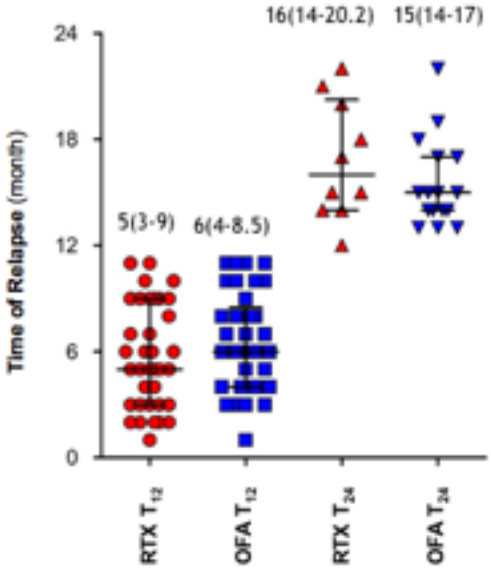

Fig. Suppl. 3 Serum levels of immunoglobulins A, G and M and circulating neutrophils counts

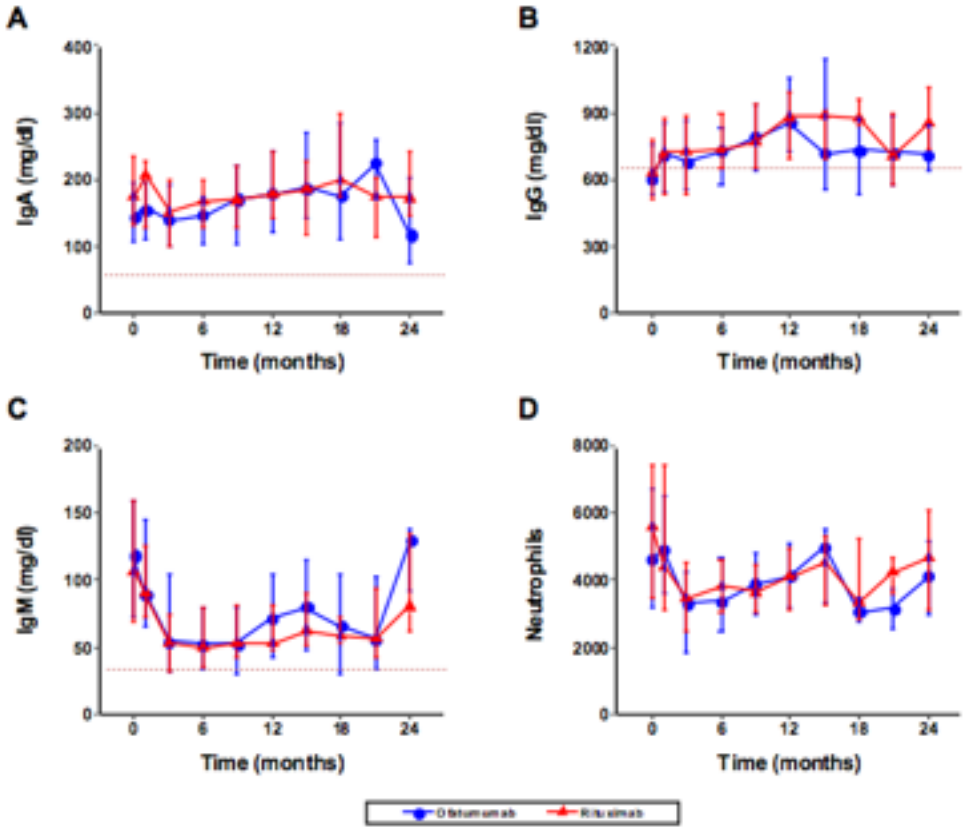

Fig. Supple. 4. Circulating levels of anti-rituximab antibodies.

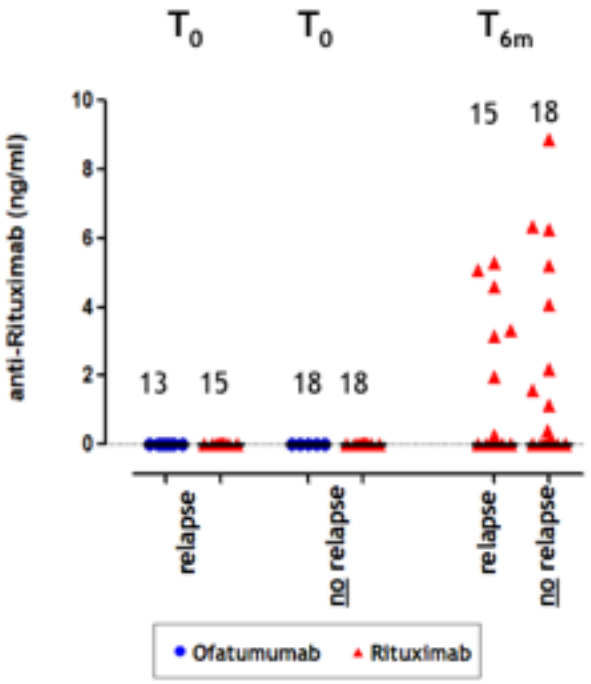

Table S1. Participants timeline

			STUDY PERIOD							
	Enrol-ment	Alloca-tion and treat-ment	Follow up							Close-out
TIMEPOINT**	*-1*	0	t_1	t_3	t_6	t_9	t_{12}	t_{15}	t_{18}	t_{24}
ENROLMENT:										
Relevant medical history	X									
Eligibility screen	X									
Project Illustration	X									
Informed consent	X									
Instruction about immunosuppressive drugs tapering	X	X								
Allocation		X								
INTERVENTIONS:										
ofatumumab)/ rituximab administration		X								
ASSESSMENTS:										
Dosage on 24-Hours Urine Collection	X	X	X	X	X	X	X	X	X	X
Physical Examination & Vital Signs	X	X	x	X	X	X	X	X	X	X

Haematology and Biochemistry (Complete blood count, kidney function, plasma proteins immunoglobulins, lipid status -cholesterol and triglycerides-, albumin, lymphocyte subpopulations -for CD 20 lymphocytes B count-)	X	X	x	X	X	X	X	X	X	X
Adverse Events data records		X	x	X	X	X	X	X	X	X

Table S2: circulating B and T-cell subpopulations expressed aspercentage of total lymphocytes at baseline and after ofatumumab or rituximab treatment.

