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Clinical Infectious Diseases

EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis --Manuscript Draft--

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Short Title:	EBV complications in Auto-HSCT for MS
Article Type:	Major Article
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Manuscript Region of Origin:	UNITED KINGDOM
Abstract:	<p>Introduction Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing across Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus reactivation (EBV-R) following AHSCT with ATG for severe autoimmune conditions is an under-recognised complication relative to T-cell deplete transplants performed for haematological diseases. This retrospective study reports EBV-R associated significant clinical sequelae in MS patients undergoing AHSCT with rabbit ATG.</p> <p>Methods Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising EBV viral load, M-protein and associated clinical sequelae were captured from clinical records.</p> <p>Results All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term follow-up, with a number of them developing high EBV viral load & associated lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some with significant neurological consequences with high M-protein and EBV-R. Six patients required anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms. Receiver operating characteristics (ROC) estimated a peak EBV viraemia of >500,000 DNA copies/ml correlated with high sensitivity (85.5%) & specificity (82.5%) (AUC-0.87; p-0.004) in predicting EBV-R related significant clinical events.</p> <p>Conclusion Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG be mandated in MS patients in the first 3 months post AHSCT.</p>
Response to Reviewers:	<p>To Dr Barbara D Alexander M.D. Associate Editor Clinical Infectious Diseases</p> <p>Dated: 30th Dec 2018</p> <p>Dear Dr Alexander</p> <p>Subject: Response to Reviewers</p> <p>Manuscript Title: EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.</p> <p>We would like to thank the journal for provisionally accepting our work. Considering the reviewer's comments, we have made revisions to the manuscript with responses outlined for each of the queries raised by the reviewer, as below:</p> <p>The Authors simply must have the manuscript edited for English grammar as many of the mistakes change the meaning of the sentence. Some (but not all) of the issues are as follows: Response: Please accept our apologies for the grammatical errors in the manuscript. We have reviewed and edited these errors where appropriate including the ones highlighted below.</p> <p>Line 138 and line 357. deleted " be mandated". Cohort is too small to warrant "mandate"..but your data can lead to recommendation.. Response: We have edited and replaced the word 'mandate' from the phrase.</p> <p>Line 212-215. Please include the conversion factor for your assay to IU/ml in the methods section i.e 10 EBV DNA copies/ml=10 IU/ml</p>

Response:
This has been rephrased within methods section; line 202-203

line 282: HAS versus IS?...I think "is"
Response:
Correction made to "is"

Line 298 and 300- not sure systemic sclerosis needs to be capitalized. But if so, needs to be so throughout manuscript
Response:
We have edited and removed un-necessary capitalisation for similar errors across the manuscript.

Lines 309-312. This sentence is not understandable based on current punctuation. Please address. ????This is further corroborated by the fact that similar LPD risk has not been observed in other ADs managed with ATG in our center. For example, among patients with Crohn' disease treated with ATG-AHSCT and those with severe aplastic anemia treated with ATG/cyclosporin, only 52% (x/x) developed EBV-R (unpublished data) and none had LPD, suggesting that the problem may not be ATG specific.
Response:
Thank you for the suggestion. We have rephrased this to reflect our experience with other Autoimmune diseases (lines 310-315).

Line 319 delete the words "may still have"
Response:
correction made.

Line 330 seems to "be"?
Response:
correction made.

Line 340 "copies"/ml
Response:
correction made.

Thank you again for your review of the revised manuscript. We hope these revisions are satisfactory and will allow formal acceptance for publication.

Yours sincerely

On behalf of all co-authors:

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To
Professor Robert Schooley, M.D.
The Editor-in-Chief
Clinical Infectious Diseases

Dated: 20th Dec 2018

Dear Professor Schooley (Editor-in-Chief) and Dr Alexander (Associate Editor)

We are pleased to submit our revised article entitled; **"EBV & Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis"** for consideration of publication in your internationally reputed journal, Clinical Infectious Diseases.

Just to summarise again: Autologous Stem Cell Transplants (AHSCT) with anti-thymocyte globulin ATG) based conditioning is a novel approach to treatment of active multiple sclerosis (MS) and recent data from MIST study collaborators (Burt et al; Clinical Trial Registry: NCT00273364) have shown some exciting preliminary results showing superiority of AHSCT over established disease modifying therapies, confirming results from other UK and international studies in this field. However, as the evidence builds, safety aspects of these procedures needs to be seriously considered.

This study reports rates of Epstein Barr virus (EBV) reactivation and associated clinical sequelae with monoclonal gammopathy (M-protein), in cohort of Multiple Sclerosis patients who underwent ATG conditioned immunosuppressive AHSCT in a single centre. We report a significantly higher proportion of MS patients had detectable EBV DNA post-AHSCT; were more likely to develop clinically significant EBV viraemia of >500,000 DNA copies/ml and develop de-novo M-protein of clinical significance with clinical events ranging from probable lymphoproliferative disorders and disabling neurological complications, unrelated to MS. This report of significant clinical complications related to EBV and M-protein, possibly reflect underlying altered immunopathological state of MS disease and its interactions with reactivation of EBV virus, which if monitored and treated pre-emptively may reduce associated morbidity and improve outcomes.

To help readers, we have also described two interesting clinical vignettes as a supplementary to this report, highlighting significant risk of neurological events following development of M-protein, triggered following EBV reactivations in MS patients.

We can confirm that this manuscript has not been published and is not under consideration for publication elsewhere. All authors have seen, approved and contributed to this work. We have no conflicts of interest to disclose. We believe that this report fits well within the scope of your journal, highlighting important clinical message about EBV complications in ATG conditioned AHSCT for MS and will appeal to journal's readers interested in infectious complications related to immunosuppressive therapies including AHSCTs for autoimmune conditions, with a potential to change clinical practice in this area. We have provided point to point responses to the reviewer's comments.

Thank you for your consideration of this revised manuscript and looking forward to your acceptance.

Yours Sincerely

On behalf of all co-authors:

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EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.

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62 Running Title: EBV complications in Auto-HSCT for MS
63

64 Summary: EBV reactivation is common post-transplant with ATG for multiple sclerosis
65 (MS), with significant lymphoproliferative & neurological sequelae associated with rising M-
66 protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as is pre-
67-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.

81 **Abstract**

82 **Introduction**

83 Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin
84 (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing
85 across Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus
86 reactivation (EBV-R) following AHSCT with ATG for severe autoimmune conditions is an
87 under-recognised complication relative to T-cell deplete transplants performed for
88 haematological diseases. This retrospective study reports EBV-R associated significant
89 clinical sequelae in MS patients undergoing AHSCT with rabbit ATG.

90 **Methods**

91 Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College
92 Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and
93 serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising
94 EBV viral load, M-protein and associated clinical sequelae were captured from clinical
95 records.

96 **Results**

97 All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term
98 follow-up, with a number of them developing high EBV viral load & associated
99 lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some
100 with significant neurological consequences with high M-protein and EBV-R. Six patients
101 required anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms.
102 Receiver operating characteristics (ROC) estimated a peak EBV viraemia of >500,000 DNA

103 copies/ml correlated with high sensitivity (85.5%) & specificity (82.5%) (AUC-0.87; p-0.004)
104 in predicting EBV-R related significant clinical events.

105 **Conclusion**

106 Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-
107 AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG in
108 MS patients in the first 3 months post AHSCT

109 **Key Words:**

110 **Multiple Sclerosis; Autologous Hematopoietic Stem Cell Transplantation, Epstein-**
111 **Barr Virus Infection; Monoclonal Gammopathy; Post-transplant Lymphoproliferative**
112 **Disorder**

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120 INTRODUCTION:

121 Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, demyelinating disease of
122 the central nervous system[1][2], with a relapsing-remitting (RRMS) presentation in the
123 majority of patients at diagnosis. Recovery from relapses may be complete or partial[3][4].
124 After a variable period of time, people with RRMS may develop a more progressive
125 disability accumulation with or without superimposed relapses; termed secondary
126 progressive multiple sclerosis (SPMS). A minority experience progressive disability from the
127 onset of disease, termed primary progressive multiple sclerosis (PPMS)[4]. A number of
128 immunomodulatory disease modifying therapies (DMTs) are currently licensed for treatment
129 of RRMS with an aim of reducing number of relapses and accrual of disability, although with
130 variable efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation
131 (AHSCT) has been a novel approach for MS management, using immunoablation followed
132 by immunomodulation mechanisms, with evidence of significant suppression of
133 inflammatory activity and qualitative changes in the reconstituted immune system (immune
134 reset theory)[6–8]. AHSCT appears most effective for MS patients with evidence of
135 inflammatory activity on MRI, younger age, a shorter disease duration, low to moderate
136 disability levels (Expanded Disability Status Scale [EDSS] <6 or up to 6.5 if recent
137 progression) and failure of at least 1 highly active DMT (natalizumab or alemtuzumab) with
138 no significant comorbidities[9–11]. Recently reported preliminary results of randomised
139 MIST study[12] found AHSCT to be superior to standard disease modifying therapy (DMT)

140 for RRMS with respect to both treatment failure and disability progression.

141

142 However, risk of subsequent rise in opportunistic infections following such
143 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing
144 AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of
145 immunosuppressive rabbit anti-thymocyte globulin (rATG) to their conditioning regimen may
146 confer a higher risk of viral reactivation in these patients. The number of AHSCTs
147 performed for MS is rising significantly in Europe[14] and as more centres perform AHSCT
148 for this indication, it is increasingly important to recognise the unique problems faced by
149 these patients post AHSCT. This retrospective study reports for the first time, EBV-R
150 associated neurological sequelae and lymphoproliferative disorder (LPD) in MS patients
151 undergoing rATG conditioned AHSCT in our centre.

152

153 **METHODS**

154 **Patients and procedures**

155 Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT
156 between February 2012 and February 2017 at Kings College Hospital, London. Peripheral
157 blood stem cells were collected following standard mobilisation strategy consisting of
158 cyclophosphamide 4g/m² over 2 days and granulocyte colony-stimulating factor for 7 days.
159 Thirty-five (97%) patients were conditioned using standard protocol of cyclophosphamide

160 (50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion
161 followed by stem cell infusion. One patient was conditioned with
162 carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG
163 (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned was
164 $7.17 \times 10^6/\text{kg}$ (range $4.0\text{-}17.1 \times 10^6/\text{kg}$).

165

166 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen;
167 VCA IgG). EBV DNA load monitoring was performed on whole blood samples by
168 standardised quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-
169 Gene™ (Qiagen) assay of EBV BZLF1 DNA. This assay was adapted from our published
170 assay using LightCycler (Roche)[15] and since been validated against the recently
171 published WHO standard, with our lab's EBV DNA quantification of 10 copies/ml considered
172 equivalent to 10 IU/ml DNA reported with the WHO reference method[16]. EBV-R was
173 defined as rising EBV DNA load of >10 copies/millilitre (ml) detected on two consecutive
174 tests based on our assay sensitivity. Symptomatic EBV-R was captured by peak blood EBV
175 DNA load, presence of B symptoms (defined by presence of either unexplained weight loss,
176 recurrent fever, night sweats); which was in-turn defined by clinical, radiological and/or
177 histological evidence based on recent ECIL-6 guidelines[17]. In addition, significant 'clinical
178 events' were also defined as new & persistent organ dysfunction (e.g. neurological events)
179 temporally associated with rising EBV viraemia in MS patients. Serum protein
180 electrophoresis was routinely tested around 3 months post HSCT as part of our institutional
181 practice, with immunoglobulin subclasses identified by immunofixation electrophoresis.
182 Patient outcomes were assessed at last follow up as of April 2017.

