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CD19 mRNA quantification improves rituximab treatment-to-target approach: A proof of concept study

Fabiana Marnetto [a,b,*], Letizia Granieri [a,b], Paola Valentino [a,b], Marco Capobianco [b], Marisa Pautasso [c], Antonio Bertolotto [a,b]

[a] Clinical Neurobiology Unit, Regional Referring Multiple Sclerosis Centre (CRESM), Neuroscience Institute Cavalieri Ottolenghi (NICO), University Hospital San Luigi Gonzaga, Orbassano, Turin, Italy
[b] Clinical Neurology Unit, Regional Referring Multiple Sclerosis Centre (CReSM), University Hospital San Luigi Gonzaga,Orbassano, Turin, Italy
[c] Clinical Pathology Unit, Laboratory of Clinical and Microbiological Analyses, University Hospital San Luigi Gonzaga, Orbassano, Turin, Italy

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ABSTRACT

We compared pre-amplification (PA) RT-PCR blood CD19 mRNA quantification with flow cytometry (FC), to personalize rituximab re-treatment in neuromyelitis optica spectrum disorders (NMOSDs) patients. 47 blood samples from 3 NMOSDs patients were studied. PA-RT-PCR quantified CD19 in all samples, and a positivity threshold was defined, whereas CD19+ B cells were under threshold in 31/47 samples by FC. In all samples where CD19+ B cells were above FC threshold, they resulted above the PA-RT-PCR threshold. CD19 mRNA was above threshold in 8 other samples, resulted negative by FC, and preceded the FC positivity in 7/8 samples by 1–3 months, showing major sensitivity.

1. Introduction

Rituximab (RTX) is a chimeric monoclonal antibody directed against CD20, a B-cell surface antigen, used for treatment of non-Hodgkin's lymphoma and several autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), primary Sjogren's syndrome (pSS), idiopathic thrombocytopenic purpura (ITP), autoimmune hemolytic anemia, cryoglobulin disease, acquired Factor VIII Abs, IgM polyneuropathies, glomerulonephropathies, pemphigus vulgaris, and inflammatory myositis (Edwards and Cambridge, 2006). RTX has been shown to limit relapses and is a promising drug for the treatment of demyelinating diseases of the central nervous system (CNS) such as relapsing remitting multiple sclerosis (RRMS) and neuromyelitis optica spectrum disorders (NMOSDs) (Greenberg et al., 2012). Several studies on NMO patients showed that a single cycle of RTX was not sufficient to suppress disease activity (Kim et al., 2011). NMO relapses can be reduced only with repeated treatment with RTX. Questions remain about how and when patients should receive further treatment (Kim et al., 2013). Finding an optimal dosing and monitoring regimen of RTX treatment is an urgent challenge for the management of patients with relapsing autoimmune disorders of CNS. This means maximizing the efficacy of the RTX treatment and reducing overtreatment, and the cost and risks of severe adverse events (McKeon and Pittock, 2013).

Three different approaches for re-treatment can be conceived based on the rheumatoid arthritis (RA) model. 1. "On demand re-treatment approach," based on the increase of disease activity. This, however, inevitably results in temporary deterioration of disease activity and inferior disease control and is possibly associated with a worse outcome (van Herwaarden et al., 2013). This approach is unethical in NMO and MS because, unlike in RA, a single relapse can cause severe permanent neurological impairment. 2. "Fixed re-treatment schedule approach": the repeated treatment with RTX at 6- to 9-month intervals. This strategy in some patients is not sufficient to prevent the recurrence of NMO (Kim et al., 2011), in others it demands unnecessary infusions, which also means a higher cost of treatment. 3. "Treatment-to-target approach": a treatment based on monitoring the percentage of memory B cells in Peripheral Blood Mononuclear Cells (PBMCs), evaluating CD19 antigen by flow cytometry (FC). This seems to be a promising strategy to individualize RTX treatment in NMOSDs patients, but relapses occur despite the CD19 antigen remaining under the detection limit (Kim et al., 2011, 2013; Pellkofer et al., 2011; Greenberg et al.,

* Corresponding author at: Clinical Neurobiology Unit, Regional Referring Multiple Sclerosis Centre (CRESM), Neuroscience Institute Cavalieri Ottolenghi (NICO), University Hospital San Luigi Gonzaga, Regione Gonzole 10, I-10043 Orbassano (Torino), Italy. Tel.: +39 011 6706601; fax: +39 011 6705448.
E-mail addresses: fabiana.marnetto@gmail.com (F. Marnetto), letiziagranieri@libero.it (L. Granieri), paolaval81@hotmail.com (P. Valentino), m.capobianco@alice.it (M. Capobianco), m.pautasso@sanluigi.piemonte.it (M. Pautasso), antonio.bertolotto@gmail.com (A. Bertolotto).

