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# Natalizumab in cerebrospinal fluid and breastmilk of patients with multiple sclerosis

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***Abstract***

**Background:** Natalizumab is a highly effective monoclonal antibody for the treatment of multiple sclerosis (MS), which can diffuse in different anatomical compartments, including cerebrospinal fluid (CSF) and milk.

**Objectives:** Starting from incidental detection of natalizumab in the CSF of MS patients, the objective of this study was to develope a flow-cytometry-based assay and apply it to quantify natalizumab in body fluids, including milk collected from nursing patients over 180 days and in patients with neutralizing antibodies against natalizumab.

**Methods:** CSF, milk and sera samples from patients with multiple sclerosis were tested by flow-cytometry for binding to a VLA-4 expressing cell line or to a control cell line. A standard curve was prepared by incubating the same cells with natalizumab at 50 µg/ml and serially diluted to 0.005 ng/ml. Binding specificity was confirmed using an anti-natalizumab neutralizing antibody.

**Results:** Our assay was sensitive enough to detect natalizumab in CSF, with a lower detection limit of 1.5 ng/ml. Neutralizing antibodies against natalizumab inhibited binding to the cell line. In breastmilk, the peak concentration was observed during the first 2 weeks after infusion and the average concentration over the observation time was 173.3 ng/ml, with a trend toward increased average milk concentration over subsequent administrations.

**Conclusion:** Routine use of such an assay would enable a better understanding of the safety of therapeutic antibody administration during pregnancy and lactation.

***Keywords:*** breastfeeding, cerebrospinal fluid, milk, multiple sclerosis, Natalizumab

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## Introduction

Natalizumab is a recombinant, humanized antibody approved for the treatment of multiple sclerosis (MS), which binds to the integrin α4β1 (or very late antigen-4, VLA-4), thus preventing the adhesion and migration of lymphocytes across the blood–brain barrier (BBB). As a monoclonal antibody of the $IgG_4$ class, its molecular weight is thought to limit crossing of the BBB or diffusion into other anatomical compartments, such as breastmilk. However, in diseases characterized by BBB disruption, large pharmacological molecules may diffuse into the cerebrospinal fluid (CSF) in a less predictable way,[1] and depending on the concentration gradient, monoclonal antibodies, as well as other therapeutic molecules can passively diffuse into milk.[2] In this study, we initially detected natalizumab in the CSF of treated patients while looking for antibodies binding to the peripheral neuroendocrine tumor cell line PFSK1 that expresses the VLA-4. Starting from this finding, we developed a flow cytometry-based assay and used it to determine natalizumab concentration in CSF, serum, and breastmilk of patients with MS.

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**Table 1.** Demographic features of the two independent CSF cohorts.

| | Basel cohort | Graz cohort |
|---|---|---|
| *N* samples | 150 | 100 |
| CIS | 1 | 1 |
| CDMS | 62 | 32 |
| Non-MS | 87 | 67 |
| Age at sampling (average ± *SD*) | 49 ± 16 | 35 ± 11 |
| Sex (female %) | 63% | 60% |
| Under therapy at LP | 15 | 4 |
| Natalizumab | 3 | 2 |
| 4-week treatment interval | 100% | 100% |

CDMS, clinically definite MS; CIS, clinically isolated sydrome; CSF, cerebrospinal fluid; LP, lumbar puncture; MS, multiple sclerosis.

## Methods

### *Sample collection*

CSF samples were collected from diagnostic lumbar punctures from 250 patients from two independent cohorts ($N=150$ for cohort 1, $N=100$ for cohort 2; Table 1). Milk samples were collected from a 35-year-old woman, affected by relapsing–remitting multiple sclerosis (RRMS), under natalizumab treatment for the past 14 years. Informed consent for publication was provided by the patient. Natalizumab administration was stopped 6 weeks before delivery and resumed 4 weeks post-partum, with an infusion every 6 weeks after conception. Eighteen milk samples were collected: before the first post-partum infusion (T0); and then on a weekly basis for the following 125 days (average interval between samples 8 days ± 1). Matching serum samples were collected after 112 and 122 days from the first post-partum infusion (9 and 19 days after the third post-partum infusion, and before the fourth). Additional longitudinal milk samples from an independent published cohort (Universitätsklinikum Carl Gustav Carus, Dresden[3]) were used to compare assay performance. Milk samples from the Dresden cohort were collected under the University Hospital Dresden ethical approval (EK348092014). All the patients who donated the additional milk samples received standard treatment interval. Serum samples included: samples from untreated donors tested as negative controls ($N=17$), samples from patients with MS treated with natalizumab and previously tested positive ($N=22$) or negative ($N=24$) for anti-natalizumab antibodies, by an enzyme-linked immunosorbent assay (ELISA) established and certified in our laboratory. Serum samples were kept at –80°C, CSF and milk samples were aliquoted and kept at –20°C until analysis.