183

Statistics

The database of transplants and outcomes was built in Microsoft Excel 2016 and statistical analyses were performed using IBM SPSS Statistics version 24.0. Patient characteristics are presented as medians (with inter-quartile ranges; IQR) for data with non-normal distribution. Comparisons of baseline characteristics used Mann-Whitney U test, Fisher's exact test, or Chi-squared test for trend as appropriate. Receiver Operating characteristics (ROC) curve was obtained correlating LPD and clonal gammopathy associated clinical events with rising EBV viraemia (copies/ml).

RESULTS

Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment, indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS patients were lost to long-term follow up for EBV monitoring. The median time to first EBV DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days) to lymphocyte recovery (defined by total lymphocyte count $>1.0 \times 10^6/\text{ml}$) following AHSCT (**See Figure 1**). A high proportion (86%; n=25/29) of the MS patients in active follow-up recovered lymphocyte counts around D56 with a median

206 lymphocyte count of $1.56 (10^6 \text{ cells/ml})$; Four patients remained lymphopenic at last follow
207 up.

208

209 All patients were stratified into following 3 groups according to peak rise/burden of EBV
210 DNA-aemia (copies/ml): $<100,000 (<100k) \text{ copies/ml}$, $100,001-500,00 (100k-500k)$
211 copies/ml and $>500,000 (>500k) \text{ copies/ml}$ to identify any specific thresholds for clinically
212 significant events related to rising EBV-R (Table 1). The majority of patients (76%) with
213 rising EBV viral load $>100k \text{ copies/ml}$ were routinely screened by computed tomographic
214 (CT) scans to assess for evidence of LPD, in line with our institutional policy. One third
215 (34.5%) of patients developed peak EBV viraemia of $>500k \text{ copies/ml}$. Eight patients
216 (27.6%) developed symptomatic EBV-R; defined as persistent fever, lymphadenopathy
217 and/or B symptoms. Of these 8 patients, only 1 (12.5%) had a peak EBV viraemia
218 $<100k \text{ DNA copies/ml}$ with the remaining 7 (87.5%) patients having a peak EBV viraemia of
219 $>500k \text{ copies/ml}$. Three patients with rising EBV viraemia $>500k \text{ copies/ml}$ had findings
220 consistent with probable LPD on CT imaging; however, none had definitive histological
221 diagnosis. Three MS patients had worsening neurological symptoms concurrent with rising
222 EBV viraemia $>500k \text{ copies/ml}$ and clonal gammopathy, as described below.

223

224 Interestingly, we also observed frequent de novo monoclonal gammopathy (MG or M-
225 protein) in 18 MS patients (62.4%) following rising EBV viraemia; the majority (n-16) of

226 whom developed IgG subtype and the remaining 2 developed IgA and IgM M-protein.
227 Concerningly two of these patients developed clinically significant M-Protein burden; one
228 patient with IgG Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological
229 symptoms mimicking MS relapse, requiring plasma exchange. Another patient developed
230 significant lumbosacral radiculopathy with rising EBV-R and high IgM lambda M-protein
231 (IgM 48.5g/L) (**see supplementary case vignettes**). **Figure 2** highlights the association of
232 neurological symptom onset following rising EBV viraemia (log copies), falling lymphocyte
233 counts ($\times 10^6/\text{ml}$) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient
234 developed painful lower limb paraesthesia following rising EBV viraemia $>500\text{k}$ copies/ml,
235 although did not have any M-protein detected. Their symptoms persisted at last follow up
236 despite no evidence of MS related new disease activity.

237

238 Six patients were treated with anti-CD20 antibody, rituximab (375mg/m² weekly up to 4
239 weeks), due to clinical severity of EBV reactivations and leading to reduction in EBV viral
240 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis
241 (**Figure 3**) confirmed EBV viraemia of $>500\text{k}$ copies/ml correlated with high sensitivity
242 (85.5%) and specificity (82.5%) (AUC-0.87; 95%CI-0.73-1.0; p-0.004) in predicting
243 significant EBV related clinical events (evidence of LPD and/or neurological symptoms) that
244 may require treatment with rituximab. The sensitivity dropped significantly on lower
245 estimates for events below 500k copies/ml.

246

247 The median time to resolution of EBV viraemia post-rituximab was 21 days (IQR 19-124
248 days) in 5 patients with >500k copies/ml (one patient was treated for late onset persistent
249 symptomatic clonal gammopathy, despite fall in EBV levels). No significant adverse events
250 were noted in the treated group. Nine patients had a persistent low level EBV viraemia
251 detectable at last follow up. All patients who underwent AHSCT were alive as of April 2017.

252

253 **DISCUSSION:**

254 MS as an autoimmune disorder (AD) is theorised to have generally similar underlying
255 pathophysiological immune dysregulation mechanisms[18–22] relative to other chronic
256 autoimmune conditions. Epstein-Barr virus (EBV) is increasingly implicated in the
257 pathogenesis of MS by virtue of epidemiological and serological evidence, impaired CD8+
258 T cell immune responses to EBV and possible underlying genetic susceptibility for
259 autoimmunity (with EBV encoded protein interactions), as recently described by Harley et al
260 and others[1,23–25].

261

262 Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid
263 organ transplants treated with immunosuppressive therapy, often with a significant impact
264 on organ function and overall survival[26–30]. It is observed that reduced intensity allo-
265 HSCT for malignant haematological conditions using alemtuzumab have a relatively lower

266 overall risk of LPD compared to ATG based treatments, possibly mediated by more
267 effective pan-B & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell
268 repertoire with delayed EBV specific CD8+ T cell recovery[31]. Clinically significant
269 endogenous viral infections including EBV following ATG conditioned AHSCT for severe
270 ADs such as Crohn's disease and systemic sclerosis is increasingly recognised, but the
271 development of lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33].
272 Nash et al[32] concerningly reported 2 deaths (1 MS and 1 systemic sclerosis patient) from
273 EBV related LPD in 56 ATG conditioned AHSCT for autoimmune diseases. Additionally,
274 EBV associated haemophagocytosis in ATG-AHSCT for ADs have also been reported[34],
275 with one resulting in death of the patient[35].

276

277 Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher
278 than published reports with allo-HSCTs (4.5-7%)[36,37] and our own centre's unpublished
279 T-cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying
280 immunopathological state of MS itself[38]. This is further corroborated by the fact that
281 similar LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with
282 ATG -AHSCT in this centre. Another example from our centre's experience of severe
283 aplastic anaemia (n=40) treated with ATG/ciclosporin, only 52% (n=21/40) developed EBV-R
284 (unpublished data) and none had LPD or required any treatment, suggesting that the
285 problem may not be ATG specific.

286

287 Our study's observation of significant persistent neurological events (with no evidence of
288 new MS disease activity) associated with clonal gammopathy suggest a potentially new
289 clinical syndrome, described for the first time in ATG conditioned AHSCTs in MS and
290 possibly induced by clonal B cell dysregulation following EBV-R. It could be hypothesised
291 that any remaining EBV infected latent B cells, surviving despite high doses of
292 cyclophosphamide (given with mobilisation and conditioning in MS ASHCT)[8] and
293 compounded by depletion of CD8+ T cells by ATG, may serve as potential source for EBV
294 escape while interacting abnormally within the host immune micro-environment[39] and
295 leading to rise in M-protein, LPD and neuro-inflammatory insults in some of the MS patients
296 post AHSCT. Reports of lower incidence of EBV-R and LPD in another commonly used
297 protocol for MS-AHSCT using BEAM-ATG (Ricardo Saccardi, Carregi University Hospital,
298 Florence; personal communication) may reflect the greater myeloablative effect of BEAM
299 chemotherapy which could further deplete the residual B cell pool and thus lower potential
300 for EBV proliferation. It is plausible that dose of rATG is critical, given we have not seen
301 similar reports from other centres where less rATG doses were given for MS-AHSCT (range
302 between 5.0-6.5 mg/Kg; personal communication) but there seems to be some variability in
303 prospective serial EBV monitoring in these patients.

304

305 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is
306 widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is
307 significantly associated with probable LPD and neurological events in MS patients with high
308 sensitivity (85.5%) and specificity (82.5%) (p=0.004) (**Fig 3, ROC curve**). Our ROC curve
309 estimates are potentially limited by the relatively small number of events analysed but this
310 has consistently been useful in our MS-AHSCT experience for predicting clinical events
311 with high EBV load. Our EBV PCR assay has been validated against the recently defined
312 standard WHO reference method (i.e. 10 copies/ml=10 IU/ml EBV DNA) [16] and thus this
313 EBV threshold for pre-emptive treatment with Rituximab, can potentially be applied in
314 relevant clinical context in other centres using similar validated essays. Rituximab treatment
315 delivered good overall response in our symptomatic patients, with resolution of EBV related
316 clinical symptoms and no subsequent viral or bacterial infections at last follow up. The role
317 of prophylactic or pre-transplant rituximab in MS-AHSCT is also a potential area of interest
318 in reducing risk of EBV-LPD in stem cell transplants as observed by Burns et al[36] and
319 future randomised studies are required to investigate its potential benefit.

320

321 Our study limitations include its retrospective nature and that no suspected LPD patients
322 had histological confirmation, mainly related to patient refusal or technical difficulties. Seven
323 MS patients were lost to follow up for EBV monitoring following discharge, which limits the

324 findings of this study. Additionally, our numbers were too small to identify any association of
325 EBV related clinical events with previous DMT exposure in MS patients.

326

327 In conclusion, symptomatic EBV reactivation increases risk of neurological sequelae and
328 LPD in MS-AHSCT. Regular monitoring for rising EBV viraemia, as recommended by
329 Snowden et al [10] and Muraro et al [11], and serum electrophoresis for M-protein should
330 be considered in the first 3 months post-AHSCT for MS. We recommend persistent high
331 EBV viraemia > 500k DNA copies/ml as potential trigger for consideration of pre-emptive
332 anti-CD20 therapy and potentially reduce associated morbidity.

