Introduction

Treatment of Multiple sclerosis (MS) with natalizumab (Nat) is associated with progressive multifocal leuкоencephalopathy (PML). The presence of antibodies against the causative JC virus (JCV) increases the risk to develop Nat-associated PML (Nat-PML).\(^1\)

The level of antibody reactivity is proposed to refine the risk to develop Nat-PML diagnosis,\(^5\) persistently high anti-JCV antibody indices (AIs) prior to diagnosis have been reported.\(^2,6\)

We examine persistence high anti-JCV antibody indices (AIs) prior to diagnosis have been reported.\(^2,6\) We examine persistence in samples before Nat-PML diagnosis than in seropositive Nat-MS (2.4 (1.0–3.4), \(n = 298, p = 0.010\)). AIs \(\geq 3.0\) were associated with a 14.5-fold (95% CI 2.3–90.4) increased PML risk \((p = 0.002)\). Groups with an AI below 1.5 exhibit higher variability or even serostatus fluctuation. AI dynamics require further investigation.

Methods

Ethics approval was obtained (Ruhr-University Bochum, registration-no. 3814-10). The anti-JCV AI was retrospectively determined by STRATIFY JCV\(^\text{TM} \) DxSelect\(^\text{TM} \)\(^7\) in the following cohorts: a longitudinal cohort of Nat-treated MS patients in the post-marketing setting (Nat-MS, \(n = 468\), Table 1); Nat-PML patients \((n = 15, \text{Table 2})\); patients with PML of other etiology (lymphoma: \(n = 2\); HIV: \(n = 1\); Fumaderm\(^\text{®} \) treatment\(^8\): \(n = 1\); Table 3, all PML diagnosed according to ref. 9).

Clinical data of 10/15 Nat-PML patients (Table 2, patients 1–10) were presented in our previous study;\(^3\) 4/15 (patients 2, 6–8) were included in a previous study\(^2\). Samples were available prior to Nat-PML \((n = 9 \text{ patients})\) and longitudinally (before/at/after PML-diagnosis, \(n = 8 \text{ patients, Table 2})\). Of the multiple samples taken before PML diagnosis, the earliest was included in the cross-sectional analyses.

Statistical analyses were performed using SPSS 22 with \(p\)-values < 0.05 considered significant. Data are presented as median (25th to 75th percentile) or (95% confidence interval (CI)). Odds ratios (ORs) of the CI were calculated with the Cox–Hinkley–Miettinen–Nurminen method.

Results

**Nat-MS cohort**

63.7% \((n = 298)\) were anti-JCV seropositive at first sampling. Irrespective of serostatus, the anti-JCV AI
Table 1. Demographic and clinical characteristics of longitudinal Nat-MS cohort.

| Total cohort \( (n = 468) \) | \( n \) | Median | 25th to 75th percentile |
|-----------------------------|-------|--------|------------------------|
| \( n \) | |        |                        |
| Age at MS diagnosis, y     | 415   | 28.6   | 21.6–35.3              |
| MS duration at Nat-initiation, y | 413   | 6.6    | 3.0–10.8               |
| Nat-duration at 1st sampling, m | 449   | 5.0    | 3.2–6.5                |
| Interval between 1st and 2nd sampling, m | 463   | 5.1    | 3.0–7.6                |

| \( n \) | Median (IQR) | \( n \) | Median (IQR) | \( n \) | Median (IQR) | \( p \)-Value\(^3\) |
|--------|--------------|--------|--------------|--------|--------------|----------------|
| Age at 1st sampling, y     | 465   | 38.6 (30.0–44.3) | 297   | 39.3 (31.1–45.2) | 168   | 36.1 (28.9–42.9) | 0.005 |

| \( n \) | \% \(^3\) | \( n \) | \% \(^3\) | \( n \) | \% \(^3\) |
|--------|-------|--------|-------|--------|-------|
| Gender |       |        |       |        |       |
| male   | 126   | 27.0   | 86    | 29.1   | 40    | 23.5   | n.s.   |
| female | 340   | 73.0   | 210   | 70.9   | 130   | 76.5   |