### *Cell lines*

PFSK1 cells and HEK293 cells were purchased from ATCC (LGC, Wesel, Germany; CRL-2060 and CRL-11268, respectively). Cells were used at passages 8–12. Stability of target expression was assessed by measuring natalizumab binding on PFSK1 cells at different passages.

### *RNA sequencing*

PFSK1 and HEK293/T17 cells were lysed in TRIzol, and RNA extracted using standard column chromatography. RNA was quality-checked on the TapeStation instrument (Agilent Technologies) using the RNA ScreenTape (Agilent, 5067-5576) – Average RINe (RNA Integrity Number, version TapeStation) was 9.8 ± 0.3, indicating RNA of high quality and consistency. RNA quantified by fluorometry using the QuantiFluor RNA System (Promega, E3310). Library preparation was performed, starting from 70 ng total RNA, using the TruSeq Stranded mRNA Library Kit (Illumina, 20020595) and the TruSeq RNA UD Indexes (Illumina, 20022371). Fifteen cycles of polymerase chain reaction (PCR) were performed. Libraries were quality-checked on the fragment analyzer (Advanced Analytical) using the Standard Sensitivity NGS Fragment Analysis Kit (Advanced Analytical, DNF-473) revealing excellent quality of libraries (average concentration was 83 ± 13 nmol/l and average library size was 329 ± 6 base pairs).

Samples were pooled to equal molarity. The pool was quantified by Fluorometry using the QuantiFluor ONE dsDNA System (Promega, E4871). Libraries were sequenced Paired-End 38 bases (in addition: eight bases for index 1 and eight bases for index 2) using the NextSeq 500 High Output Kit 75 cycles (Illumina, FC-404-1005) loaded at 2.0 pM and including 1% PhiX.

Primary data analysis was performed with the Illumina RTA version 2.4.11 and Basecalling Version bcl2fastq v2.20.0.422. Two NextSeq runs were performed to compile enough reads (on average per sample: 507 ± 15.5 million pass-filter reads).

### *Flow cytometry*

Before measurement, milk samples were thawed and centrifuged at 2000× g for 10 min for lipid separation. For the assay, 50,000 PFSK1 cells were distributed into each well of 96-well plates. For the first experiment a serial two-fold dilution of milk was done, from undiluted until 1:512 dilution. A 1:4 dilution of milk was chosen for the following three independent replicates. CSF was tested at 1:4 dilution. Serum was tested using a serial two-fold titration starting from 1:20. A standard curve was prepared by incubating the same cells with T0 milk, spiked with natalizumab starting from 50 µg/ml and serially diluted to 0.005 ng/ml. To test antibody specificity, PFSK1 cells were incubated with either natalizumab, milk, or serum in presence of an anti-natalizumab neutralizing antibody (Bio-Rad HCA250). Cells were incubated on ice for 30 min, washed twice in phosphate-buffered saline (PBS) and labeled with 1:200 dilution of Alexafluor-488-conjugated anti-human immunoglobulin G (IgG) (JIR 109-096-098) or anti-$IgG_4$ (Southern Biotech, 9200-30) for 30 min on ice. After two washes in PBS, cells were resuspended in 4% paraformaldehyde and acquired using a Beckman Coulter CytoFLEX flow cytometer equipped with a 96-well plate reader.