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369 REFERENCES

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- 371 1. Pender MP, Burrows SR. Epstein–Barr virus and multiple sclerosis: potential opportunities for
372 immunotherapy. *Clinical & Translational Immunology* **2014**; 3:e27. Available at:
373 <http://doi.wiley.com/10.1038/cti.2014.25>.
- 374 2. Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. *New England Journal of Medicine* **2018**;
375 378:169–180. Available at: <http://www.nejm.org/doi/10.1056/NEJMra1401483>.
- 376 3. Compston A, Coles A. Multiple sclerosis. *The Lancet* **2008**; 372:1502–1517. Available at:
377 <http://linkinghub.elsevier.com/retrieve/pii/S0140673608616207>.
- 378 4. Lublin FD, Reingold SC, Cohen JA, et al. Defining the clinical course of multiple sclerosis: The 2013
379 revisions. *Neurology* **2014**; 83:278–286. Available at:
380 <http://www.neurology.org/cgi/doi/10.1212/WNL.0000000000000560>.
- 381 5. Comi G, Radaelli M, Soelberg Sørensen P. Evolving concepts in the treatment of relapsing multiple
382 sclerosis. *The Lancet* **2017**; 389:1347–1356. Available at:
383 <https://linkinghub.elsevier.com/retrieve/pii/S0140673616323881>.
- 384 6. Muraro PA, Douek DC, Packer A, et al. Thymic output generates a new and diverse TCR repertoire
385 after autologous stem cell transplantation in multiple sclerosis patients. *The Journal of Experimental*
386 *Medicine* **2005**; 201:805–816. Available at: <http://www.jem.org/lookup/doi/10.1084/jem.20041679>.
- 387 7. Muraro PA, Robins H, Malhotra S, et al. T cell repertoire following autologous stem cell transplantation
388 for multiple sclerosis. *J Clin Invest* **2014**; 124:1168–1172. Available at:
389 <http://www.ncbi.nlm.nih.gov/pubmed/24531550>.
- 390 8. Cull G, Hall D, Fabis-Pedrini M, et al. Lymphocyte reconstitution following autologous stem cell
391 transplantation for progressive MS. *Multiple Sclerosis Journal – Experimental, Translational and*
392 *Clinical* **2017**; 3:205521731770016. Available at: <https://doi.org/10.1177/2055217317700167>.
- 393 9. Sormani MP, Muraro PA, Schiavetti I, et al. Autologous hematopoietic stem cell transplantation in
394 multiple sclerosis. *Neurology* **2017**; 88:2115–2122. Available at:
395 <http://www.neurology.org/lookup/doi/10.1212/WNL.0000000000003987>.
- 396 10. Snowden JA, Saccardi R, Allez M, et al. Haematopoietic SCT in severe autoimmune diseases:
397 updated guidelines of the European Group for Blood and Marrow Transplantation. *Bone Marrow*
398 *Transplantation* **2012**; 47:770–790. Available at: <http://www.nature.com/articles/bmt2011185>.
- 399 11. Muraro PA, Pasquini M, Atkins HL, et al. Long-term Outcomes After Autologous Hematopoietic Stem
400 Cell Transplantation for Multiple Sclerosis. *JAMA Neurology* **2017**; 74:459. Available at:
401 <http://archneur.jamanetwork.com/article.aspx?doi=10.1001/jamaneurol.2016.5867>.
- 402 12. Burt RK, Balabanov R, Snowden JA, Sharrack B, Oliveira MC, Burman J. Non-myeloablative
403 hematopoietic stem cell transplantation (HSCT) is superior to disease modifying drug (DMD) treatment
404 in highly active Relapsing Remitting Multiple Sclerosis (RRMS): interim results of the Multiple Sclerosis

International Stem cell Transp. Neurology **2018**; 90. Available at:
http://n.neurology.org/content/90/15_Supplement/S36.004.abstract.

13. Daikeler T, Tichelli A, Passweg J. Complications of autologous hematopoietic stem cell transplantation for patients with autoimmune diseases. *Pediatric Research* **2012**; 71:439–444. Available at:
<http://www.nature.com/doi/10.1038/pr.2011.57>.

14. Snowden JA, Badoglio M, Labopin M, et al. Evolution, trends, outcomes, and economics of hematopoietic stem cell transplantation in severe autoimmune diseases. *Blood Advances* **2017**; 1:2742–2755. Available at:
<http://www.bloodadvances.org/lookup/doi/10.1182/bloodadvances.2017010041>.

15. Patel S, Zuckerman M, Smith M. Real-time quantitative PCR of Epstein–Barr virus BZLF1 DNA using the LightCycler. *Journal of Virological Methods* **2003**; 109:227–233. Available at:
<http://linkinghub.elsevier.com/retrieve/pii/S0166093403000764>.

16. Semenova T, Lupo J, Alain S, et al. Multicenter Evaluation of Whole-Blood Epstein-Barr Viral Load Standardization Using the WHO International Standard. *Journal of Clinical Microbiology* **2016**; 54:1746 LP-1750. Available at: <http://jcm.asm.org/content/54/7/1746.abstract>.

17. Styczynski J, van der Velden W, Fox CP, et al. Management of Epstein-Barr Virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: Sixth European Conference on Infections in Leukemia (ECIL-6) guidelines. *Haematologica* **2016**; 101:803–811. Available at:
<http://www.haematologica.org/cgi/doi/10.3324/haematol.2016.144428>.

18. Dejaco C, Duftner C, Grubeck-Loebenstien B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* **2006**; 117:289–300. Available at:
<http://doi.wiley.com/10.1111/j.1365-2567.2005.02317.x>.

19. Arellano G, Acuña E, Reyes LI, et al. Th1 and Th17 Cells and Associated Cytokines Discriminate among Clinically Isolated Syndrome and Multiple Sclerosis Phenotypes. *Frontiers in immunology* **2017**; 8:753. Available at: <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00753/full>.

20. Jha S, Srivastava SY, Brickey WJ, et al. The Inflammasome Sensor, NLRP3, Regulates CNS Inflammation and Demyelination via Caspase-1 and Interleukin-18. *Journal of Neuroscience* **2010**; 30:15811–15820. Available at: <http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.4088-10.2010>.

21. Beynon V, Quintana FJ, Weiner HL. Activated Human CD4+CD45RO+ Memory T-Cells Indirectly Inhibit NLRP3 Inflammasome Activation through Downregulation of P2X7R Signalling. *PLoS ONE* **2012**; 7:e39576. Available at: <https://dx.plos.org/10.1371/journal.pone.0039576>.

22. Fernández-Menéndez S, Fernández-Morán M, Fernández-Vega I, Pérez-Álvarez A, Villafani-Echazú J. Epstein–Barr virus and multiple sclerosis. From evidence to therapeutic strategies. *Journal of Neurological Sciences* **2016**; 361:213–219. Available at:
https://www.clinicalkey.com.ezsecureaccess.balamand.edu.lb/service/content/pdf/watermarked/1-s2.0-S0022510X16300132.pdf?locale=en_US.

23. Pender MP. The Essential Role of Epstein-Barr Virus in the Pathogenesis of Multiple Sclerosis. *The Neuroscientist* **2011**; 17:351–367. Available at:
<http://journals.sagepub.com/doi/10.1177/1073858410381531>.
24. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Annals of Neurology* **2007**; 61:288–299. Available at: <http://doi.wiley.com/10.1002/ana.21117>.
25. Harley JB, Chen X, Pujato M, et al. Transcription factors operate across disease loci, with EBNA2 implicated in autoimmunity. *Nature Genetics* **2018**; 50:699–707. Available at:
<http://www.nature.com/articles/s41588-018-0102-3>.
26. Loren AW, Porter DL, Stadtmauer EA, Tsai DE. Post-transplant lymphoproliferative disorder: a review. *Bone Marrow Transplantation* **2003**; 31:145–155. Available at:
<http://www.nature.com/articles/1703806>.
27. Dotti G, Fiocchi R, Motta T, et al. Lymphomas occurring late after solid-organ transplantation: influence of treatment on the clinical outcome. *Transplantation* **2002**; 74:1095–102. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/12438953>.
28. Nagle SJ, Reshef R, Tsai DE. Posttransplant Lymphoproliferative Disorder in Solid Organ and Hematopoietic Stem Cell Transplantation. *Clinics in Chest Medicine* **2017**; 38:771–783. Available at:
<https://linkinghub.elsevier.com/retrieve/pii/S0272523117300874>.
29. Meijer E, Dekker AW, Weersink AJL, Rozenberg-Arska M, Verdonck LF. Prevention and treatment of epstein–barr virus-associated lymphoproliferative disorders in recipients of bone marrow and solid organ transplants. *British Journal of Haematology* **2002**; 119:596–607. Available at:
<http://dx.doi.org/10.1046/j.1365-2141.2002.03887.x>.
30. Dierickx D, Habermann TM. Post-Transplantation Lymphoproliferative Disorders in Adults. *New England Journal of Medicine* **2018**; 378:549–562. Available at:
<http://dx.doi.org/10.1056/NEJMr1702693>.
31. Meij P. Impaired recovery of Epstein-Barr virus (EBV)--specific CD8+ T lymphocytes after partially T-depleted allogeneic stem cell transplantation may identify patients at very high risk for progressive EBV reactivation and lymphoproliferative disease. *Blood* **2003**; 101:4290–4297. Available at:
<http://www.bloodjournal.org/cgi/doi/10.1182/blood-2002-10-3001>.
32. Nash R a, Dansey R, Storek J, et al. Epstein-Barr virus-associated posttransplantation lymphoproliferative disorder after high-dose immunosuppressive therapy and autologous CD34-selected hematopoietic stem cell transplantation for severe autoimmune diseases. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **2003**; 9:583–91. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14506660>.
33. Daikeler T, Tzankov A, Hoenger G, et al. Minimal T-cell requirements for triggering haemophagocytosis associated with Epstein–Barr virus-driven B-cell proliferation: a clinical case study. *Annals of the Rheumatic Diseases* **2011**; 70:1338 LP-1339. Available at:
<http://ard.bmj.com/content/70/7/1338.abstract>.

- 479 34. Daikeler T, Tzankov A, Hoenger G, et al. Minimal T-cell requirements for triggering
480 haemophagocytosis associated with Epstein-Barr virus-driven B-cell proliferation: a clinical case study.
481 *Annals of the Rheumatic Diseases* **2011**; 70:1338–1339. Available at:
482 <http://ard.bmj.com/cgi/doi/10.1136/ard.2010.139246>.
- 483 35. Brinkman DMC, de Kleer IM, ten Cate R, et al. Autologous stem cell transplantation in children with
484 severe progressive systemic or polyarticular juvenile idiopathic arthritis: Long-term followup of a
485 prospective clinical trial. *Arthritis & Rheumatism* **2007**; 56:2410–2421. Available at:
486 <http://doi.wiley.com/10.1002/art.22656>.
- 487 36. Burns DM, Rana S, Martin E, et al. Greatly reduced risk of EBV reactivation in rituximab-experienced
488 recipients of alemtuzumab-conditioned allogeneic HSCT. *Bone Marrow Transplantation* **2016**; 51:825–
489 832. Available at: <http://www.nature.com/articles/bmt201619>.
- 490 37. van Esser JWJ. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell
491 transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-
492 depleted SCT. *Blood* **2001**; 98:972–978. Available at:
493 <http://www.bloodjournal.org/cgi/doi/10.1182/blood.V98.4.972>.
- 494 38. Tørring C, Andreassen C, Gehr N, Bjerg L, Petersen T, Höllsberg P. Higher incidence of Epstein-Barr
495 virus-induced lymphocyte transformation in multiple sclerosis. *Acta Neurologica Scandinavica* **2014**;
496 130:90–96. Available at: <http://doi.wiley.com/10.1111/ane.12249>.
- 497 39. Martinez OM, Krams SM. The Immune Response to Epstein Barr Virus and Implications for
498 Posttransplant Lymphoproliferative Disorder. *Transplantation* **2017**; 101:2009–2016. Available at:
499 <http://insights.ovid.com/crossref?an=00007890-201709000-00018>.