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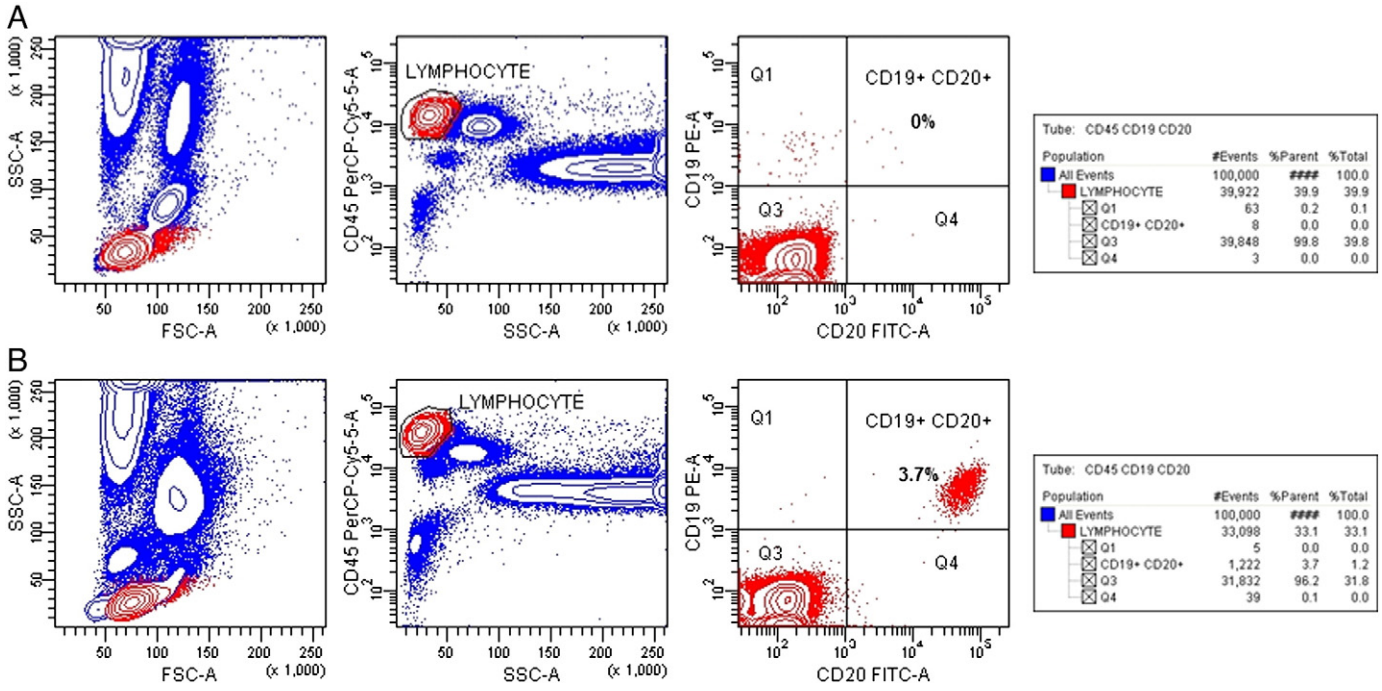


Fig. 1. Flow cytometry gating strategy for CD45 + CD19 + CD20 + B cell analysis. Density plot shows that lymphocytes were gated on CD45 + cells (red). B cells of interest were then identified as CD19 + CD20 + cells: all samples reported as B cells CD19 + CD20 + < 0.1% of total lymphocytes have no detectable CD19 + CD20 + B cells, because they don't appear as a defined cluster. A: sample in which CD19 + B cells were undetectable (reported as <0.1% of total lymphocytes); B: sample in which CD19 + B cells were detectable (reported as 3.7% of total lymphocytes).

2012). Likely, the FC method has a limited sensitivity in detecting CD19 antigen expression. To improve sensitivity, Kim and co-authors suggested monitoring circulating B cells by CD27 antigen detection together with CD19, but this approach needs to be confirmed by future studies (Kim et al., 2013).