### *Statistical analysis*

Concentrations were calculated using the quadrantic interpolator from dcr package in R and by noncompartmental pharmacokinetic methods, provided by GraphPad Prism (GraphPad Software, La Jolla, California), subtracting the geometric mean of the fluorescent intensity (GMFI) of cells incubated with T0 milk to the GMFI of the standard curve. Average milk concentration over a 6-week period was calculated excluding the concentration obtained during the first 2 weeks after infusion to eliminate the peak effect. The relative infant dose (RID) was calculated using a method described by Bennett with the assumption that the infant will consume approximately 150 ml/kg of breastmilk per day (Bennet, 1988).[4]

## Results

### *Free natalizumab is detected in CSF, serum, and milk of treated patients*

While screening a cohort of 150 CSF samples for antibodies binding to a panel of neural derived cell lines, we identified a subset of MS patients ($n=3$ out of 63 MS patients) with CSF IgG binding to PFSK1 cells. None of the non-inflammatory controls ($n=87$) showed IgG reactivity against this cell line (Figure 1a). The test of an independent CSF cohort ($n=100$) (Neurologie, Medizinische Universität Graz) and analysis of the clinical features of positive patients, revealed that all PFSK1-binding IgG-positive patients ($n=5$ from the two cohorts) were under natalizumab treatment (Figure 1(b)). We confirmed that the identified PFSK1 reactivity was due to the detection of free natalizumab using an anti-$IgG_4$ secondary antibody and by testing binding of natalizumab on the same cell line (Figure 2(a)). We next tested 22 CSF and 20 serum samples in a blind fashion: all three patients under natalizumab showed $IgG_4$ binding to PFSK1 in both CSF and serum while none of the untreated patients showed this reactivity (Figure 1(c)–(d)). Average CSF natalizumab concentration was 63.6 ± 32.1 ng/ml (mean ± *SE*). CSF natalizumab content correlated with the time from previous infusion but did not correlate with treatment duration. Expression of VLA-4 by PFSK1 cells and minimal expression by HEK293/T17 cells was confirmed by RNA seq (1934 reads *versus* 71, respectively). Binding of natalizumab on both cell lines was further assessed by flow cytometry, demonstrating PFSK1-specific binding (Figure 2(a)). The use of the neutralizing anti-natalizumab antibody HCA250 further confirmed $IgG_4$-binding specificity on PFSK1 cells (Figure 2(b)). VLA-4 expression stability was confirmed by incubating PFSK1 cells coming from seven different passages with natalizumab at 10 µg/ml (Figure 2(c)). $IgG_4$ binding to PFSK1 was reduced in 21 of 22 samples from patients treated with natalizumab and positive for neutralizing anti-natalizumab antibodies by standard ELISA (Figure 2(d)).

Having demonstrated the feasibility of measuring natalizumab concentration with a lower detection limit of 1.5 ng/ml, we applied the technique to measuring free natalizumab in breastmilk (Figure 3(a)). $IgG_4$ binding from the milk at T0, obtained after 70 days from the last pre-delivery administration, was comparable with the one obtained incubating the cells only with the secondary

