Erythroblastaemia in natalizumab-treated patients with multiple sclerosis

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INTRODUCTION

Natalizumab is a monoclonal antibody that significantly reduces the occurrence of relapses in relapse-remitting multiple sclerosis (RRMS) patient.(1) It is a recombinant antibody directed against the α4 subunit of the α4 β1 (VLA-4) integrin of the lymphocytes. It prevents the binding between VLA-4 and vascular endothelium cell adhesion molecule 1 (VCAM-1), which, over time, decreases α4 expression, resulting in a reduced extravasation of inflammatory immune cells across the blood-brain barrier into the central nervous system.(2)
Due to modifications of the bone marrow vascular niche and the interference of natalizumab with the homing of hemotopoietic stem cells, the major hematologic finding in patients treated with natalizumab relates to the number of CD34+cells, which rapidly egress from the bone marrow cavity into the peripheral blood.(3)
VLA-4 also play a critical role in erythropoiesis(4), being essential for the terminal proliferation and differentiation of erythroid progenitor cells. Erythroid cells specifically express fibronectin receptors α4 β1 and the engagement of α4 β1 integrin by fibronectin provides signals necessary for the terminal expansion of differentiating erythroblasts.(5)
Early papers on the clinical use of natalizumab in RRMS patients reported erythroblastemia as occasional and transient.(6-7) Recently, a report on 14 RRMS patients treated with natalizumab observed on blood smears a high prevalence of nucleated red blood cells (NRBCs) (93%) versus a negative value in 14 interferon treated patients.(8)
Clear data of prevalence on wider populations of patients compared with other treatment options and healthy controls is lacking. This study aimed to determine the prevalence and count of NRBCs in peripheral blood of natalizumab, fingolimod, interferon, glatiramer acetate and treatment naïve patients and healthy controls of the same geographic area using the same lab equipment.

METHODS

Study population
Peripheral blood samples were consecutively selected from RRMS patients of our Multiple Sclerosis center between November and December 2014. The samples of 209 patients were included and classified according to pharmacological treatment, as follows: 26 subjects receiving natalizumab (NTZ), 76 receiving interferon (IFN), 44 receiving glatiramer acetate (GA), 16 receiving fingolimod (FTY) and 47 treatment naïve patients (NA). Patients with previous immunosuppressive or previous natalizumab treatment were not included.
Furthermore, two-hundred forty healthy subjects (HS) were randomly selected among healthy blood donors of the same geographic area to be healthy controls for our RRMS patients.
Sample preparation and analysis
A total number of 800 peripheral whole blood samples (560 RRMS patients and 240 HS) collected in K3EDTA blood tubes (Becton Dickinson, Franklin Lakes, NJ) and processed on XN-9000 (Sysmex Co., Kobe, Japan) were analyzed. The cell blood count (CBC) and extended leukocyte differential count was always performed within 2 hour from sample collection.
XN-9000 analyzer has a specific channel (WNR) for NRBCs counting based on optical fluorescence system associated with a specific lysing agent which is responsible for selective lysis of red blood cells (RBCs). NRBCs counts are expressed both as percent (%) and absolute (#) count (x109/L).(9)
The imprecision of NRBC automated count method was also assessed according to the Clinical and Laboratory Standards Institute (CLSI) EP5-A2 guideline (10), by analyzing three different levels (1, 2 and 3) of control material (XN-CHECK; Streck Laboratories Inc., Omaha, NE, USA) in duplicate for 40 consecutive working days. The imprecision was below 9.5%. The blood smears were also automatically prepared with Autoslider SP-10 (Sysmex Co., Kobe, Japan) and May-Grünwald-Giemsa stained (Carlo Erba Reagents S.p.A. Milano, Italy), and the blood smear review process was performed with DI60 (Sysmex Co., Kobe, Japan). Both Autoslider SP-10 and DI60 were physically connected with the XN-9000 analyzer.
The digital images were then reevaluated by a skilled specialist in laboratory hematology, according to the CLSI standard H20-A2 (11) and ICSH guideline (12). Samples are confirmed positive in microscopic review when NRBCs ≥ 1/200 white blood cells (WBCs) (or ≥0.5%) in accordance with the criteria described in Standard International Consensus Group for Hematology.(13)
In the NTZ group, we carried out an additional analysis on a subsequent blood sample in order to count the CD34+ cells as markers of bone marrow mobilization. The cell enumeration was performed using the BD™ Stem Cell Enumeration Kit (Becton Dickinson., cat 344563 CE IVD). We incubated 100 µl of peripheral blood with 20 µL of CD45 FITC/CD34 PE reagent and 20 µL of 7-AAD at room temperature. The BD FACSCanto II Flow Cytometer acquired the data, which were analyzed by the BD FACSCanto Software, meeting the ISHAGE guidelines for cell count in single platform. (14)
Statistical analysis
The statistical analysis was executed with Analyse-it (Analyse-it Software Ltd, Leeds, UK). The values distribution was assessed with Shapiro-Wilk test. In the event of a non-normal distribution, the results were reported as median values for each class of subjects. The statistical difference was then evaluated with the nonparametric Kruskal-Wallis and Steel-Dwass-Critchlow-Fligner (pair comparison) tests. P-Values lower than 0.05 were considered statistically significant.
Standard protocol approvals, registration, and patient consent
The study was conducted in accordance with Helsinki Declaration and under the terms of all relevant local legislations. The investigation was based on pre-existing samples, approval from local ethical standards committee was obtained.