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520 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**
521 **EBV DNA-aemia burden.**

Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender		M-Protein (n)	11	0	7
Male	19 (52.8%)				
Female	17 (47.2%)				
Disease Type (n; %)		Median EBV DNA log value at peak (IQR)			
Relapsing Remitting MS	22 (61.1%)		4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Secondary Progressive MS	10 (27.8%)				
Primary Progressive MS	4 (11.1%)				
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n)		Symptomatic EBV (n)			
Natalizumab	22		1	0	7
Alemtuzumab	8				
Both	6				
Median EDSS (range)	6.0 (2.5 – 8.0)	LPD diagnosis (CT/Biopsy) (n)	0	0	3 by CT alone
Median follow up post AHSCT in days (range)	436 (188 – 785)	Neuro/autoimmune complications (n)	0	0	3
No. of patients with prior EBV exposure (n; %)	36 (100%)	Treated with Rituximab (n)	0	0	6
No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
No. of patients lost to long term follow up (n; %)	7 (19.5%)	Detectable EBV DNA at last follow up (n)	9	1	3
Median Time to EBV detection post AHSCT in days (IQR)	30 (23-46)	Median time for EBV resolution (IQR in days)	67 days (44-155)	47 days (N/A)	63 days (45 - 170)
Median Time to peak EBV DNA levels in days (IQR)	32 (31-53)	Median Time to peak EBV DNA levels in days (IQR)	40 days (25-85)	30 days (N/A)	39 days (32-43)

523

524 **Abbreviations:**

525 AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography;
526 DMT- Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded
527 Disability Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-
528 Protein: Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis

Figure Legends

Figure 1: Trend of Lymphocyte count recovery following ATG in MS patients.

Legend: This figure shows trends of lymphocyte count from baseline to recovery post AHSCT for MS patients. Majority of patients had normal baseline lymphocyte counts pre-HSCT and became lymphopenic post ATG with counts recovering towards d+28 and >85% of patients recovered counts by d+56, with some overshooting from their baseline, possibly reflective of EBV related lymphoproliferation in some of these patients.

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

Figure 2: Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT.

Legend: This figure demonstrates trends of EBV copies (log), paraprotein levels (g/l) and Lymphocyte levels (counts $\times 10^6/\text{ml}$) in two MS patients with significant neurological symptoms following EBV reactivation. Both patients had significant EBV viraemia ($\log > 5.2$ or $> 500,000$ copy number) and developed significant paraproteinaemia, which was only noted after persistent unexplained neurological symptoms. The trend reversed following anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.

Legend: ROC curve demonstrating significant correlation between high EBV levels and clinical events (LPD & neurological events) in MS-AHSCT patients, with highest sensitivity and specificity noted with peak EBV viraemia of $> 500,000$ copies/ml ($p=0.0004$).

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

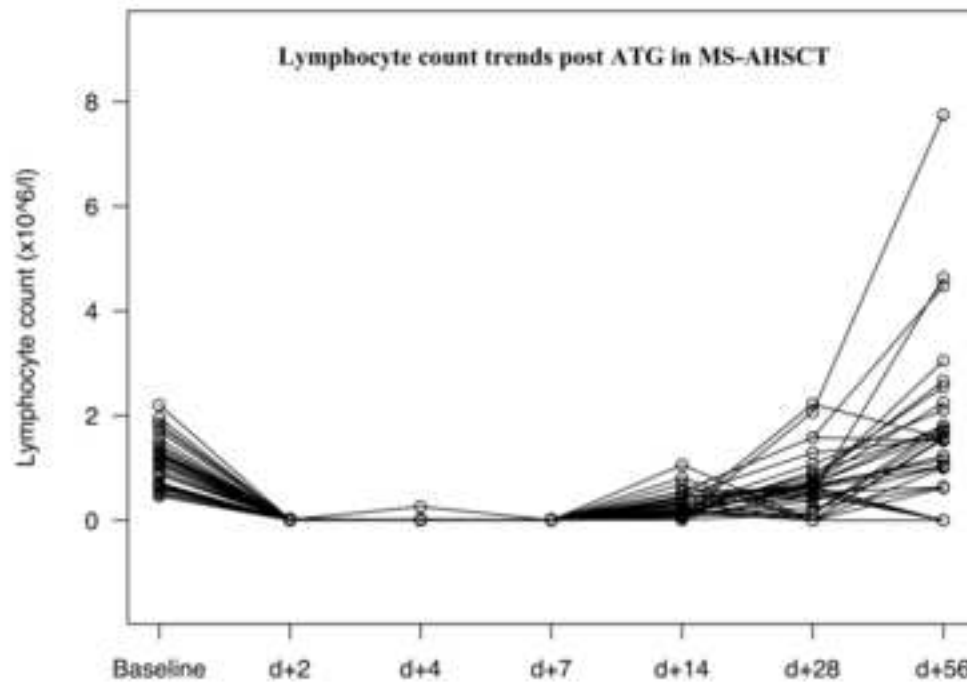
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Abbreviations:

AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography; DMT- Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded Disability Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-Protein: Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis

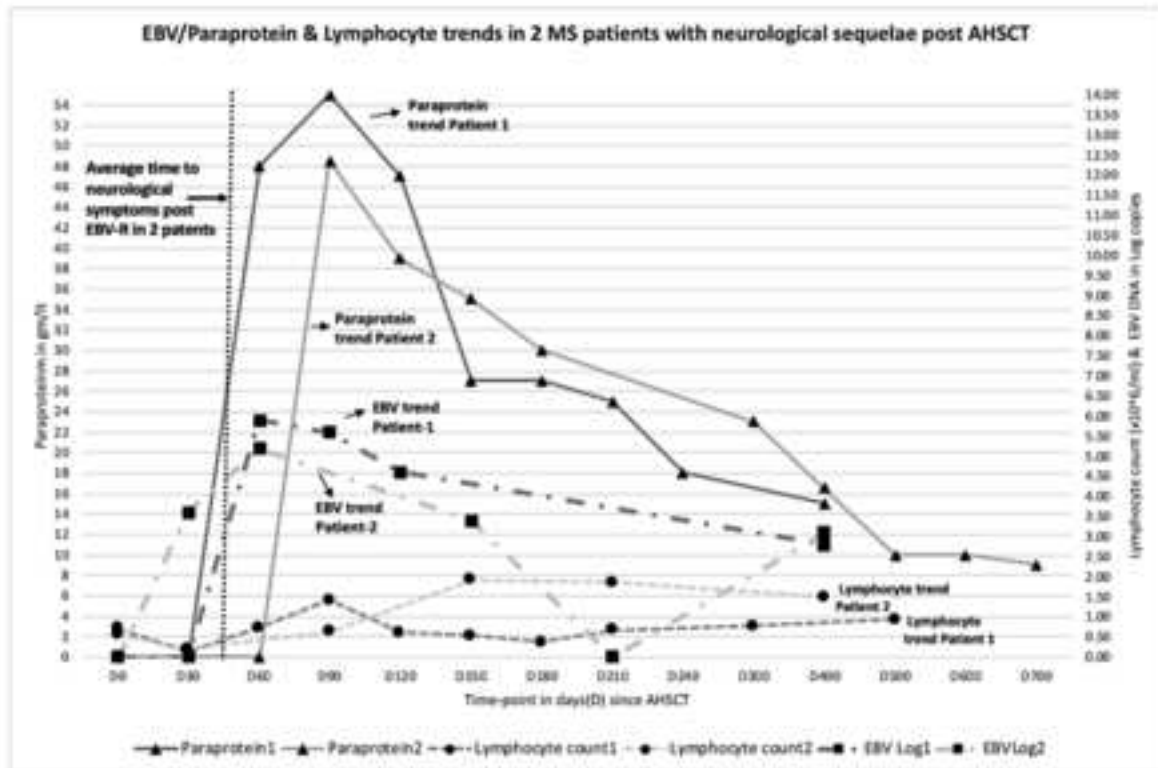
Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.



Abbreviations:

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

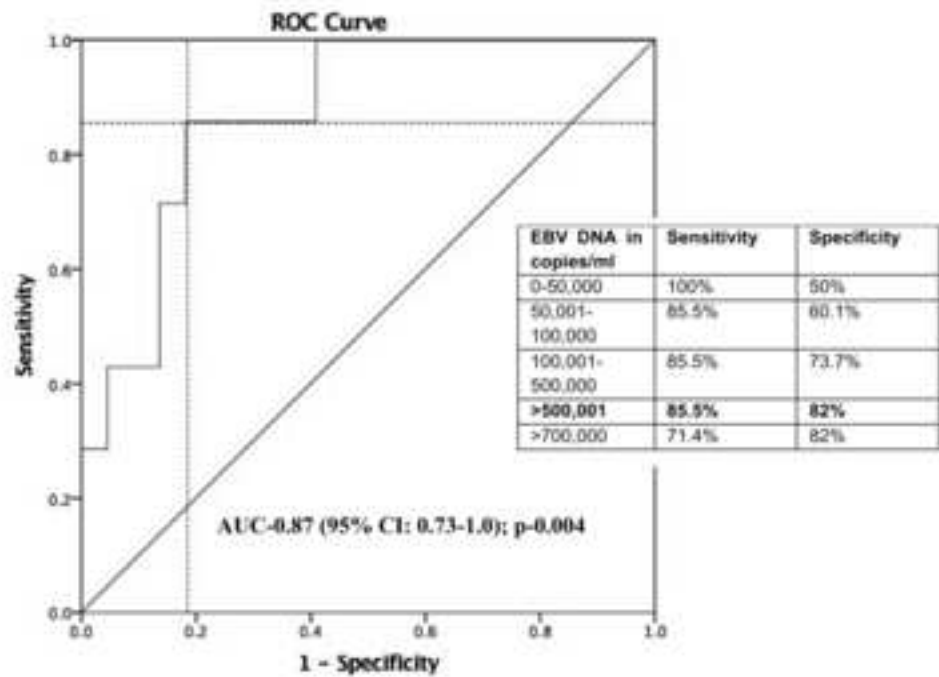
Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT



Abbreviations:

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.



Abbreviations:

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics.

EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.

Running Title: EBV complications in Auto-HSCT for MS

SUPPLEMENTARY:

Case vignettes of 2 MS patients describing EBV-related significant paraproteinaemia and neurological sequelae.