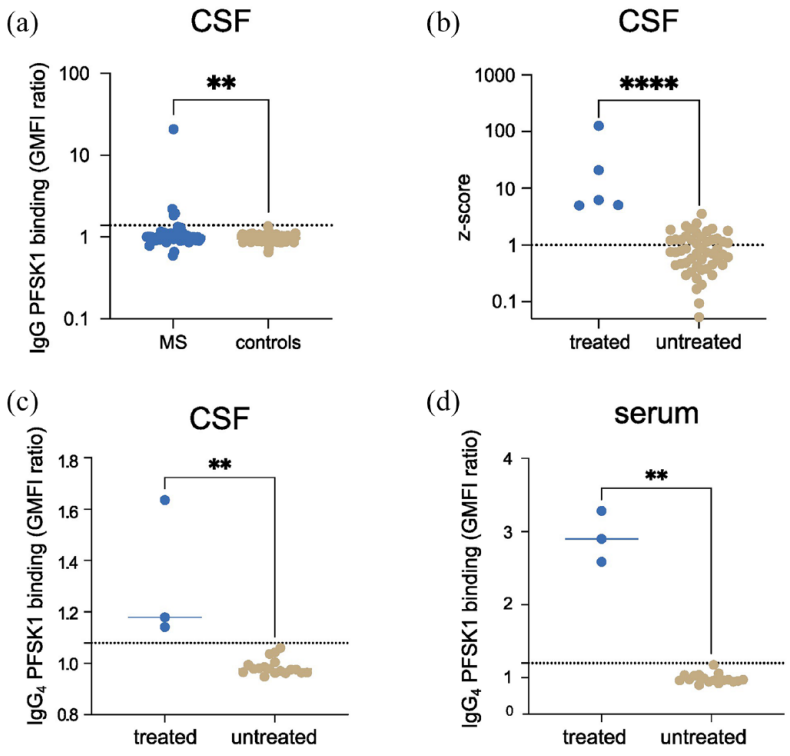


**Figure 1.** (a) IgG binding on PFSK1 cells from CSF of MS donors compared with non-inflammatory controls ($p = 0.008$, Mann–Whitney test) using an anti-IgG antibody. Each dot corresponds to the binding of IgG on PFSK1 (ratio of geometric mean of the florescence intensity – GMFI – of cells incubated with the sample to cells incubated only with secondary antibody) from each CSF sample. (b) IgG binding on PFSK1 cells in natalizumab treated compared with untreated patients; results from two independently tested cohorts are pooled and expressed as the number of standard deviations by which each sample is above or below the mean value of the control samples ($p < 0.0001$, Mann–Whitney test). Each dot corresponds to one sample. (c) Natalizumab detection in CSF from treated patients compared with untreated patients ($p < 0.0015$, Mann–Whitney test) using an anti-$IgG_4$ antibody. Each dot corresponds to the binding of $IgG_4$ on PFSK1 (ratio of geometric mean of the florescence intensity – GMFI – of cells incubated with the sample to cells incubated only with secondary antibody) from each serum sample. (d) Natalizumab detection in serum from treated patients compared with untreated patients ($p < 0.0018$, Mann–Whitney test) using an anti-$IgG_4$ antibody. Each dot corresponds to the binding of $IgG_4$ on PFSK1 (ratio of GMFI of cells incubated with the sample to cells incubated only with secondary antibody) from each serum sample.

antibody. Free natalizumab was detectable in all the milk samples after the first drug exposure, with a concentration of $173.3 \pm 52.7$ ng/ml (mean $\pm$ *SE*) overall. Peak natalizumab concentration was reached after 1 week from each infusion, with a maximum concentration of 878 ng/ml after the first infusion. Parallel serum samples were available at two timepoints, after 9 and 19 days from the third infusion, with an average concentration of 4639 ng/ml and a breastmilk to serum ratio of 5.38% and 1.7%, respectively. RID calculated from the average natalizumab concentration in milk over the whole observation time was equal to 1.1%, in line with recently published data and well below the 10% that is generally considered safe for breastfeeding. When comparing natalizumab concentration over each dose interval, we observed a tendency toward reduction of the peak effect and a progressive increase of the average concentration over the time, although this did not reach statistical significance (Figure 3(a) and (b)). The concentration of natalizumab in longitudinally collected milk samples from three treated patients of an independent cohort correlated and were similar to those previously reported by the independent center, except for one patient, from which we detected concentrations up to two-fold lower when compared with the original measurement (Supplementary Figure 1), suggesting a potential impact of the storage time on assay reproducibility.