| \( n \) | \% \(^3\) | \( n \) | \% \(^3\) | \( n \) | \% \(^3\) |
|--------|-------|--------|-------|--------|-------|
| Pre-treatment of any kind (immunomodulators and immunosuppressants) |       |        |       |        |       |
| yes    | 387   | 95.1   | 247   | 95.7   | 140   | 94.0   | n.s.   |
| no     | 20    | 4.9    | 11    | 4.3    | 9     | 6.0    |

| \( n \) | \% \(^3\) | \( n \) | \% \(^3\) | \( n \) | \% \(^3\) |
|--------|-------|--------|-------|--------|-------|
| Immunomodulatory pre-treatment |       |        |       |        |       |
| yes    | 364   | 89.4   | 229   | 88.8   | 135   | 90.6   | n.s.   |
| -no    | 43    | 10.6   | 29    | 11.2   | 14    | 9.4    |

| \( n \) | \% \(^3\) | \( n \) | \% \(^3\) | \( n \) | \% \(^3\) |
|--------|-------|--------|-------|--------|-------|
| Immunosuppressive pre-treatment (incl. mitoxantrone) |       |        |       |        |       |
| yes    | 60    | 14.7   | 42    | 16.3   | 18    | 12.1   | n.s.   |
| no     | 347   | 85.3   | 216   | 83.7   | 131   | 87.9   |

(continued)
showed modest fluctuation over time: intra-individual coefficient of variation (CV) 9.8% (4.8–17.6); median AIs of 0.8 (0.2–2.8) at first, 0.9 (0.2–2.8) at second time point. Initially seropositive patients exhibited an AI of 2.4 (1.0–3.4) at first testing with a CV of 7.7% (3.7–15.3). Eight of 298 seropositive patients had no AI change, 113/298 an AI increase, and 177/298 an AI decrease.

Stratified by AI thresholds,2 groups with lower AIs demonstrated higher CV (AI ≤ 0.4 (n = 193): CV 12.9% (6.7–22.6); AI > 0.4–≤ 0.9 (n = 45): CV 15.5% (8.1–26.2) versus AI > 1.5–≤ 3.0 (n = 95): CV 9.4% (4.2–15.2); AI > 3.0 (n = 103): CV 4.6% (2.4–7.8)). A significant difference in CV was also observed between AI groups > 0.9–≤ 1.5 (n = 32, CV 12.2% (4.3–27.1)) versus > 3.0 and > 1.5–≤ 3.0 versus > 3.0 (all p < 0.001, Kruskal–Wallis/Dunn’s post hoc test, no differences in sampling intervals, Figure S1 (available online)).

Serostatus change from negative (36.3% at first time point) to positive was observed in 11% over 7.6 (4.6–12.2) months. Twelve of 19 exhibited an AI ≤ 0.9 at second testing. The fastest seroconversion from negative to positive with an AI increase of 2.0 was observed over 1.8 months; a maximum AI increase from 0.1 to 3.5 was seen within 11.8 months.

Serostatus change from positive (63.7% at first time point) to negative occurred in 4% over 4.6 (2.8–7.1) months. The highest initial AI in this subgroup was 0.83. In 9/12 patients, confirmatory testing determined seronegative status at second testing.

Despite high CV of 33.3% (19.8–88.4) in patients with change in serostatus (n = 31), AIs remained low (first AI 0.3 (0.2–0.4); second AI 0.4 (0.3–0.7)).

Seropositive patients were older than seronegative patients (39.3 (31.1–45.2), n = 297, versus 36.1 (28.9–42.9), n = 168, p = 0.005, Mann–Whitney test, two-sided). Gender, pre-treatment groups (comprising any pre-treatment prior to initiation of Nat (i.e. immunomodulators and immunosuppressants), immunomodulators, immunosuppressants including mitoxantrone, mitoxantrone alone), anti-Nat antibody status were neither associated with serostatus (Table 1) nor anti-JCV AI in seropositive patients (Figure 1).