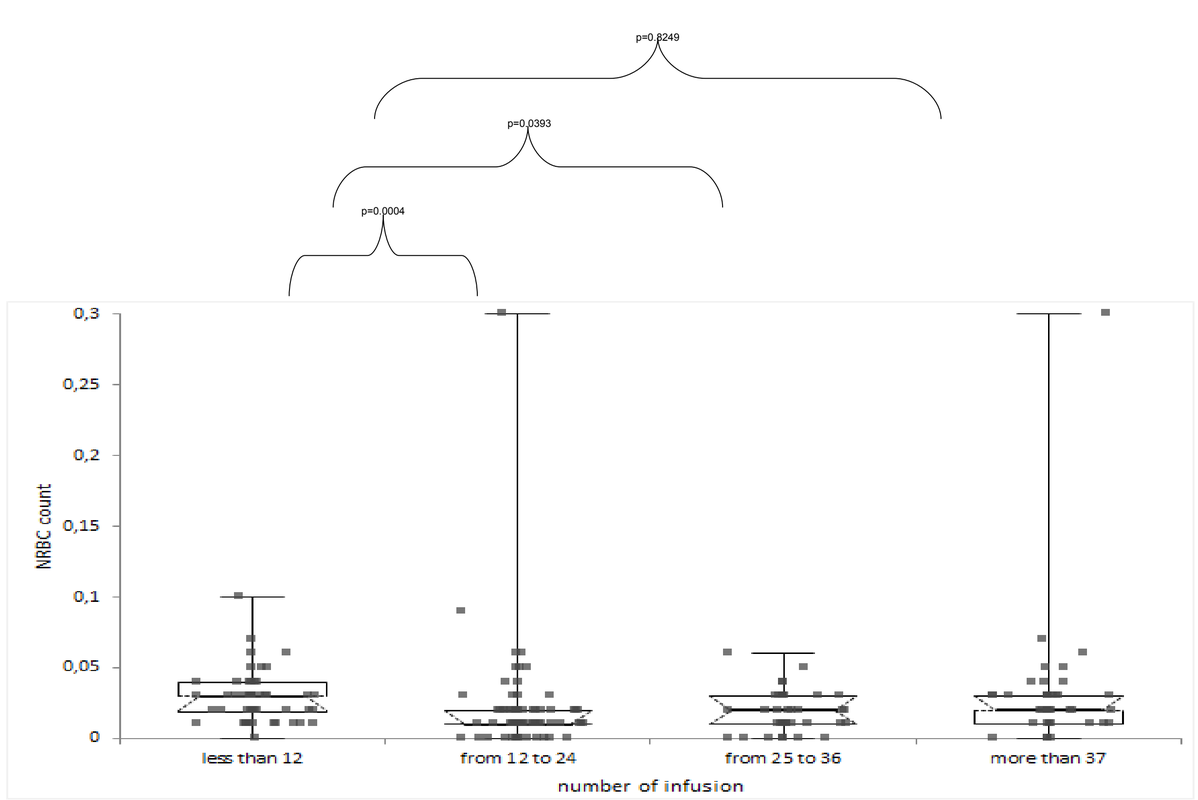
RESULTS
The rate of samples with NRBCs presence (threshold of positivity: 0.001 x109/L and confirmed by microscopic review) was significantly higher in NTZ (89.6%) compared with all the other treatment groups (0.0% for FTY, 6.6% for IFN, 9.0% for GA, 2.1% for NA) and HS (0.0%) (p<0.0001) (Table 1).
The median value of NRBCs, WBCs, RBCs, NRBCs, hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and red cell distribution width (RDW) of all subjects are shown in Table 1.
Though within a range of normality, the median values for all parameters, with the exception of MCH, were found to be significantly different between NTZ and HS (p<0.05).
The median value of NRBCs count resulted significantly higher in NTZ (median 0.020 x10^9/L, p value<0.0001) relative to all other treatment groups (Table 1).
The median values of WBCs in NTZ were significantly higher than all other groups (p<0.0001) (Table 1).
The median values of HGB did not show anemia in all groups. Moreover, HGB levels were comparable in NTZ, IFN, FTY and NA, while in GA they were significantly higher than NTZ and IFN (p value<0.05, see Table 1). HGB evaluated according to sex resulted to have the same pattern described in Table 1 (data not shown). RBCs count resulted lower in NTZ compared to FTY, GA and NA (p<0.05, see Table 1). RDW parameters (RDW-SD and RDW-CV) were higher in NTZ patients than all the other groups (p <0.001).
Nevertheless, no morphologic alterations were evident at the subsequent microscopic revision of RBCs, WBCs and platelets.
Besides, we observed a significantly increased number of absolute NRBCs within the first 12 natalizumab infusions, with a subsequent decrement (p value<0.05) and stabilization of the number of cells (Graph 1).
No relationship between the time lapse from the last natalizumab infusion to the sample draw and the NRBCs absolute count was evident (data not shown).
No significant differences were detected subgrouping NTZ patients by age or sex (data not shown).
The analysis of CD34+cells in the peripheral blood of our natalizumab-treated patients detected a median value of 8 cells/µL (range 3-21) in blood samples drawn 28 days after the last infusion, no statistically significant association was evident between CD34+ cells values and the number of natalizumab infusions.