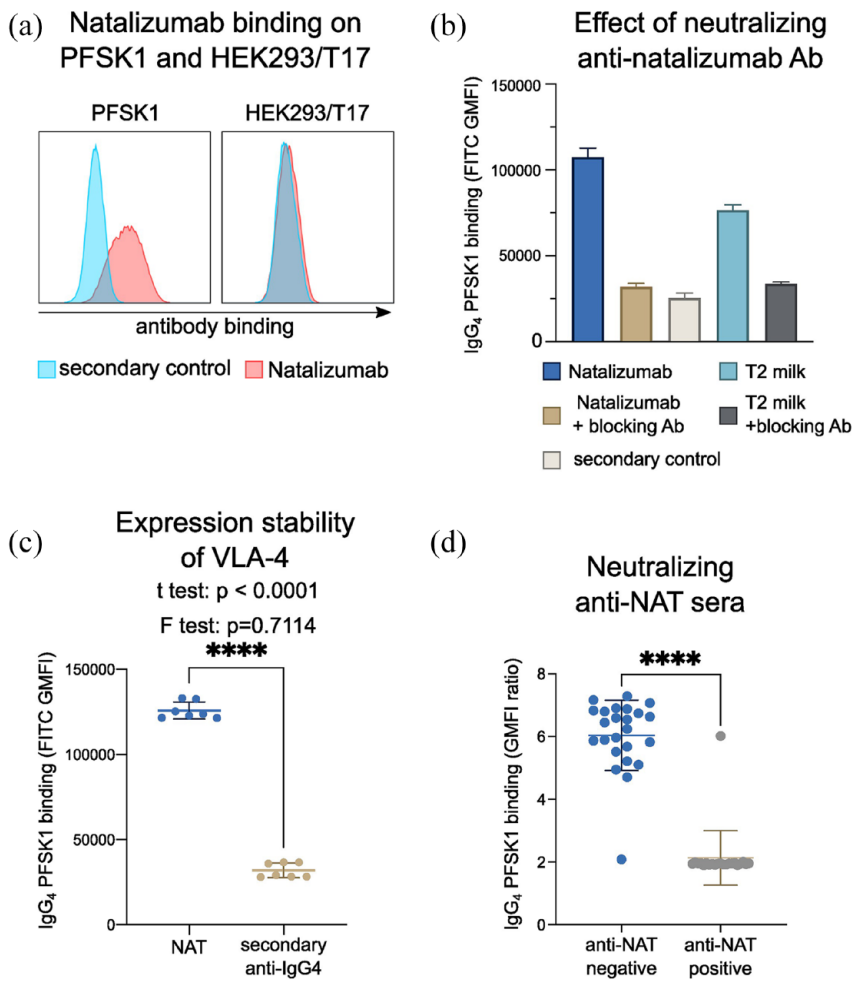


**Figure 2.** (a) Natalizumab binding on VLA-4 expressing cells (PFSK1) compared with non-expressing cells (HEK293/T17) by flow cytometry using an FITC-conjugated secondary anti-$IgG_4$. (b) Effect of natalizumab blocking antibody HCA250 on natalizumab and milk (T2) $IgG_4$ binding to PFSK1 cells. Serum was used at 1:40 dilution. Each column corresponds to average binding of $IgG_4$ on PFSK1 (geometric mean of the florescence intensity – GMFI – of cells incubated with the sample) from three replicates. Error bars are standard error. (c) Stability of expression of VLA-4 by PFSK1 cells. PFSK1 cells from passages 7 to 13 were incubated with either natalizumab at 10 µg/ml or with secondary only control. *p*-value for comparing binding of natalizumab to secondary only was calculated using unpaired *t*-test. Variances were compared using *F*-test. (d) Natalizumab detection in serum from treated patients positive for anti-natalizumab antibodies were compared with anti-natalizumab negative samples ($p < 0.0001$, Mann–Whitney test) using an anti-$IgG_4$ antibody. Each dot corresponds to the binding of $IgG_4$ on PFSK1 (ratio of GMFI of cells incubated with the sample to cells incubated only with secondary antibody) from each serum sample, tested in three independent experiments.

## Discussion

During an unbiased screening of CSF samples looking for autoantibodies binding to novel antigenic targets in MS patients, we encountered a subset of MS samples with IgG reactivity against PFSK1 cells which could have been misinterpreted as MS-specific antibodies but were actually due to a confounding variable (natalizumab treatment). We decided to apply this result to develop a flow cytometric assay to measure natalizumab in different biological samples, including serum, CSF, and milk. Diffusion of natalizumab into CSF and milk has been reported (Table 2).[3,5–10] Our newly developed assay for detection of natalizumab is easy to establish, reproducible and sensitive enough to enable measurement in two biological compartments where diffusion of monoclonal antibodies is considered low or negligible.[1] The assay was also suited to identify patients with neutralizing antibodies against natalizumab. The