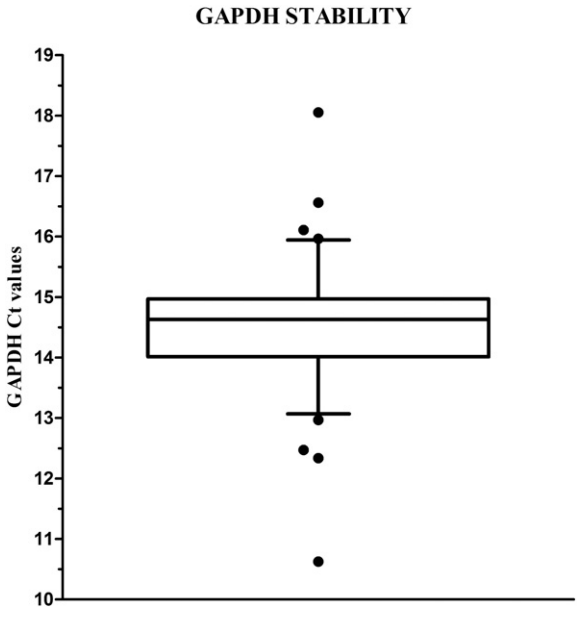


Fig. 2. Analysis of the housekeeping gene stability. Distribution of GAPDH Ct values obtained from 87 samples (tested in duplicate). The box represents 50% of the samples (25th–75th percentiles), while 90% of all samples reside within the limits of the box and its whiskers (5th–95th percentiles). The line within the box indicates the median (14.63) Ct value. The broad range of GAPDH Ct values (10.54–18.11) was due to only 2 samples, while the 90% of tested samples showed Ct values for GAPDH in a range between 13.07 (5th percentile) and 15.95 (95th percentile).

Based on this assumption, we hypothesized that real time PCR (RT-PCR), with increased sensitivity through pre-amplification (PA), may be a more sensitive method than FC in detecting CD19 + B cells, because it quantifies mRNA instead of the protein. The present study compared the performance of the PA-RT-PCR method and the traditional FC approach in paired samples obtained from NMOSDs patients.

2. Patients and methods

2.1. Patients and series characteristics

In the CRESM (Centro di Riferimento Regionale per la Sclerosi Multipla) in Orbassano, Italy, a treatment-to-target approach is applied for RTX re-treatment in NMOSDs patients. FC monitors monthly the circulating memory B cells by measuring the CD19 antigen, and RTX re-treatment (1000 mg infused twice, with a 2-week interval) is administered when CD19 + B cells exceed 0.1% of total lymphocytes (Capobianco et al., 2007).

We selected and studied 5 series of monthly blood samples from 3 NMOSDs patients, based on the following eligibility criteria: 1. Availability in CRESM bio-bank of a sample for PA-RT-PCR and a paired sample already analyzed by FC, and 2. Each series included one RTX re-infusion administered according to the above described protocol based on FC detection of CD19 antigen exceeding 0.1% of total lymphocytes. A total of 47 paired samples were available for comparison. Actually, FC analysis was performed in 48 samples (in one sample in Series 3, the PA-RT-PCR analysis did not produce results due to technical problems); 5 additional samples were tested for PA-RT-PCR only, for a total of 52 analyzed samples: 4 samples were tapped during the 2 weeks of RTX re-treatment, while for one sample in Series 1 the FC analysis was not available.

Blood samples in Series 1, 2, 3, and 4 were obtained from 2 NMOSDs patients (1 with LETM and 1 with recurrent episodes of myelitis), while samples in Series 5 were obtained from a patient with clinically and radiologically defined NMO (Wingerchuk et al., 2006). All 3 patients

Table 1
Intra-assay variability.

		GAPDH Ct	CD19 Ct	CD19 RE
Reference sample	Mean	14.68 (14.48–14.78)	20.61 (20.44–20.69)	1.169 (1–1.39)
	SD	0.13	0.10	0.15
	CV%	0.89	0.46	12.47
Commercial reference human RNA	Mean	10.39 (10.2–10.7)	31.7 (31.06–31.92)	$1.26 * 10^{-5}$ ($1.03 * 10^{-5}$ $1.76 * 10^{-5}$)
	SD	0.19	0.36	$2.97 * 10^{-6}$
	CV%	1.87	1.14	23.46
NMOSDs patient sample	Mean	15.13 (14.92–15.31)	31.86 (31.6–32.12)	$3.01 * 10^{-4}$ ($2.62 * 10^{-4}$–$4.02 * 10^{-4}$)
	SD	0.17	0.26	$5.91 * 10^{-5}$
	CV%	1.13	0.81	19.63

Three samples were tested 5 times each in the same amplification session: data about intra-assay variability are expressed as mean, standard deviation (SD) and coefficient of variation (CV%) calculated both for Ct values and relative expression (RE) values.

Table 2
Inter-assay variability.

		GAPDH Ct	CD19 Ct	CD19 RE
Reference sample	Mean	14.39 (14.03–14.71)	18.94 (18.71–19.06)	1[a]
	SD	0.34	0.19	[a]
	CV%	2.39	1.03	[a]
Commercial reference human RNA	Mean	10.12 (9.60–10.47)	30.63 (30.46–30.83)	$1.58 * 10^{-5}$ ($1.36 * 10^{-5}$–$1.88 * 10^{-5}$)
	SD	0.45	0.19	$2.66 * 10^{-6}$
	CV%	4.49	0.61	16.79

Two samples were tested 3 times each in different amplification sessions: data about inter-assay variability are expressed as mean, standard deviation (SD) and coefficient of variation (CV) calculated both for Ct values and relative expression (RE) values.
[a] For reference sample SD and CV were not calculated for CD19 RE values, as they were always conventionally = 1.

were women. The 2 NMOSDs patients were treated with RTX as first choice treatment, while the NMO patient had been previously treated with immunoglobulins. The 3 patients gave written informed consent for the use of their blood banked samples for this comparative study.