Patient 1

43-year-old female with Relapsing Remitting Multiple Sclerosis (RRMS), previously treated with natalizumab and three courses of alemtuzumab but continued to have breakthrough disease. She had a relatively mild baseline disability with an Expanded Disability Severity Scale (EDSS) of 2.5. She had an uncomplicated inpatient stay for the Autologous Haematopoietic Stem cell transplant (AHSCT) procedure and was discharged on day 15 post-transplant. A blood test on day 26 demonstrated Epstein-Barr Virus (EBV) reactivation (155,845 copies/ml). A repeat test on day 34 showed an increase in copy number to 638,634 copies/ml. She was asymptomatic, and the plan was to monitor this closely. On day 37 post-transplant she developed a significant deterioration in strength in the right lower limb and on day 42 she developed pyrexia and was admitted to a local hospital. She was found to have CMV reactivation which was treated with IV ganciclovir as well as ongoing EBV reactivation and she remained an inpatient for 4 weeks. She did not receive rituximab at the local centre but on repeat testing at day 145 the copy number was vastly reduced at 2,355 copies/ml. A high IgM paraproteinaemia was first detected at day 92 post-transplant (48.58g/L). This had not been routinely monitored previously. This paraproteinaemia was initially felt to be asymptomatic and was monitored closely, slowly improving over time. A CT scan was performed which demonstrated a single 1.7cm right hilar lymph node requiring observation.

A bone marrow aspirate showed a small excess of plasma cells (5-9%) on aspirate with no other significant findings.

The EBV reactivation initially settled at 6 months post-transplant. At one-year post transplant she had a persistent IgM paraprotein (23g/L) and her right leg weakness had continued to progress with her EDSS now at 5.0. There was also a mild recurrence of EBV (DNA at 1,335 copies/ml). It was considered that as the onset of the right leg weakness had coincided with the high level of EBV reactivation and paraproteinaemia that these factors may have driven a peripheral neuropathy. She was treated with rituximab 375mg/m² weekly for 4 weeks at 19 months post-transplant following which EBV DNA again became undetectable and the paraprotein reduced to 9g/L. Despite this, there was no improvement in strength of the right leg. Nerve conduction studies subsequently confirmed an L5-S1 radiculopathy but without a generalised polyneuropathy neuropathy. She has had no new or active demyelinating lesions on MRI head and spine post-transplant that would account for these symptoms and the slowly progressive nature of the weakness does not suggest an MS relapse. The cause of the weakness is likely an atypical IgM paraprotein associated radiculo-neuropathy was strongly suspected.

Patient 2

42-year-old male with Secondary Progressive Multiple Sclerosis (SPMS), previously treated with interferon and copaxone which were discontinued due to side effects and ongoing relapses, respectively. He was then treated with natalizumab for 2 years but continued to progress and was offered HSCT. He had a moderate level of baseline disability with an EDSS of 5.5 (walking at least 100m unaided). The transplant procedure was complicated by neutropenic sepsis which was treated successfully, and he was discharged on day 13 post-transplant with no new neurological symptoms. He was readmitted on day 17 post-transplant with pyrexia and rigors. Blood cultures grew *Stenotrophomonas maltophilia* and he was treated for line sepsis with appropriate antibiotics and fully recovered. An EBV viraemia of

58,324 copies/ml was detected for the first time on this admission. On day 22 he had developed new urinary urgency, diplopia and significant deterioration in mobility. This was felt to represent either a pseudorelapse driven by infection or a true relapse and an MRI was performed which demonstrated no new demyelinating lesions and no other significant pathology. A repeat EBV DNA assessment on day 28 demonstrated a significant rise in EBV viraemia to >10 million copies/ml (log change).

His neurological symptoms persisted and on day 34 he began spiking temperatures again; antibiotics were restarted but blood and urine cultures came back negative, but his EBV viraemia had risen to over 39 million copies/ml. He continued to experience intermittent pyrexia, which possibly was attributed to his EBV viraemia. No evidence of lymphadenopathy was noted during this period. Due to significant neurological decline, he was consequently commenced on rituximab 375mg/m² weekly for 4 weeks on day 38 post-transplant. Testing on day 51 demonstrated a reduction in EBV viraemia to DNA of 2.2 million copies/ml and on day 52, a significant IgG kappa paraproteinaemia (45.6 g/L) was identified. This had not been routinely monitored previously. It was considered that this degree of paraproteinemia and resulting hyperviscosity may have been a driver of his neurological symptoms. These values continued to improve over time with further doses of rituximab and the EBV viraemia was <100,000 copies/ml and the IgG kappa paraprotein down to 8.63 g/L by Day 87. However, due to persistence of these markers as well as his ongoing neurological symptoms, he was given a single plasma exchange on day 80 that was of minimal symptomatic benefit.

He had ongoing rehabilitation, including a short admission in a specialist neuro-rehabilitation ward. neurorehabilitation unit. At one year review he still required bilateral support to walk, putting his EDSS at 6.5. A repeat MRI at 12 months post-transplant was again stable with no new demyelinating lesions. This patient demonstrated significant deterioration in his condition post-transplant and although there may be an element of disease progression, we suspect this was in large part driven by EBV viraemia and associated paraproteinaemia/hyperviscosity.

The EBV viraemia was undetectable at the last follow up, although there was ongoing paraproteinaemia with an IgG kappa of 15 g/L.

EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.

Running Title: *EBV complications in Auto-HSCT for MS*

Varun Mehra*, Elijah Rhone*, Stefani Widya, Mark Zuckerman, Victoria Potter, Kavita Raj, Austin Kulasekararaj, Donal McLornan, Hugues de Lavallade, Nana Benson-Quarm, Christina Lim, Sarah Ware, Malur Sudhanva, Omar Malik, Richard Nicholas, Paolo A Muraro, Judith Marsh, Ghulam J Mufti, Eli Silber, Antonio Pagliuca and Majid A. Kazmi

***These authors contributed equally to this work as 1st Authors.**

Key Points:

EBV reactivation is common post-transplant with ATG for multiple sclerosis (MS), with significant lymphoproliferative & neurological sequelae associated with rising M-protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as is pre-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.

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Key Words:

Multiple Sclerosis; Autologous Hematopoietic Stem Cell Transplantation, Epstein-Barr Virus Infection; Monoclonal Gammopathy; Post-transplant Lymphoproliferative Disorder

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- 79 1. **Varun Mehra-** no competing financial interests
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111 **Abstract**

112 **Introduction**

113 Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin
114 (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing across
115 Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus reactivation (EBV-
116 R) following AHSCT with ATG for severe autoimmune conditions is an under-recognised
117 complication relative to T-cell deplete transplants performed for haematological diseases.
118 This retrospective study reports EBV-R associated significant clinical sequelae in MS patients
119 undergoing AHSCT with rabbit ATG.

120 **Methods**

121 Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College
122 Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and
123 serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising
124 EBV viral load, M-protein and associated clinical sequelae were captured from clinical
125 records.

126 **Results**

127 All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term
128 follow-up, with a number of them developing high EBV viral load & associated
129 lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some with
130 significant neurological consequences with high M-protein and EBV-R. Six patients required
131 anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms. Receiver
132 operating characteristics (ROC) estimated a peak EBV viraemia of >500,000 DNA copies/ml

133 correlated with high sensitivity (85.5%) & specificity (82.5%) (AUC-0.87; p-0.004) in
134 predicting EBV-R related significant clinical events.

135 **Conclusion**

136 Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-
137 AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG ~~be~~
138 ~~mandated~~ in MS patients in the first 3 months post AHSCT

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151 INTRODUCTION:

152 Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, demyelinating disease of the
153 central nervous system[1][2], with a relapsing-remitting (RRMS) presentation in the majority
154 of patients at diagnosis. Recovery from relapses may be complete or partial[3][4]. After a
155 variable period of time, people with RRMS may develop a more progressive disability
156 accumulation with or without superimposed relapses; termed secondary progressive multiple
157 sclerosis (SPMS). A minority experience progressive disability from the onset of disease,
158 termed primary progressive multiple sclerosis (PPMS)[4]. A number of immunomodulatory
159 disease modifying therapies (DMTs) are currently licensed for treatment of RRMS with [an](#)
160 aim of reducing number of relapses and accrual of disability, although with variable
161 efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation (AHSCT) has
162 been a novel approach for MS management, using immunoablation followed by
163 immunomodulation mechanisms, with evidence of significant suppression of inflammatory
164 activity and qualitative changes in the reconstituted immune system (immune reset theory)[6–
165 8]. AHSCT appears most effective for MS patients with evidence of inflammatory activity on
166 MRI, younger age, a shorter disease duration, low to moderate disability levels (Expanded
167 Disability Status Scale [EDSS] <6 or up to 6.5 if recent progression) and failure of at least 1
168 highly active DMT (natalizumab or alemtuzumab) with no significant comorbidities[9–11].
169 Recently reported preliminary results of randomised MIST study[12] found AHSCT to be
170 superior to standard disease modifying therapy (DMT) for RRMS with respect to both

171 treatment failure and disability progression.

172

173 However, risk of subsequent rise in opportunistic infections following such
174 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing
175 AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of
176 immunosuppressive rabbit anti-thymocyte globulin (rATG) to their conditioning regimen may
177 confer a higher risk of viral reactivation in these patients. The number of AHSCTs performed
178 for MS is rising significantly in Europe[14] and as more centres perform AHSCT for this
179 indication, it is increasingly important to recognise the unique problems faced by these
180 patients post AHSCT. This retrospective study reports for the first time, EBV-R associated
181 neurological sequelae and lymphoproliferative disorder (LPD) in MS patients undergoing
182 rATG conditioned AHSCT in our centre.

183

184 **METHODS**

185 **Patients and procedures**

186 Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT
187 between February 2012 and February 2017 at Kings College Hospital, London. Peripheral
188 blood stem cells were collected following standard mobilisation strategy consisting of
189 cyclophosphamide 4g/m² over 2 days and granulocyte colony-stimulating factor for 7 days.
190 Thirty-five (97%) patients were conditioned using standard protocol of cyclophosphamide

191 (50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion;
192 followed by stem cell infusion. One patient was conditioned with
193 carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG
194 (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned^{ed} was 7.17
195 $\times 10^6/\text{kg}$ (range 4.0-17.1 $\times 10^6/\text{kg}$).

196

197 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen; VCA
198 IgG). EBV DNA load monitoring was performed on whole blood samples by standardised
199 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-GeneTM (Qiagen)
200 assay of EBV BZLF1 DNA. This assay was, adapted from our published assay using
201 LightCycler (Roche)[15] and since been validated against the recently published WHO
202 standard, with our lab's EBV DNA quantification of 10 copies/ml considered equivalent to 10
203 IU/ml DNA reported with the WHO reference method[16]. EBV-R was defined as rising EBV
204 DNA load of >10 copies/millilitre (ml) detected on two consecutive tests based on our assay
205 sensitivity. Symptomatic EBV-R was captured by peak blood EBV DNA load, presence of B
206 symptoms (defined by presence of either unexplained weight loss, recurrent fever, night
207 sweats); which was in-turn defined by clinical, radiological and/or histological evidence based
208 on recent ECIL-6 guidelines[17]. In addition, significant 'clinical events' were also defined as
209 new & persistent organ dysfunction (e.g. neurological events) temporally associated with
210 rising EBV viraemia in MS patients. Serum protein electrophoresis was routinely tested
211 around 3 months post HSCT, as part of our institutional practice, with immunoglobulin
212 subclasses identified by immunofixation electrophoresis. Patient outcomes were assessed at
213 last follow up as of April 2017.