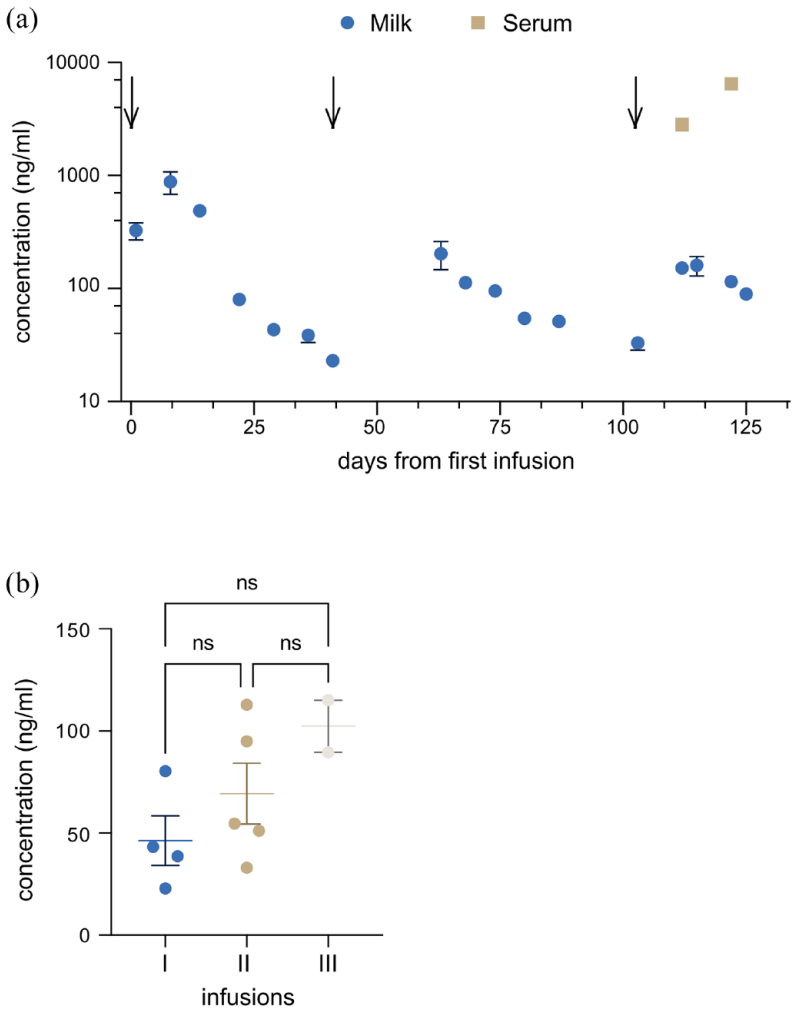


**Figure 3.** (a) Natalizumab concentration in breastmilk and paired serum samples. The horizontal axis shows the number of days from the first infusion. The vertical axis shows natalizumab concentration in ng/ml. Each dot corresponds to the mean of three replicates. Error bars are standard error. Arrows indicate single infusions. (b) Comparison of milk average concentration during three subsequent infusion intervals. Natalizumab concentration from each inter-dose timeframe is plotted, excluding the first 2-week timepoints, and compared by Mann–Whitney test, showing non-significant progressive accumulation of the drug in milk. Error bars show standard error.

measured concentrations are comparable with those reported by others (Table 2). When we measured samples from an independent cohort, which had been collected from 2017 on, we found correlating and comparable concentrations. However, the observation of lower concentrations in one of the patients suggests that parameters for long-term storage of milk samples between collection and measurement may need optimization.

Currently natalizumab administration is contraindicated during pregnancy and lactation, as are most of the disease-modifying treatments approved for MS, due to lack of safety information.[11] On the other hand, natalizumab discontinuation has been associated with MS reactivation and MS rebound activity[12] that requires close monitoring after withdrawal.

While the limited number of samples tested does not allow to make a generalized statement about natalizumab kinetics and safety, the incorporation of our assay into routine patient monitoring would provide further guidance for the management of therapeutic antibody administration during pregnancy and lactation.

**Table 2.** Summary of studies reporting measurement of natalizumab in breastmilk in women with MS or IBD treated during pregnancy and breastfeeding and in CSF of treated MS patients. $C_{max}$ is maximum measured concentration.

| Publication | Type of study | Material | NAT dose (mg) | Interval (weeks) | Analytical method | No. of patients | $C_{max}$ | Peak |
|---|---|---|---|---|---|---|---|---|
| Baker *et al.*[5] | Case report | Milk | 300 | 4 | ELISA at Biogen | 1 | 2.83 µg/ml | 50 d |
| Ciplea *et al.*[8] | Prospective | Milk | 300 | 4–6 | ELISA with anti-idiotype antibody | 2 | 140 ng/ml | 2–5 d |
| Matro *et al.*[7] | Prospective | Milk | 300 | 4 | Homogenous mobility shift assay | 2 | 460 ng/ml | 24 h |
| Proschmann *et al.*[6] | Cohort study | Milk | 300 | 4 | Flow cytometry | 4 | 412 ng/ml | 1–8 d |
| Proschmann *et al.*[3] | Prospective | Milk | 300 | 4 | Flow cytometry | 11 | 306 ng/ml | 1–8 d |
| Sehr *et al.*[9] | Prospective | CSF | 300 | 4–8 | Flow cytometry | 27 | 44.8 ng/ml | |
| Harrer *et al.*[10] | Prospective | CSF | 300 | $3.9 \pm 1.5$ | ELISA | 15 | 111 ng/ml | |