2.2. Methods

2.2.1. Blood sampling

Blood was drawn every month both in 3 ml EDTA tubes and in Tempus™ blood RNA Tubes (Life Technologies, Monza, Italy). Samples in EDTA tubes were processed within 24 h for FC analysis, while samples in Tempus™ Tubes were immediately banked at −80 °C, for mRNA analysis.

2.2.2. Flow cytometry analysis

Lymphocyte subsets were determined by triple-color immunofluorescent staining in freshly obtained blood samples, using antibodies directed against CD45/CD20/CD19 (Becton Dickinson, San Jose, CA, USA), followed by red blood cell lysis and immediate acquisition and analysis. FC data acquisition was performed on a BD FACS Canto II (Becton Dickinson). For each sample, 100,000 total events were collected for analysis. B cells were identified as CD19 + CD20 + cells. The data acquired were analyzed using BD FACS DIVA Software (Becton Dickinson). CD19 + B cell subset was defined as undetectable by FC when any cluster of cells showing CD19 + CD20 + immunophenotype was observed: this condition was reported as <0.1% of total lymphocytes (Fig. 1).

2.2.3. Real time PCR analysis

Total RNA from whole blood was obtained using the "Maxwell R 16 LEV simply RNA Tissue Kit" (Promega, Madison, USA) on the Maxwell 16 instrument (Promega, Madison, USA), following a protocol adapted by the manufacturer to Tempus™ tubes. RNA concentrations were quantified using the Nanodrop® ND-1000 spectrophotometer (Celbio, Milano, Italy). RNA from each sample was adjusted to a concentration of 5 ng/μl. Reverse transcription to cDNA from the RNA extracted was carried out using the High Capacity Reverse Transcription kit (Life

Table 3
Reproducibility of the whole procedure.

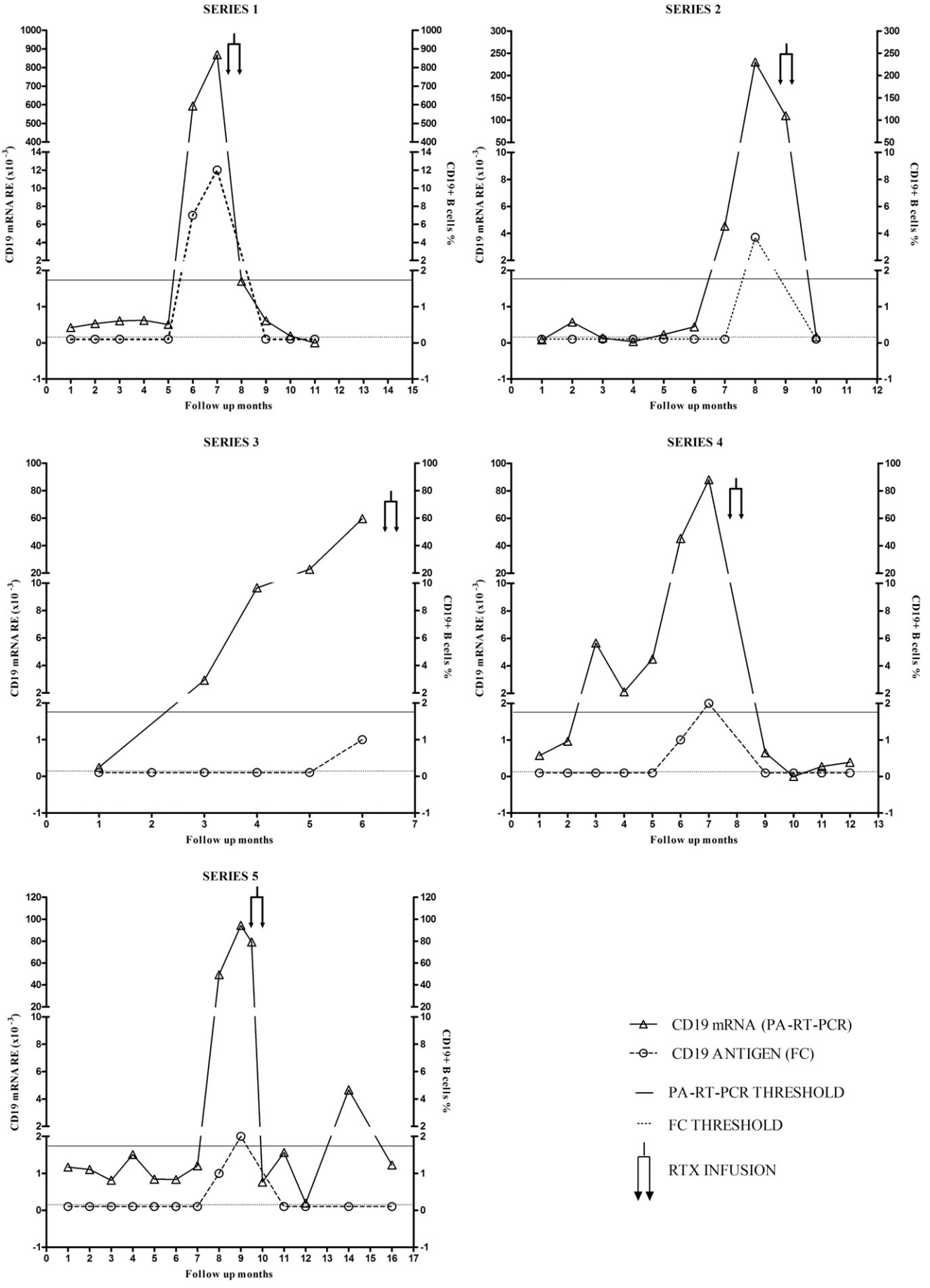
		GAPDH Ct	CD19 Ct	CD19 RE
Sample 1	Mean	14.54 (14.28–14.81)	20.21 (19.94–20.47)	1.40 (1.39–1.40)
	SD	0.37	3.37	$3.48 * 10^{-4}$
	CV%	2.57	1.85	0.025
Sample 2	Mean	14.08 (13.97–14.19)	19.92 (19.86–19.99)	1.24 (1.20–1.28)
	SD	0.16	0.95	0.052
	CV%	1.10	0.48	4.17
Sample 3	Mean	14.5 (14.19–14.81)	24.24 (24.08–24.40)	0.084 (0.075–0.092)
	SD	0.44	0.23	0.012
	CV%	3.02	0.94	14.55