214

215 **Statistics**

216 The database of transplants and outcomes was built in Microsoft Excel 2016 and statistical
217 analyses were performed using IBM SPSS Statistics version 24.0. Patient characteristics are
218 presented as medians (with inter-quartile ranges; IQR) for data with non-normal distribution.
219 Comparisons of baseline characteristics used Mann-Whitney U test, Fisher's exact test, or
220 Chi-squared test for trend as appropriate. Receiver Operating characteristics (ROC) curve
221 was obtained correlating LPD and clonal gammopathy associated clinical events with rising
222 EBV viraemia (copies/ml).

223

224 **RESULTS**

225 Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS
226 phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had
227 prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received
228 both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment,
229 indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS
230 patients were lost to long-term follow up for EBV monitoring. The median time to first EBV
231 DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a
232 median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline
233 lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days)
234 to lymphocyte recovery (defined by total lymphocyte count $>1.0 \times 10^6/\text{ml}$) following AHSCT
235 (**See Figure 1**). A high proportion (86%; n=25/29) of the MS patients in active follow-up

236 recovered lymphocyte counts around D56 with a median lymphocyte count of 1.56×10^6
237 cells/ml); Four patients remained lymphopenic at last follow up.

238

239 All patients were stratified into following 3 groups according to peak rise/burden of EBV DNA-
240 aemia (copies/ml): <100,000 (<100k) copies/ml, 100,001-500,00 (100k-500k) copies/ml and
241 >500,000 (>500k) copies/ml to identify any specific thresholds for clinically significant events
242 related to rising EBV-R (Table 1). The majority of patients (76%) with rising EBV viral load
243 >100k copies/ml were routinely screened by computed tomographic (CT) scans to assess for
244 evidence of LPD, in line with our institutional policy. One third (34.5%) of patients developed
245 peak EBV viraemia of >500k copies/ml. Eight patients (27.6%) developed symptomatic EBV-
246 R; defined as persistent fever, lymphadenopathy and/or B symptoms. Of these 8 patients,
247 only 1 (12.5%) had a peak EBV viraemia <100kDNA copies/ml with the remaining 7 (87.5%)
248 patients having a peak EBV viraemia of >500k copies/ml. Three patients with rising EBV
249 viraemia >500k copies/ml had findings consistent with probable LPD on CT imaging;
250 however, none had definitive histological diagnosis. Three MS patients had worsening
251 neurological symptoms concurrent with rising EBV viraemia >500k copies/ml and clonal
252 gammopathy, as described below.

253

254 Interestingly, we also observed frequent de novo monoclonal gammopathy (MG or M-protein)
255 in 18 MS patients (62.4%) following rising EBV viraemia; the majority (n-16) of whom

256 developed IgG subtype and the remaining 2 developed IgA and IgM M-protein. Concerningly
257 two of these patients developed clinically significant M-Protein burden; one patient with IgG
258 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking
259 MS relapse, requiring plasma exchange. Another patient developed significant lumbosacral
260 radiculopathy with rising EBV-R and high IgM lambda M-protein (IgM 48.5g/L) (**see**
261 **supplementary case vignettes**). **Figure 2** highlights the association of neurological
262 symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts (\times
263 $10^6/\text{ml}$) with significant rise in M-protein (gm/l) levels post AHSCT. A third patient developed
264 painful lower limb paraesthesia following rising EBV viraemia $>500\text{k}$ copies/ml, although did
265 not have any M-protein detected. Their symptoms persisted at last follow up despite no
266 evidence of MS related new disease activity.

267

268 Six patients were treated with anti-CD20 antibody, rituximab (375mg/m² weekly up to 4
269 weeks), due to clinical severity of EBV reactivations and, leading to 4reduction in EBV viral
270 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis
271 (**Figure 3**) confirmed EBV viraemia of $>500\text{k}$ copies/ml correlated with high sensitivity
272 (85.5%) and specificity (82.5%) (AUC-0.87; 95%CI-0.73-1.0; p-0.004) in predicting significant
273 EBV related clinical events (evidence of LPD and/or neurological symptoms) that may require
274 treatment with rituximab. The sensitivity dropped significantly on lower estimates for events
275 below 500k copies/ml.

276

277 The median time to resolution of EBV viraemia post-rituximab was 21 days (IQR 19-124 days)
278 in 5 patients with >500,000 ~~k~~ copies/ml (one patient was treated for late onset persistent
279 symptomatic clonal gammopathy, despite fall in EBV levels). No significant adverse events
280 were noted in the treated group. Nine patients had a persistent low level EBV viraemia
281 detectable at last follow up. All patients who underwent AHSCT were alive as of April 2017.

282

283 **DISCUSSION:**

284 MS as an autoimmune disorder (AD) ~~has~~ is theorised to have generally similar underlying
285 pathophysiological immune dysregulation mechanisms[18–22] relative to other chronic
286 autoimmune conditions. Epstein-Barr virus (EBV) is increasingly implicated in the
287 pathogenesis of MS by virtue of epidemiological and serological evidence, impaired CD8+ T
288 cell immune responses to EBV and possible underlying genetic susceptibility for
289 autoimmunity (with EBV encoded protein interactions), as recently described by Harley et al
290 and others[1,23–25].

291

292 Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid
293 organ transplants treated with immunosuppressive therapy, often with a significant impact
294 on organ function and overall survival[26–30]. It is observed that reduced intensity allo-HSCT
295 for malignant haematological conditions using alemtuzumab have a relatively lower overall

296 risk of LPD compared to ATG based treatments, possibly mediated by more effective pan-B
297 & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell repertoire with delayed
298 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections
299 including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease
300 and ~~s~~Systemic ~~s~~Sclerosis is increasingly recognised, but the development of
301 lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32]
302 concerning reported 2 deaths (1 MS and 1 systemic sclerosis patient) from EBV related
303 LPD in 56 ATG conditioned AHSCT for autoimmune diseases. Additionally, EBV associated
304 haemophagocytosis in ATG-AHSCT for ADs have also been reported[34], with one resulting
305 in death of the patient[35].

306

307 Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher
308 than published reports with allo-HSCTs (4.5-7%)[36,37] and our own centre's unpublished T-
309 cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying
310 immunopathological state of MS itself[38]. This is further corroborated by the fact that similar
311 LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with ATG -
312 AHSCT in this centre. ~~Another example from ouour~~ Another example from our ~~centre's~~ centre's experience ~~of in~~ severe aplastic
313 anaemia ~~aa_ (n-40), a type of AD causing severe bone marrow failure(n-40) treated & treated~~
314 with ATG/ciclosporin, ~~n,n;~~ only 52% (n-21/40) ~~patients~~ developed EBV-R (unpublished data)
315 and ~~-n~~None had LPD or required any treatment, ~~supporting the notion that it may not just be~~

~~a specific ATG-related problem suggesting that the problem may not be ATG specific.~~

Our study's observation of significant persistent neurological events (with no evidence of new MS disease activity) associated with clonal gammopathy, suggest a potentially new clinical syndrome, described for the first time in ATG conditioned AHSCTs in MS and, possibly induced by clonal B cell dysregulation following EBV-R. It could be hypothesised that any remaining EBV infected latent B cells, ~~which may still have survived~~surviving despite high doses of cyclophosphamide (given with mobilisation and conditioning in MS ASHCT)[8] and compounded by depletion of CD8+ T cells by ATG, may serve as potential source for EBV escape while interacting abnormally within the host immune micro-environment[39] and leading to rise in M-protein, LPD and neuro-inflammatory insults in some of the MS patients post AHSCT. Reports of lower incidence of EBV-R and LPD in another commonly used protocol for MS-AHSCT using BEAM-ATG (Ricardo Saccardi, Carregi University Hospital, Florence; personal communication) may reflect the greater myeloablative effect of BEAM chemotherapy which could further deplete the residual B cell pool and thus lower potential for EBV proliferation. It is plausible that dose of rATG is critical, given we have not seen similar reports from other centres where less rATG doses were given for MS-AHSCT (range between 5.0-6.5 mg/Kg; personal communication), but there seems to be some variability in prospective serial EBV monitoring in these patients.

336 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is
337 widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is
338 significantly associated with probable LPD and neurological events in MS patients with high
339 sensitivity (85.5%) and specificity (82.5%) (p=0.004) (**Fig 3, ROC curve**). Our ROC curve
340 estimates are potentially limited by the relatively small number of events analysed but this
341 has consistently been useful in our MS-AHSCT experience for predicting clinical events with
342 high EBV load. Our EBV PCR assay has been validated against the recently defined standard
343 WHO reference method (i.e. 10 copies/ml=10 IU/ml EBV DNA) [16] and thus this EBV
344 threshold for pre-emptive treatment with Rituximab, can potentially be applied in relevant
345 clinical context in other centres using similar validated assays. Rituximab treatment delivered
346 good overall response in our symptomatic patients, with resolution of EBV related clinical
347 symptoms and no subsequent viral or bacterial infections at last follow up. The role of
348 prophylactic or pre-transplant rituximab in MS-AHSCT is also a potential area of interest in
349 reducing risk of EBV-LPD in stem cell transplants as observed by Burns et al[36] and future
350 randomised studies are required to investigate its potential benefit.

351

352 Our study limitations include its retrospective nature and that no suspected LPD patients had
353 histological confirmation, mainly related to patient refusal or technical difficulties. Seven MS
354 patients were lost to follow up for EBV monitoring following discharge, which limits the

355 findings of this study. Additionally, our numbers were too small to identify any association of
356 EBV related clinical events with previous DMT exposure in MS patients.

357

358 In conclusion, symptomatic EBV reactivation increases risk of neurological sequelae and
359 LPD in MS-AHSCT. Regular monitoring for rising EBV viraemia, as recommended by
360 Snowden et al [10] and Muraro et al [11], and serum electrophoresis for M-protein should be
361 ~~considered mandated~~ in the first 3 months post-AHSCT for MS, ~~and~~ We recommend
362 persistent high EBV viraemia > 500k DNA copies/ml as potential trigger for consideration of
363 pre-emptive anti-CD20 therapy and potentially reduce associated morbidity.