Parameter	**Unit**	**Ofatumumab**							**Rituximab**						
Time	**Months**	**0**	**1**	**3**	**6**	**9**	**12-15**	**p-value**	**0**	**1**	**3**	**6**	**9**	**12-15**	**p-value**
$CD19^+$ B cells	**Median**	7.6	0***	0.1***	3.2***	3.5**	6.7	<0.001	9.2	0.1***	0.3***	3.2***	6*	5.6	<0.001
	IQR	5.2-12.3	0.0-0.2	0.1-0.3	0.3-6.3	1.0-9.7	2.9-11.8		6.3-13.8	0.0-0.2	0.1-2.0	0.6-6.9	3.1-10.7	3.0-12.6	
Transitional B cells	**Median**	0.8	0***	0***	1.4	2.8*	3.1**	<0.001	0.6	0***	0.3	1.3	1.8	1.2	<0.001
	IQR	0.3-1.3	0.0-0.0	0.0-0.0	0.1-3.8	1.3-3.7	1.8-3.9		0.3-0.9	0.0-0.0	0.0-3.5	0.3-3.6	0.1-3.1	0.7-3.5	
Mature B cells	**Median**	10.3	0***	0***	4.1*	6.8	11.9	<0.001	10.1	0***	0.2***	5.4	11.3	8.2	<0.001
	IQR	7.3-13.3	0.0-0.0	0.0-0.1	0.2-7.6	2.1-15.5	8.9-14.3		6.2-17.8	0.0-0.0	0.0-2.0	2.7-18.6	2.6-15.8	6.3-10.0	
Total memory	**Median**	3.5	0***	0***	0.4***	0.5***	0.8**	<0.001	4.3	0***	0.2***	0.8***	0.6***	1**	<0.001

B cells	**IQR**	1.8-5.2	0.0-0.0	0.0-0.1	0.1-1.0	0.3-0.9	0.5-1.2		2.3-6.2	0.0-0.0	0.1-0.4	0.3-1.1	0.4-1.9	0.8-2.2	
Switched memory	**Median**	1.3	0***	0***	0.1***	0.2***	0.3**	<0.001	1.7	0***	0***	0.4***	0.2***	0.5*	<0.001
B cells	**IQR**	0.8-2.4	0.0-0.0	0.0-0.0	0.0-0.7	0.1-0.5	0.2-0.5		1.0-3.0	0.0-0.0	0.0-0.1	0.2-0.6	0.1-1.0	0.3-0.9	
IgMmemory	**Median**	1.6	0***	0***	0.2***	0.3***	0.4*	<0.001	2.1	0***	0.1***	0.3***	0.4***	0.6**	<0.001
B cells	**IQR**	0.8-2.5	0.0-0.0	0.0-0.1	0.1-0.3	0.1-0.5	0.3-0.8		1.1-3.1	0.0-0.0	0.0-0.3	0.1-0.5	0.2-0.7	0.3-0.8	
Plasmablasts	**Median**	0.1	0***	0**	0.1	0.1	0.1	<0.001	0.1	0***	0.1	0.1	0.1	0.1	<0.05
	IQR	0.0-0.1	0.0-0.0	0.0-0.1	0.0-0.2	0.0-0.2	0.1-0.2		0.0-0.1	0.0-0.0	0.0-0.2	0.0-0.1	0.0-0.2	0.0-0.2	
CD3⁺	**Median**	70.1	83.9***	79.4***	75.2*	75.6*	73.9	<0.001	69.1	86.4**	79.4***	76.8**	74.6	73.2	<0.001
T cells	**IQR**	62.8-76.1	79.5-87.2	69.7-85.5	70.3-80.6	69.3-79.5	69.2-81		63.2-75.9	69.4-91.8	74.3-81.9	73.4-80.5	63.2-80.7	65.8-81.5	
CD56⁺	**Median**	7.8	10.5	13.2***	13.2**	9.9	10.2	<0.001	8.4	9.2	11.4***	10.7	8.7	8	<0.05
NK cells	**IQR**	4.3-11.3	6.4-13.7	7.9-22.7	6.3-19.2	6.2-14.6	5.5-14.7		4.5-13.7	4.8-26.3	9-15.4	6.7-13.1	5-13	6.8-12.1	
CD4⁺/CD8⁺ T cellRatio	**Median**	1.8	2.1	1.9	1.9	1.8	1.7	ns	2	1.9	1.9	2.2	2	2.2	ns
	IQR	1.4-2.3	1.6-2.4	1.2-2.9	1.4-2.2	1.5-2.4	1.5-1.9		1.3-2.5	1.2-2.7	1.5-2.5	1.6-2.8	1.4-2.6	1.8-2.5	
Treg cells	**Median**	1.8	1.6	2.8***	2.3*	2.4**	2.6**	<0.001	1.6	2.7*	2.8***	2.6***	2.2*	2.7***	<0.001
	IQR	0.9-2.3	1-3.4	1.6-3.5	1.9-3.1	1.8-3.0	1.9-3.0		1.1-2.3	1.6-3.3	2.1-3.4	2.2-3.6	1.7-3.0	2.0-3.9	