Three paired samples undergoing the full assay procedure (from RNA extraction to final PA-RT-PCR quantification) were tested: data about reproducibility of the whole procedure are expressed as mean, standard deviation (SD) and coefficient of variation (CV) calculated both for Ct values and relative expression (RE) values.

Table 4
Analysis of CD19 + B cells detection by flow cytometry (FC) and pre-amplification-real time PCR (PA-RT-PCR) on 47 paired blood samples.

	FC +	FC-	TOT
PA-RT-PCR +	8	8	16
PA-RT-PCR −	0	31	31
TOT	8	39	47

In all 8 samples in which CD19 + B cells were detected by FC, CD19 mRNA RE was over the threshold. Additional 8 samples tested positive for CD19 + B cells by PA-RT-PCR, but were negative when analyzed by FC: 7 out of 8 of these anticipated by 1–3 months the CD19 + B cell increment detected by FC, while 1 out 8 samples was a false positive result.
FC +: CD19 + B cells above the flow cytometry threshold.
FC −: CD19 + B cells under the FC threshold.
PA-RT-PCR +: CD19 + B cells above the pre amplification-real time-PCR threshold.
PA-RT-PCR −: CD19 + B cells under the PA-RT-PCR threshold.


SERIES 1
SERIES 2
SERIES 3
SERIES 4
SERIES 5
CD19 mRNA RE (x10 -3)
CD19+ B cells %
Follow up months
CD19 mRNA (PA-RT-PCR)
CD19 ANTIGEN (FC)
PA-RT-PCR THRESHOLD
FC THRESHOLD
RTX INFUSION

Technologies, Monza, Italy), according to the manufacturer's instructions. 20 μl of RNA was transcribed to a final volume of 40 μl. Since previous experiments performed in our laboratory showed that the traditional RT-PCR technique was not sensitive enough to detect CD19 mRNA (data not shown), we performed a pre-amplification for the analyzed targets. Amplification efficiency was evaluated for three different genes (GAPDH, CASC 3 and HPRT 1), in order to choose the best performing housekeeping gene.

The inventoried Taq-Man® gene expression assays for CD19 (Hs00174333_m1, Life Technologies, Monza, Italy), GAPDH (Hs99999905_m1, Life Technologies, Monza, Italy) and CDKN1B (Hs00153277_m1, Life Technologies, Monza, Italy) were used for both pre-amplification and RT-PCR reactions. For pre-amplification, the pooled TaqMan® assays (including fluorescent probes) were diluted with 1 × Tris-EDTA (TE) buffer, so that each assay was at a final concentration of 0.2 fold in the Pre Amp assay pool, according to the manufacturer's instructions. 6.25 μl cDNA was amplified in a 25 μl reaction consisting of 12.5 μl Taq-Man® PreAmp Master Mix (Life Technologies, Monza, Italy) and 6.25 μl pooled assay mix (0.2×, each assay) (Noutsias et al., 2008). Pre-amplification of this gene assay pool was carried out for 14 cycles on a TC-512 Programmable Thermal Controller (Techne, Barloworld Scientific, Staffordshire, UK) as follows: denaturation at 95 °C for 10 min and 14 cycles of amplification (15 s at 95 °C, 4 min at 60 °C). The pre-amplified products were then diluted with TE buffer at a ratio of 1:5 (resulting volume 125 μl) and were used as templates for the RT-PCR analysis.