Table 1. Demographic and lab results of the analyzed patients.
Notes: significant p-values thresholds are reported for comparisons of NTZ with each single other group; †: p<0.001, ‡: p< 0.05.

	Natalizumab	Fingolimod	Interferon	Glatiramer acetate	Treatment naïve	Healthy subjects
N. subjects	26	16	76	44	47	240
N. samples	218	63	174	58	47	240
Age years, mean (SD)	34 (9)	40 (10)	41‡ (11)	41‡ (9)	41‡ (10)	41† (12)
NRBCs presence (% of patients)	89.6	0.0†	6.6†	9.0†	2.1†	0.0†
NRBCs x109/L median (95% CI)	0.020 (0.020-0.020)	0.000† (0.000-0.000)	0.000 † (0.000-0.000)	0.000 † (0.000-0.000)	0.000 † (0.000-0.000)	0.000† (0.000-0.000)
WBCs x109/L median (95% CI)	9.23 (8.79-9.78)	3.81† (3.73-4.31)	5.85† (5.55-6.19)	6.23† (5.85-6.68)	6.28† (6.11-7.16)	6.03† (5.89-6.23)
HGB g/L median (95% CI)	131 (130-133)	140 (134-144)	136 (134-137)	142† (135-150)	140 (135-147)	146† (144-148)
RBCs x1012/L median (95% CI)	4.5 (4.5-4.6)	4.8‡ (4.6-5.0)	4.7 (4.6-4.7)	4.9† (4.7-5.1)	4.9‡ (4.7-5.1)	4.9 † (4.9-5.0)
MCV fL median (95% CI)	87.5 (87.0-88.0)	87.7 (85.7-89.7)	87.2 (86.2-88.0)	86.5 (85.7-88.5)	87.5 (85.4-88.6)	88.6† (88.3-89.1)
MCH pg median (95% CI)	29.50 (29.20-29.90)	29.50 (28.80-30.00)	29.65 (29.20-29.90)	29.15 (28.70-29.70)	29.30 (28.90-29.80)	29.60 (29.30-29.70)
MCHC g/L median (95% CI)	336 (333-338)	335 (332-339)	337 (335-338)	336 (333-339)	335 (331-338)	333‡ (332-333)
RDW-SD fL median (95% CI)	43.050 (42.800-43.600)	41.100 † (40.000- 41.400)	40.650† (40.000-41.200)	40.650† (39.500-41.100)	40.400† (39.600-42.100)	42.050† (41.400-42.500)
RDW-CV % median (95% CI)	13.60 (13.50-13.70)	12.90 † (12.70-13.10)	12.80† (12.60-13.00)	13.00† (12.50-13.10)	13.00 † (12.60-13.20)	12.90 † (12.80-13.00)

DISCUSSION

Our data show that the prevalence of positivity and the levels of erythroblastaemia are significantly higher in patients treated with natalizumab in respect to fingolimod, interferon, glatiramer acetate, treatment naïve and healthy subjects.
These observations are consistent with the report by Robier et al (8) on a smaller sample of patients and differs from previous papers (6,7), where erythroblastemia was considered occasional. Another more recent 18-month longitudinal study (15) on natalizumab-treated patients reported a lower presence of erythroblasts in peripheral blood (only 16%); though apparently quite different from our results, the two studies are scarcely comparable due to a different study design, timing of data collection and instrumental acquisition. We used a newer automated NRCBs enumeration analyzer, which has a higher performance and sensitivity than previous instruments. (16,17,18)