**PML cohorts**

Nat-PML and PML patients of other etiology tested seropositive at all time points (Tables 2 and 3). In samples 26.3 (19.2–34.5) months before Nat-PML

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### Table 1. Continued.

|                  | anti-JCV negative (1st sample) | anti-JCV positive (1st sample) |
|------------------|-------------------------------|-------------------------------|
|                  | n                             | n                             |
|                  | %3                            | %3                            |
| Mitoxantrone pre-treatment |                  |                  |
| yes              | 43                            | 364                           |
| no               | 188                           | 208                           |
| Anti-Nat antibody status |                  |                  |
| positive         | 19                            | 45                            |
| negative         | 169                           | 369                           |
| Refer to table 2 for an explanation of the footnotes.
### Table 2. Demographic and clinical characteristics of Nat-PML patients.

| Case no. | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 |
|----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Sex      | F  | F  | F  | M  | F  | F  | F  | F  | M  | F  | M  | F  | M  | F  | F  |
| Age at PML-diagnosis, y | 35 | 40 | 35 | 42 | 45 | 58 | 30 | 34 | 41 | 33 | 46 | 43 | 46 | 52 |
| Sample collection in relation to PML-diagnosis (- before), m | 2.9 a) | -29.5 b) | -17.7 | -36.7 a) | -33.6 b) | -26.3 | a) 6.8 b) | 15.1 | a) -35.3 b) -27.8 c) 0.2 | 0.9 b) 0.1 c) 12.5 | a) -23.2 b) -8.0 c) 0.4 |
| No. of Nat infusions at PML-diagnosis | 31 | 29 | n/a | 24 | n/a | 39 | 31 | 30 | 27 | 37 | n/a | 30 | 38 | 40 | >70 |
| Immunosuppressive pre-treatment | yes | no | no | no | no | no | yes | no | no | no | no | no | no | no | no |
| AI | 3.6 a) | 3.6 b) | 1.8 a) | 3.4 b) | 3.2 | a) 3.0 b) | 3.9 | a) 3.1 b) 3.5 c) 0.2 | 3.3 a) 3.9 b) 2.9 c) 3.9 | a) 3.95 b) 4.43 c) 3.32 |
| JCV DNA, copies per ml (CSF) | 72 | 120 | 24 | 29,750 | 9 | 660 | 255 | 37 | 8 | 22 | 544 | neg | 6,930 | 120 |
| JCV DNA, copies per ml (s) | n/a | n/a | 30 | neg | 66 | n/a | neg | 533 | neg | 2 | 1435 | neg | 270 | neg |

1 Indicates data available for number of patients, due to the retrospective character, diverging numbers are explained by missing data. 2 Mann-Whitney test, two-sided; 3 Refer to the respective subgroup. 4 Fisher’s exact test, two-sided. 5 For samples < 1 month after diagnosis, effects of plasma exchange (PLEX) may be considered, PLEX dates not known. 6 Collected at the time point of diagnosis, if not indicated otherwise. 7 3 months after diagnosis. 8 4 months after diagnosis. 9 6 months after diagnosis. AI: antibody index; CSF: cerebrospinal fluid; DNA: deoxyribonucleic acid; F: female; IQR: interquartile range; JCV: John Cunningham virus; m: months; M: male; n/a: not available; n.s.: not significant; Nat: natalizumab; neg: negative; no.: number; PML: progressive multifocal leukoencephalopathy; s: serum; y: years.
(n = 9), the AI was higher (3.4 (3.1–3.6)) than in seropositive Nat-MS (2.4 (1.0–3.4), n = 298, \( p = 0.010, \) Mann–Whitney test, two-sided). The AI before occurrence of Nat-PML was \( \geq 3.0 \) in eight out of nine patients resulting in a 14.5 higher (95% CI 2.3–90.4) PML risk with an AI threshold dichotomized as \( \geq 3.0 \) versus \(< 3.0\) (\( p = 0.002, \) Fisher’s exact test, two-sided). The lowest AI threshold with increased PML risk was \( \geq 2.2\) (OR 6.9 (95% CI 1.1–42.0); \( p = 0.044\)).

Longitudinal Nat-PML samples demonstrated persistently high AIs \( \geq 3.0\). AIs remained \( \geq 3.0\) in all but one sample at/after Nat-PML diagnosis (Table 2). PML of other etiology exhibited an AI of 3.7 (1.2–3.9) around time point of diagnosis (Table 3).

### Discussion

Seroprevalence in this Nat-MS cohort was higher (63.7%) than in other studies\(^3,5,6,10\) but proportions of patients with serostatus change were similar.\(^3,5\) Seropositivity was consistently associated with age.\(^3,10\) Significant associations of AI levels with age, gender, pre-treatment, and anti-Nat antibodies within the seropositive cohort were absent.