The RT-PCR reactions for TaqMan® gene expression assays contained 2.5 μl cDNA, 6.25 μl Universal Master Mix (Life Technologies, Monza, Italy) and 0.625 μl TaqMan® gene expression assay. Reactions were carried out, up to a final volume of 12.5 μl with RNAse-free water. All experiments were performed in duplicate on an ABI StepOne Plus real-time PCR System (Applied Biosystems, Monza, Italy).

The RT-PCR protocol was the following: denaturation by a hot start at 95 °C for 10 min, followed by 40 cycles of a two-step program (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min).

CD19 relative expression (RE) was calculated applying the $2^{-\Delta\Delta Ct}$ formula: GAPDH was used as housekeeping gene, while a pre-amplified cDNA obtained from a pool of 5 healthy subjects was used as reference sample.

Pre-amplification uniformity related to CDKN1B (reference gene suggested by Life Technologies for the determination of PA uniformity) and GAPDH was calculated according to the Manufacturer's instructions: a ΔΔCt close to zero indicates ideal PA uniformity. Life Technologies suggests a range between −1.5 and +1.5 PA uniformity values as acceptable.

The assay performance was evaluated in terms of inter- and intra-assay variability and housekeeping gene stability. Finally, the reproducibility of the method was evaluated by testing paired samples undergoing the full assay procedure from RNA extraction to final PA-RT-PCR quantification.

2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 5.0 (GraphPad) and R software, version 3.0.1 (for outliers' analysis). Data were expressed as means ± standard deviation (SD), the coefficient of variation (CV) was calculated according to the formula: SD/mean × 100 (CV was considered acceptable if <25%). The positivity threshold for CD19 RE was calculated following the formula: mean + 2SD CD19 RE of 11 samples collected within 12 weeks after RTX infusion.

3. Results

3.1. Flow cytometry analysis

The threshold for detection of circulating CD19+ B cells was set at <0.1% of total lymphocytes. Based on this cut off, CD19 antigen resulted above the cut-off in 8/48 (17%) of analyzed samples (Table 4).

3.2. Set up of pre-amp real time-PCR (PA-RT-PCR)

3.2.1. Choosing the housekeeping gene

The amplification efficiency of CD19 and 3 other genes (GAPDH, CASC 3 and HPRT 1) was evaluated in order to determine the best suited housekeeping gene for this method. GAPDH showed 99.89% of amplification efficiency, while CASC 3 and HPRT 1 showed amplification efficiency values of 98.13% and 98.36%, respectively (data not shown); these data suggested that GAPDH was the best suited housekeeping gene among the analyzed genes in terms of amplification efficiency, consequently it was chosen for all the experiments.

3.2.2. Pre-amplification

CD19 mRNA expression was initially investigated in RT-PCR without pre-amplification in 50 samples from RTX-treated patients: CD19 mRNA resulted as undetectable (or Ct > 35) in 30/50 (60%) samples, indicating that the sensitivity of traditional RT-PCR was too low (data not shown).

Pre-amplification of cDNA with the investigated housekeeping gene assays resulted in a mean Ct improvement of 7.57 (range: 7.36–7.78) for CDKN1B, and in a mean improvement of 7.48 Ct values (range: 7.21–7.75) for GAPDH. The mean PA uniformity for GAPDH related to CDKN1B was 0.0925 (range: 0.035–0.15). With respect to the investigated target gene assay CD19, PA yielded a mean improvement of 7.71 Ct values (range: 7.48 to 7.94). The PA uniformity for CD19 related to CDKN1B was −0.135 (range: −0.11 to −0.16). This data suggested that the 2 targets involved in our assay (GAPDH and CD19) were pre-amplified uniformly, without interferences between each other.

3.2.3. Evaluation of the housekeeping gene stability

In the PA condition, considering the threshold Ct for low expression levels >35, GAPDH Ct values were always <35: in particular, GAPDH mean Ct value was 14.56 (range 10.54–18.11, SD = 0.94, CV = 6.45%), calculated in 87 total samples (tested in duplicate). Of note, the broad range of GAPDH Ct values was due to 2 out 87 samples showing Ct values of 10.54 and 18.11 (confirmed as outliers by statistical analysis), respectively, while 90% of tested samples showed Ct values for GAPDH ranging from 13.07 to 15.95 (Fig. 2). The two samples were maintained in the analyses, because they were not technical outliers (the amplification reactions were technically correct). These data, supported by a good SD and a good CV, confirmed that GAPDH was acceptable as housekeeping gene in terms of stability.