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376 REFERENCES

377

- 378 1. Pender MP, Burrows SR. Epstein–Barr virus and multiple sclerosis: potential opportunities for
379 immunotherapy. *Clinical & Translational Immunology* **2014**; 3:e27. Available at:
380 <http://doi.wiley.com/10.1038/cti.2014.25>.
- 381 2. Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. *New England Journal of Medicine* **2018**;
382 378:169–180. Available at: <http://www.nejm.org/doi/10.1056/NEJMra1401483>.
- 383 3. Compston A, Coles A. Multiple sclerosis. *The Lancet* **2008**; 372:1502–1517. Available at:
384 <http://linkinghub.elsevier.com/retrieve/pii/S0140673608616207>.
- 385 4. Lublin FD, Reingold SC, Cohen JA, et al. Defining the clinical course of multiple sclerosis: The 2013
386 revisions. *Neurology* **2014**; 83:278–286. Available at:
387 <http://www.neurology.org/cgi/doi/10.1212/WNL.0000000000000560>.
- 388 5. Comi G, Radaelli M, Soelberg Sørensen P. Evolving concepts in the treatment of relapsing multiple
389 sclerosis. *The Lancet* **2017**; 389:1347–1356. Available at:
390 <https://linkinghub.elsevier.com/retrieve/pii/S0140673616323881>.
- 391 6. Muraro PA, Douek DC, Packer A, et al. Thymic output generates a new and diverse TCR repertoire
392 after autologous stem cell transplantation in multiple sclerosis patients. *The Journal of Experimental*
393 *Medicine* **2005**; 201:805–816. Available at: <http://www.jem.org/lookup/doi/10.1084/jem.20041679>.
- 394 7. Muraro PA, Robins H, Malhotra S, et al. T cell repertoire following autologous stem cell transplantation
395 for multiple sclerosis. *J Clin Invest* **2014**; 124:1168–1172. Available at:
396 <http://www.ncbi.nlm.nih.gov/pubmed/24531550>.
- 397 8. Cull G, Hall D, Fabis-Pedrini M, et al. Lymphocyte reconstitution following autologous stem cell
398 transplantation for progressive MS. *Multiple Sclerosis Journal – Experimental, Translational and*
399 *Clinical* **2017**; 3:205521731770016. Available at: <https://doi.org/10.1177/2055217317700167>.
- 400 9. Sormani MP, Muraro PA, Schiavetti I, et al. Autologous hematopoietic stem cell transplantation in
401 multiple sclerosis. *Neurology* **2017**; 88:2115–2122. Available at:
402 <http://www.neurology.org/lookup/doi/10.1212/WNL.0000000000003987>.
- 403 10. Snowden JA, Saccardi R, Allez M, et al. Haematopoietic SCT in severe autoimmune diseases:
404 updated guidelines of the European Group for Blood and Marrow Transplantation. *Bone Marrow*
405 *Transplantation* **2012**; 47:770–790. Available at: <http://www.nature.com/articles/bmt2011185>.
- 406 11. Muraro PA, Pasquini M, Atkins HL, et al. Long-term Outcomes After Autologous Hematopoietic Stem
407 Cell Transplantation for Multiple Sclerosis. *JAMA Neurology* **2017**; 74:459. Available at:
408 <http://archneur.jamanetwork.com/article.aspx?doi=10.1001/jamaneurol.2016.5867>.
- 409 12. Burt RK, Balabanov R, Snowden JA, Sharrack B, Oliveira MC, Burman J. Non-myeloablative
410 hematopoietic stem cell transplantation (HSCT) is superior to disease modifying drug (DMD) treatment
411 in highly active Relapsing Remitting Multiple Sclerosis (RRMS): interim results of the Multiple Sclerosis

- International Stem cell Transp. Neurology **2018**; 90. Available at:
http://n.neurology.org/content/90/15_Supplement/S36.004.abstract.
13. Daikeler T, Tichelli A, Passweg J. Complications of autologous hematopoietic stem cell transplantation for patients with autoimmune diseases. *Pediatric Research* **2012**; 71:439–444. Available at:
<http://www.nature.com/doi/10.1038/pr.2011.57>.
 14. Snowden JA, Badoglio M, Labopin M, et al. Evolution, trends, outcomes, and economics of hematopoietic stem cell transplantation in severe autoimmune diseases. *Blood Advances* **2017**; 1:2742–2755. Available at:
<http://www.bloodadvances.org/lookup/doi/10.1182/bloodadvances.2017010041>.
 15. Patel S, Zuckerman M, Smith M. Real-time quantitative PCR of Epstein–Barr virus BZLF1 DNA using the LightCycler. *Journal of Virological Methods* **2003**; 109:227–233. Available at:
<http://linkinghub.elsevier.com/retrieve/pii/S0166093403000764>.
 16. Semenova T, Lupo J, Alain S, et al. Multicenter Evaluation of Whole-Blood Epstein-Barr Viral Load Standardization Using the WHO International Standard. *Journal of Clinical Microbiology* **2016**; 54:1746 LP-1750. Available at: <http://jcm.asm.org/content/54/7/1746.abstract>.
 17. Styczynski J, van der Velden W, Fox CP, et al. Management of Epstein-Barr Virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: Sixth European Conference on Infections in Leukemia (ECIL-6) guidelines. *Haematologica* **2016**; 101:803–811. Available at:
<http://www.haematologica.org/cgi/doi/10.3324/haematol.2016.144428>.
 18. Dejaco C, Duftner C, Grubeck-Loebenstien B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* **2006**; 117:289–300. Available at:
<http://doi.wiley.com/10.1111/j.1365-2567.2005.02317.x>.
 19. Arellano G, Acuña E, Reyes LI, et al. Th1 and Th17 Cells and Associated Cytokines Discriminate among Clinically Isolated Syndrome and Multiple Sclerosis Phenotypes. *Frontiers in immunology* **2017**; 8:753. Available at: <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00753/full>.
 20. Jha S, Srivastava SY, Brickey WJ, et al. The Inflammasome Sensor, NLRP3, Regulates CNS Inflammation and Demyelination via Caspase-1 and Interleukin-18. *Journal of Neuroscience* **2010**; 30:15811–15820. Available at: <http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.4088-10.2010>.
 21. Beynon V, Quintana FJ, Weiner HL. Activated Human CD4+CD45RO+ Memory T-Cells Indirectly Inhibit NLRP3 Inflammasome Activation through Downregulation of P2X7R Signalling. *PLoS ONE* **2012**; 7:e39576. Available at: <https://dx.plos.org/10.1371/journal.pone.0039576>.
 22. Fernández-Menéndez S, Fernández-Morán M, Fernández-Vega I, Pérez-Álvarez A, Villafani-Echazú J. Epstein–Barr virus and multiple sclerosis. From evidence to therapeutic strategies. *Journal of Neurological Sciences* **2016**; 361:213–219. Available at:
https://www.clinicalkey.com.ezsecureaccess.balamand.edu.lb/service/content/pdf/watermarked/1-s2.0-S0022510X16300132.pdf?locale=en_US.

- 449 23. Pender MP. The Essential Role of Epstein-Barr Virus in the Pathogenesis of Multiple Sclerosis. *The*
450 *Neuroscientist* **2011**; 17:351–367. Available at:
451 <http://journals.sagepub.com/doi/10.1177/1073858410381531>.
- 452 24. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: The role of infection.
453 *Annals of Neurology* **2007**; 61:288–299. Available at: <http://doi.wiley.com/10.1002/ana.21117>.
- 454 25. Harley JB, Chen X, Pujato M, et al. Transcription factors operate across disease loci, with EBNA2
455 implicated in autoimmunity. *Nature Genetics* **2018**; 50:699–707. Available at:
456 <http://www.nature.com/articles/s41588-018-0102-3>.
- 457 26. Loren AW, Porter DL, Stadtmauer EA, Tsai DE. Post-transplant lymphoproliferative disorder: a review.
458 *Bone Marrow Transplantation* **2003**; 31:145–155. Available at:
459 <http://www.nature.com/articles/1703806>.
- 460 27. Dotti G, Fiocchi R, Motta T, et al. Lymphomas occurring late after solid-organ transplantation: influence
461 of treatment on the clinical outcome. *Transplantation* **2002**; 74:1095–102. Available at:
462 <http://www.ncbi.nlm.nih.gov/pubmed/12438953>.
- 463 28. Nagle SJ, Reshef R, Tsai DE. Posttransplant Lymphoproliferative Disorder in Solid Organ and
464 Hematopoietic Stem Cell Transplantation. *Clinics in Chest Medicine* **2017**; 38:771–783. Available at:
465 <https://linkinghub.elsevier.com/retrieve/pii/S0272523117300874>.
- 466 29. Meijer E, Dekker AW, Weersink AJL, Rozenberg-Arska M, Verdonck LF. Prevention and treatment of
467 epstein–barr virus-associated lymphoproliferative disorders in recipients of bone marrow and solid
468 organ transplants. *British Journal of Haematology* **2002**; 119:596–607. Available at:
469 <http://dx.doi.org/10.1046/j.1365-2141.2002.03887.x>.
- 470 30. Dierickx D, Habermann TM. Post-Transplantation Lymphoproliferative Disorders in Adults. *New*
471 *England Journal of Medicine* **2018**; 378:549–562. Available at:
472 <http://dx.doi.org/10.1056/NEJMra1702693>.
- 473 31. Meij P. Impaired recovery of Epstein-Barr virus (EBV)--specific CD8+ T lymphocytes after partially T-
474 depleted allogeneic stem cell transplantation may identify patients at very high risk for progressive
475 EBV reactivation and lymphoproliferative disease. *Blood* **2003**; 101:4290–4297. Available at:
476 <http://www.bloodjournal.org/cgi/doi/10.1182/blood-2002-10-3001>.
- 477 32. Nash R a, Dansey R, Storek J, et al. Epstein-Barr virus-associated posttransplantation
478 lymphoproliferative disorder after high-dose immunosuppressive therapy and autologous CD34-
479 selected hematopoietic stem cell transplantation for severe autoimmune diseases. *Biology of blood*
480 *and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation*
481 **2003**; 9:583–91. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14506660>.
- 482 33. Daikeler T, Tzankov A, Hoenger G, et al. Minimal T-cell requirements for triggering
483 haemophagocytosis associated with Epstein–Barr virus-driven B-cell proliferation: a clinical case
484 study. *Annals of the Rheumatic Diseases* **2011**; 70:1338 LP-1339. Available at:
485 <http://ard.bmj.com/content/70/7/1338.abstract>.

- 486 34. Daikeler T, Tzankov A, Hoenger G, et al. Minimal T-cell requirements for triggering
487 haemophagocytosis associated with Epstein-Barr virus-driven B-cell proliferation: a clinical case study.
488 *Annals of the Rheumatic Diseases* **2011**; 70:1338–1339. Available at:
489 <http://ard.bmj.com/cgi/doi/10.1136/ard.2010.139246>.
- 490 35. Brinkman DMC, de Kleer IM, ten Cate R, et al. Autologous stem cell transplantation in children with
491 severe progressive systemic or polyarticular juvenile idiopathic arthritis: Long-term followup of a
492 prospective clinical trial. *Arthritis & Rheumatism* **2007**; 56:2410–2421. Available at:
493 <http://doi.wiley.com/10.1002/art.22656>.
- 494 36. Burns DM, Rana S, Martin E, et al. Greatly reduced risk of EBV reactivation in rituximab-experienced
495 recipients of alemtuzumab-conditioned allogeneic HSCT. *Bone Marrow Transplantation* **2016**; 51:825–
496 832. Available at: <http://www.nature.com/articles/bmt201619>.
- 497 37. van Esser JWJ. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell
498 transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-
499 depleted SCT. *Blood* **2001**; 98:972–978. Available at:
500 <http://www.bloodjournal.org/cgi/doi/10.1182/blood.V98.4.972>.
- 501 38. Tørring C, Andreassen C, Gehr N, Bjerg L, Petersen T, Höllsberg P. Higher incidence of Epstein-Barr
502 virus-induced lymphocyte transformation in multiple sclerosis. *Acta Neurologica Scandinavica* **2014**;
503 130:90–96. Available at: <http://doi.wiley.com/10.1111/ane.12249>.
- 504 39. Martinez OM, Krams SM. The Immune Response to Epstein Barr Virus and Implications for
505 Posttransplant Lymphoproliferative Disorder. *Transplantation* **2017**; 101:2009–2016. Available at:
506 <http://insights.ovid.com/crossref?an=00007890-201709000-00018>.