AI levels in our seropositive Nat-MS patients are higher than in the initial report\(^2\), but similar to a more recent study.\(^5\) The reasons are not clear; our cohort and the latter with open-label treatment in different clinical settings may reflect an unbiased, mixed collective.

Of note, variation of anti-JCV AI is common, with only eight out of 298 seropositive patients exhibiting no AI change at all. Yet, there are differences in the magnitude of variability. Stratified by anti-JCV AI, groups with low AI (\( \leq 0.4; > 0.4–< 0.9\)) exhibited higher variability partially leading to change in serostatus despite advances of the second generation assay.\(^6,7\) Especially in these subgroups, determination of serostatus may be less predictive than absolute AI level to determine low PML-risk.

The lowest AI variability was seen in AI-groups \( > 1.5\) versus \( \geq 1.5\) but \( > 3.0\); however, for AIs above 2.5 the assay signal is saturated, partially explaining lower CV in high AI groups.

As an AI threshold of \( \leq 1.5\) versus \( > 1.5\) is proposed to provide a conservative risk estimate,\(^2\) evaluation of AI fluctuation in the group of \( > 1.5\) may help to refine PML risk assessment: individual dynamics of anti-JCV AI exceeding the projected inter-test variability defined as more than twofold median CV in this group, i.e. 18.8%, may point to an underlying biological process.

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### Table 3.

Demographic and clinical characteristics of patients with PML of different etiology.

| Case no. | 1        | 2        | 3        | 4        |
|----------|----------|----------|----------|----------|
| PML etiology | Fumaderm \(^6\) | HIV | lymphoma | lymphoma |
| Sex      | M        | M        | M        | M        |
| Age at PML-diagnosis, y | 69        | 45        | 64        | 56        |
| Sample collection in relation to PML-diagnosis, m | 0.5        | 0        | 3.8        | 0        |
| Immunosuppressive pre-treatment | no        | no        | yes        | yes        |
| AI       | 3.6      | 3.9      | 1.2      | 3.8      |
| JCV DNA, copies per ml (CSF) \(^1\) | 16        | 2400     | neg\(^2\) | 4125     |
| JCV DNA, copies per ml (s) | neg        | n/a      | n/a      | n/a      |

\(^1\) Collected at the time point of diagnosis, if not indicated otherwise. \(^2\) 4 months after diagnosis. AI: antibody index; CSF: cerebrospinal fluid; DNA: deoxyribonucleic acid; HIV: human immunodeficiency virus; JCV: John Cunningham virus; m: months; M: male; n/a: not available; neg: negative; PML: progressive multifocal leukoencephalopathy; s: serum; y: years
remains unclear taking into account higher AIs in comparison with previous observations and the small number of samples before PML diagnosis \( (n = 9) \) in our study. Still, even a more conservative threshold, e.g., at an AI level of 1.5, can only reflect statistical risk estimations that need to be combined with individual risk-assessment.

Whether determination of AI dynamics, especially in AIs > 1.5, may be additionally helpful in PML risk assessment, deserves further investigation in a prospective setting. Our study supports repeated determination of anti-JCV-AI in addition to serostatus in Nat-PML risk stratification.

**Conflict of interest**

A.S. received personal compensation for activities with Novartis, Sanofi and Almirall Hermal GmbH. N.v.A. and A.K.T. report no disclosures. R.H. received research and travel grants from Biogen and Novartis. T.P. and M.S. are employees of Biogen and hold stocks. G.K. was a Biogen employee at the time work was completed and holds stocks at Biogen. R.G. received personal compensation for activities with Bayer Healthcare, Biogen, Merck Serono, Teva Neuroscience, Novartis and from the German Ministry for Education and Research (BMBF, “German Competence Network Multiple Sclerosis” (KKNMS), CONTROL MS, 01GI0914). A.C. received consulting fees, speaker honoraria (Almirall, Bayer Schering, Biogen, Genzyme, Merck Serono, Novartis, Sanofi, Teva); research support (Biogen, Genzyme, Novartis); and research grants from the German Ministry for Education and Research (BMBF, “German Competence Network Multiple Sclerosis” (KKNMS), CONTROL MS, 01GI0914).

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