3.2.4. Inter- and intra-assay variability

The pool of RNA used as reference sample, one of the NMOSDs patients' sample and a commercial reference standard human RNA (Universal Human Reference RNA, Agilent Technologies, California, USA) were tested 5 times each in the same amplification session, in order to evaluate the intra-assay variability of the PA-RT-PCR: data

Fig. 3. CD19+ B cell detection by PA-RT-PCR and FC during RTX treatment. Association of CD19+ B cells detected by flow cytometry (FC) and pre-amplification-real time-PCR (PA-RT-PCR) with rituximab (RTX) re-infusions in 5 series of paired blood samples from 3 patients with NMOSD. The positivity thresholds for FC and PA-RT-PCR were <0.1% of total lymphocytes and $<1.739 \times 10^{-3}$ RE, respectively. Note: RTX re-infusions were performed as follows: 1000 mg RTX infused twice, 2 weeks apart. In Series 2 and 5 blood samples for PA-RT-PCR analysis were available also before the second infusion of RTX, and showed a non-complete depletion of CD19+ B cells.

about Ct means of GAPDH and CD19, SD ad CV of both Ct values and CD19 RE values showed a good intra-assay reproducibility (Table 1).

Similarly, the inter-assay variability of the PA-RT-PCR was evaluated by testing the pool of RNA used as reference sample and the commercial reference standard RNA 3 times in independent amplification sessions: data about Ct means of GAPDH and CD19, SD ad CV of both Ct values and CD19 RE values showed a good inter-assay reproducibility (Table 2).

3.2.5. Reproducibility of the whole procedure

Reproducibility of the whole procedure has been evaluated by testing 3 paired samples undergoing the full assay procedure, from RNA extraction to final PA-RT-PCR quantification: data expressed as Ct means of GAPDH and CD19, SD ad CV of both Ct values and CD19 RE values showed a good reproducibility of the method (Table 3).

3.3. CD19 mRNA relative expression in RTX-treated patients

Unlike FC, the PA-RT-PCR method showed a detectable amount of CD19 mRNA even in the first weeks after RTX infusion. Thus, a threshold of "CD19 mRNA positivity" had to be set to detect a significant increase of CD19 mRNA that required RTX re-infusion. Several studies on RTX treatment reported a B cell depletion within 2–12 weeks after RTX infusion (Feldman and Razzaque, 2011). In our series, 11 samples collected within 12 weeks after RTX infusions were used to establish a cut-off limit of RTX-induced B cell depletion based on the formula: (mean CD19 mRNA RE of 11 time-points after RTX infusion) + (2SD). The resulting value for the threshold of "CD19 mRNA positivity" was defined accordingly as RE $> 1.739 \times 10^{-3}$.

Based on this cut-off, CD19 mRNA resulted over the threshold in 18/52 (35%) of analyzed samples obtained from RTX-treated patients (Table 4).

3.4. Analysis of CD19 positivity in paired samples (FC and PA-RT-PCR)

In all 8 samples in which an increase of CD19+ B cells was observed by FC (CD19+ B cells was set at <0.1% of total lymphocytes), CD19 RE resulted over the threshold of 1.739×10^{-3}. CD19 positivity was also found by PA-RT-PCR in 8 other paired samples, which resulted negative for CD19 antigen by FC (Table 4). In 3 series out of 5, PA-RT-PCR was able to find CD19 positivity between one and three months before FC did (Fig. 3). In series 5, CD19 mRNA expression appeared to have increased in one isolated time-point, preceded and followed by a negative time-point, resulting in false positivity (Fig. 3).

4. Discussion

The aim of the present study was to evaluate the sensitivity of a RT-PCR method to quantify CD19+ circulating B cells, compared to the FC method.

As traditional RT-PCR was unable to detect CD19 mRNA in all the samples obtained during RTX treatment, we improved the sensitivity of the method by pre-amplification (PA). The resulting PA-RT-PCR was set up following the manufacturer's instructions: uniformity of PA was evaluated and resulted in acceptable values with respect to the manufacturer's indications (PA uniformity for CD19 and GAPDH related to CDKN1B were −0.135 and 0.0925, respectively). The performance of the method was evaluated by analyzing housekeeping gene stability and inter- and intra-assay variability; finally, the reproducibility of the whole procedure was evaluated by testing paired samples undergoing the full assay procedure: data obtained by these analyses showed that PA-RT-PCR is a reliable and reproducible method for quantification of CD19 mRNA.

The method was able to detect CD19 mRNA in all analyzed samples, including those in the first 12 weeks after RTX infusion, which lie under FC threshold of detection.

The detection of CD19 mRNA in all samples required to establish a threshold that was empirically determined as RE 1.739×10^{-3}, using 11 samples tapped in the 12 weeks after RTX infusion. This threshold, that must be verified with a larger sampling, seems to work. In fact at only one time-point out of 47, in Series 5, PA-RT-PCR showed an increment of CD19 mRNA that was preceded and followed by values under the threshold. This can represent a false positive result.