CSF, cerebrospinal fluid; IBD, inflammatory bowel disease; MS, multiple sclerosis; NAT, natalizumab.

## Declarations

### *Ethics approval and consent to participate*

All procedures were approved by the Ethical Committee of Northwest Switzerland (study protocol 2021-00908) and by the Ethical Committee of the Medical University of Graz, Austria (study protocol 17-046 ex 05/06 and 31-432 ex 18/19). Milk samples from the Dresden cohort were collected under the University Hospital Dresden ethical approval (EK348092014). Written informed consent was obtained from all the study participants.

### *Consent for publication*

Informed consent for publication was provided by the patient donating milk samples.

### *Author contributions*

**Ilaria Callegari:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing – original draft.

**Mika Schneider:** Investigation; Writing – original draft.

**Vera Aebischer:** Investigation; Writing – original draft.

**Margarete M. Voortman:** Investigation; Writing – original draft.

**Undine Proschmann:** Investigation; Writing – review & editing.

**Tjalf Ziemssen:** Investigation; Writing – review & editing.

**Raija Lindberg:** Investigation; Writing – review & editing.

**Bettina Fischer-Barnicol:** Investigation; Writing – original draft.

**Michael Khalil:** Investigation; Methodology; Writing – original draft.

**Ludwig Kappos:** Conceptualization; Writing – original draft.

**Jens Kuhle:** Investigation; Writing – original draft.

**Nicholas S.R. Sanderson:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing – original draft.

**Tobias Derfuss:** Conceptualization; Funding acquisition; Investigation; Methodology; Supervision; Writing – original draft.

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### *Competing interests*

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: U.P. received speaker fee from Merck, Biogen and Bayer and personal compensation from Biogen and Roche for consulting service. B.F.B. received financial compensation for participation in advisory boards for Biogen. M.K. has received funding for attending meetings or travel from Merck and Biogen, honoraria for lectures or presentations from Novartis and Biogen and speaker serves on scientific advisory boards for Biogen, Merck, Roche, Novartis, Bristol-Myers Squibb, and Gilead. He received research grants from Biogen and Novartis. L.K. discloses research support to his institution (University Hospital Basel): steering committee, advisory board, and consultancy fees (Actelion, Bayer HealthCare, Biogen, BMS, Genzyme, Janssen, Merck, Novartis, Roche, Sanofi, Santhera, and TG Therapeutics); speaker fees (Bayer HealthCare, Biogen, Merck, Novartis, Roche, and Sanofi); support of educational activities (Allergan, Bayer HealthCare, Biogen, CSL Behring, Desitin, Genzyme, Merck, Novartis, Pfizer, Roche, Sanofi, Shire, and Teva); license fees for Neurostatus products; and grants (Bayer HealthCare, Biogen, European Union, InnoSwiss, Merck, Novartis, Roche, Swiss MS Society, and Swiss National Research Foundation). J.K. received speaker fees, research support, travel support, and/or served on advisory boards by Swiss MS Society, Swiss National Research Foundation (320030_189140/1), University of Basel, Progressive MS Alliance, Bayer, Biogen, Bristol Myers Squibb, Celgene, Merck, Novartis, Octave Bioscience, Roche, Sanofi. T.D. received financial compensation for participation in advisory boards, steering committees, and data safety monitoring boards, and for consultation for Alexion, Novartis Pharmaceuticals, Merck, Biogen, Celgene, GeNeuro, MedDay, Mitsubishi Tanabe Pharma, Roche, and Sanofi Genzyme. T.D. also received research support from Alexion, Roche, Biogen, National Swiss Science Foundation, European Union, and Swiss MS Society.

### *Availability of data and materials*

All data and material used for this manuscript are available upon request.

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## Supplemental material

Supplemental material for this article is available online.

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