The use of the same fully automated system and a microscopic verification for all our samples according to the CLSI standard H20-A2 (11), ICSH (12) and ISLH (13) strengthens our results. We did not detect any morphologic alteration of RBCs such as teardrop-shaped cells, schistocytes or inclusion bodies (p.e. Jolly bodies, basophilic stippling or Cabot rings), nor signs of dysplasia in white blood cells and platelets according to ICSH grading.(19)
The absence of hemoglobin pathologic values or red blood cells morphologic alteration rejects the hypothesis of natalizumab-induced erythroblastaemia due to hypoxic stress.
The comparison with different RRMS treatment groups and a healthy control population of the same geographic area further reinforces our results.
Considering the frequency of erytrhoblastaemia, it might be justifiable to refrain from further diagnostic procedures if it were an isolated observation without any other laboratory results suggesting underlying disorders. Nevertheless, it is not yet clear whether it might have long term implications on these patients. The values of CD 34+ cells detected in NTZ patients are consistent with previous reports (3,20) and suggests a natalizumab-triggered mobilization of hematopoietic stem cells from the bone marrow. The cross-sectional higher levels of NRBCs counts detected within the first 12 infusions need further confirmation from longitudinal observations. The overall absence of differences of NRBCs counts in natalizumab-treated patients according to treatment duration (no significant difference between the first 12 infusions and more than 36 infusions) and to the time lapse from the previous infusion enhances the hypothesis that hematopoietic stem cells concentrations do not differ significantly between the first doses and the chronic treatment.(3,21)
In conclusion, we confirm erythroblastaemia as a frequent finding in natalizumab treated RRMS patients. A more extended knowledge and an adequate long-term observation of this phenomenon are essential to better understand any pathological long term implication. Larger populations of natalizumab-treated patients along with pre-treatment information on erythrobastaemia and concomitant CD34+ cell count might be important additional elements to acquire for a better interpretation of the on-treatment findings.



Graph 1. NRBCs absolute count (x109/L)in natalizumab-treated patients by number of infusions.

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