CD19 mRNA quantification was more sensitive than the traditional FC approach, in fact in 3 out of 5 series CD19 RE value above the threshold anticipated the FC method by 1 to 3 months in detecting a significant increment of CD19+ B cells (Fig. 3). Moreover, using FC as gold standard, no false negative results were found as PA-RT-PCR was over the established threshold in all samples in which FC showed an increase of CD19+ cells.

In conclusion, this proof of concept study showed that PA-RT-PCR is a reliable method for the quantification of CD19+ circulating B cells in the blood of RTX-treated patients, and seems to be more sensitive than traditional FC in detecting CD19+ B cell increment. These are preliminary data, obtained by analyzing a limited number of paired samples, and it should be confirmed and validated by future studies with a greater number of patients and time-points. Despite these limitations, the present study indicates that the PA-RT-PCR method could improve the treatment-to-target approach of RTX re-treatment in NMOSDs. This strategy could be applied to other RTX treated diseases and to other monoclonal antibodies directed against CD20+ B cells.

Conflict of interest

Fabiana Marnetto, Letizia Granieri and Paola Valentino received refunds from Biogen Idec and Merck Serono for attending several courses.

Antonio Bertolotto and Marco Capobianco received refunds from Farmades, Aventis, Serono, and Dompé Biotec for attending several conferences. Antonio Bertolotto received: fees for lectures by Serono, Aventis, Dompé Biotec and Biogen Idec; funds for research and for staff members from Serono, Aventis and Dompé Biotec.

Marisa Pautasso has no potential financial conflict of interest related to this manuscript.

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References

Capobianco, M., Malucchi, S., di Sapio, A., Gilli, F., Sala, A., Bottero, R., Marnetto, F., Doriguzzi Bozzo, C., Bertolotto, A., 2007. Variable responses to rituximab treatment in neuromyelitis optica (Devic's disease). Neurol. Sci. 28, 209–211.

Edwards, J.C., Cambridge, G., 2006. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat. Rev. Immunol. 6, 394–403.

Feldman, R.J., Razzaque, A.A., 2011. Relevance of Rituximab Therapy in Pemphigus Vulgaris. Analysis of Current Data and the Immunologic Basis for Its Observed Responses. Expert. Rev. Clin. Immunol. 7, 529–541.

Greenberg, B.M., Graves, D., Remington, G., Hardeman, P., Mann, M., Karandikar, N., Stuve, O., Monson, N., Frohman, E., 2012. Rituximab dosing and monitoring strategies in neuromyelitis optica patients: creating strategies for therapeutic success. Mult. Scler. 18, 1022–1026.

Kim, S.H., Kim, W., Li, X.F., Jung, I.J., Kim, H.J., 2011. Repeated treatment with rituximab based on the assessment of peripheral circulating memory B cells in patients with relapsing neuromyelitis optica over 2 years. Arch. Neurol. 68, 1412–1420 (Nov).

Kim, S.H., Huh, S.Y., Lee, S.J., Joung, A., Kim, H.J., 2013. A 5-year follow-up of rituximab treatment in patients with neuromyelitis optica spectrum disorder. JAMA Neurol. 70, 1110–1117.

McKeon, A., Pittock, S., 2013. Individualized rituximab treatment for neuromyelitis Optica spectrum disorders. JAMA Neurol. 70, 103–104.

Noutsias, M., Rohde, M., Block, A., Klippert, K., Lettau, O., Blunert, K., Hummel, M., Kühl, U., Lehmkuh, l.H., Hetzer, R., Rauch, U., Poller, W., Pauschinger, M., Schultheiss, H.P., Volk,

H.D., Kotsch, K., 2008. Preamplification techniques for real-time RT-PCR analyses of endomyocardial biopsies. BMC Mol. Biol. 9, 3 (14).
Pellkofer, H.L., Krumbholz, M., Berthele, A., Hemmer, B., Gerdes, L.A., Havla, J., Bittner, R., Canis, M., Meinl, E., Hohlfeld, R., Kuempfel, T., 2011. Long-term follow-up of patients with neuromyelitis optica after repeated therapy with rituximab. Neurology 76, 1310–1315.
van Herwaarden, N., van der Maas, A., Jansen, T.L., Dutmer, E.A., Hartkamp, A., van Riel, P.L., Kievit, W., van den Bemt, B.J., den Broeder, A.A., 2013. Can response duration after the first rituximab treatment be used in timing of rituximab re-treatment? Scand. J. Rheumatol. 42, 251–252.
Wingerchuk, D.M., Lennon, V.A., Pittock, S.J., Lucchinetti, C.F., Weinshenker, B.G., 2006. Revised diagnostic criteria for neuromyelitis optica. Neurology 23, 1485–1489.