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527 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**
528 **EBV DNA-aemia burden.**
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Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender		M-Protein (n)	11	0	7
Male	19 (52.8%)				
Female	17 (47.2%)				
Disease Type (n; %)		Median EBV DNA log value at peak (IQR)			
Relapsing Remitting MS	22 (61.1%)		4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Secondary Progressive MS	10 (27.8%)				
Primary Progressive MS	4 (11.1%)				
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n)		Symptomatic EBV (n)			
Natalizumab	22		1	0	7
Alemtuzumab	8				
Both	6				
Median EDSS (range)	6.0 (2.5 – 8.0)	LPD diagnosis (CT/Biopsy) (n)	0	0	3 by CT alone
Median follow up post AHSCT in days (range)	436 (188 – 785)	Neuro/autoimmune complications (n)	0	0	3
No. of patients with prior EBV exposure (n; %)	36 (100%)	Treated with Rituximab (n)	0	0	6
No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
No. of patients lost to long term follow up (n; %)	7 (19.5%)	Detectable EBV DNA at last follow up (n)	9	1	3
Median Time to EBV detection post AHSCT in days (IQR)	30 (23-46)	Median time for EBV resolution (IQR in days)	67 days (44-155)	47 days (N/A)	63 days (45 - 170)
Median Time to peak EBV DNA levels in days (IQR)	32 (31-53)	Median Time to peak EBV DNA levels in days (IQR)	40 days (25-85)	30 days (N/A)	39 days (32-43)

530

531 **Abbreviations:**

532 AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography; DMT-
533 Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded Disability
534 Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-Protein:
535 Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis

Figure Legends

Figure 1: Trend of Lymphocyte count recovery following ATG in MS patients.

Legend: This figure shows trends of lymphocyte count from baseline to recovery post AHSCT for MS patients. Majority of patients had normal baseline lymphocyte counts pre-HSCT and became lymphopenic post ATG with counts recovering towards d+28 and >85% of patients recovered counts by d+56, with some overshooting from their baseline, possibly reflective of EBV related lymphoproliferation in some of these patients.

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

Figure 2: Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT.

Legend: This figure demonstrates trends of EBV copies (log), paraprotein levels (g/l) and Lymphocyte levels (counts $\times 10^6/\text{ml}$) in two MS patients with significant neurological symptoms following EBV reactivation. Both patients had significant EBV viraemia ($\log > 5.2$ or $> 500,000$ copy number) and developed significant paraproteinaemia, which was only noted after persistent unexplained neurological symptoms. The trend reversed following anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

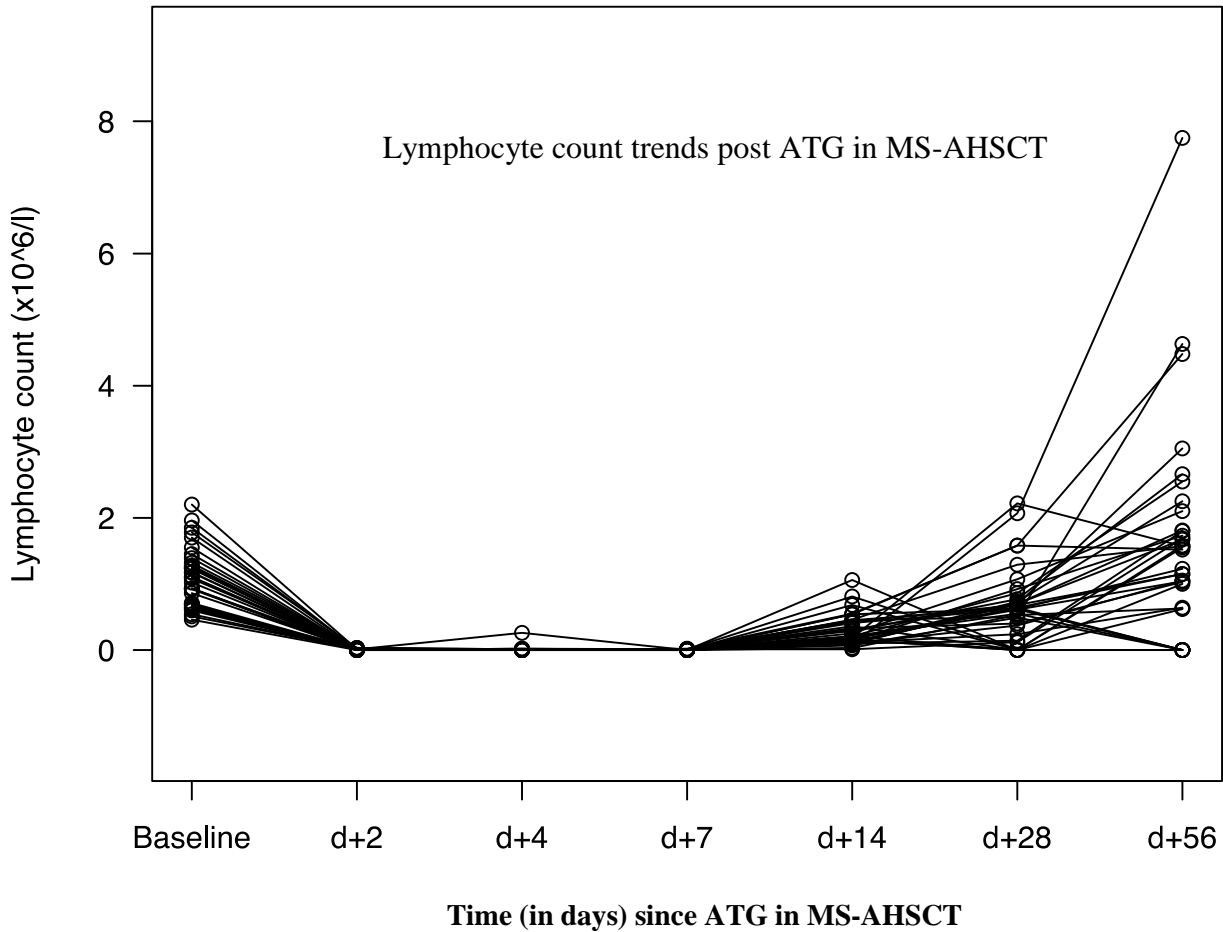
Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.

Legend: ROC curve demonstrating significant correlation between high EBV levels and clinical events (LPD & neurological events) in MS-AHSCT patients, with highest sensitivity and specificity noted with peak EBV viraemia of $> 500,000$ copies/ml ($p = 0.0004$).

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

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Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.

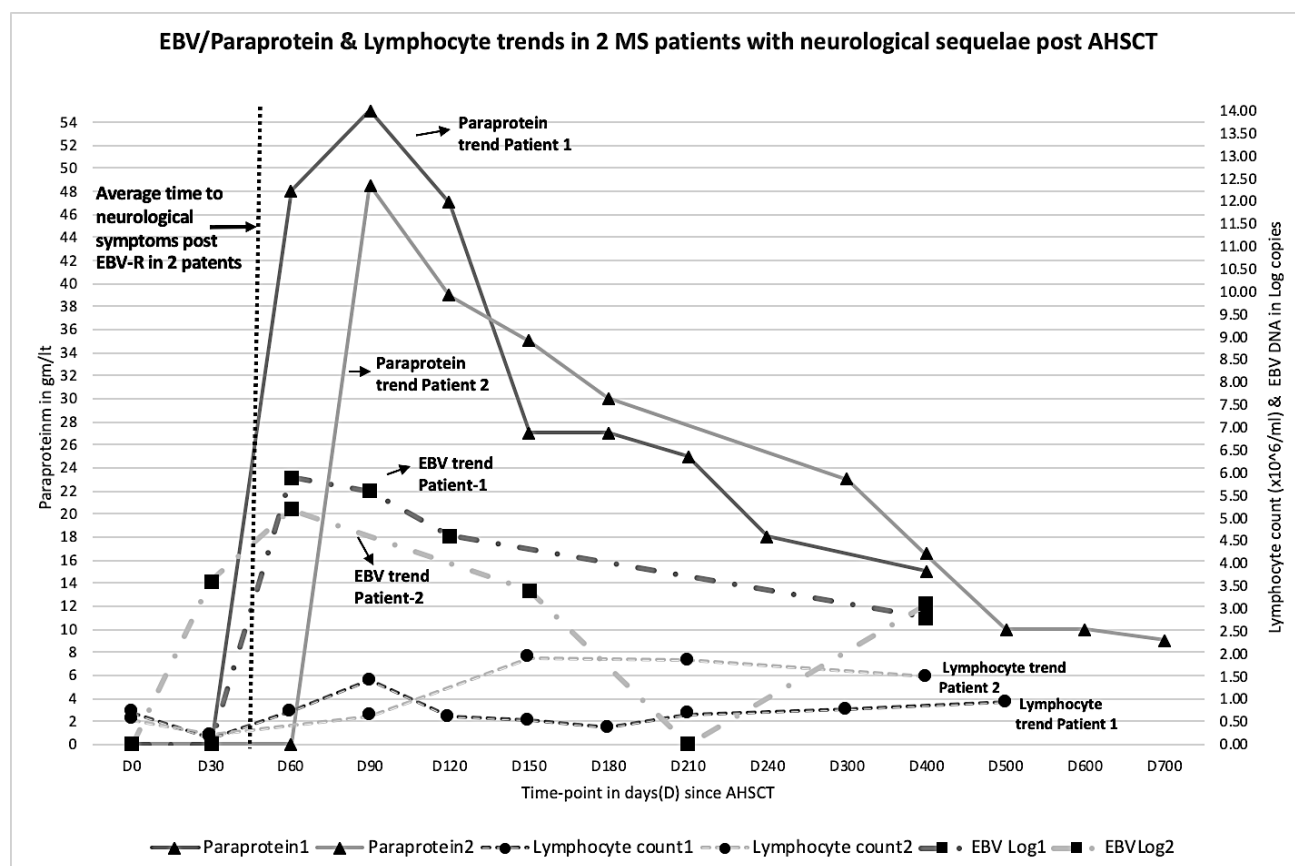


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Abbreviations:

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

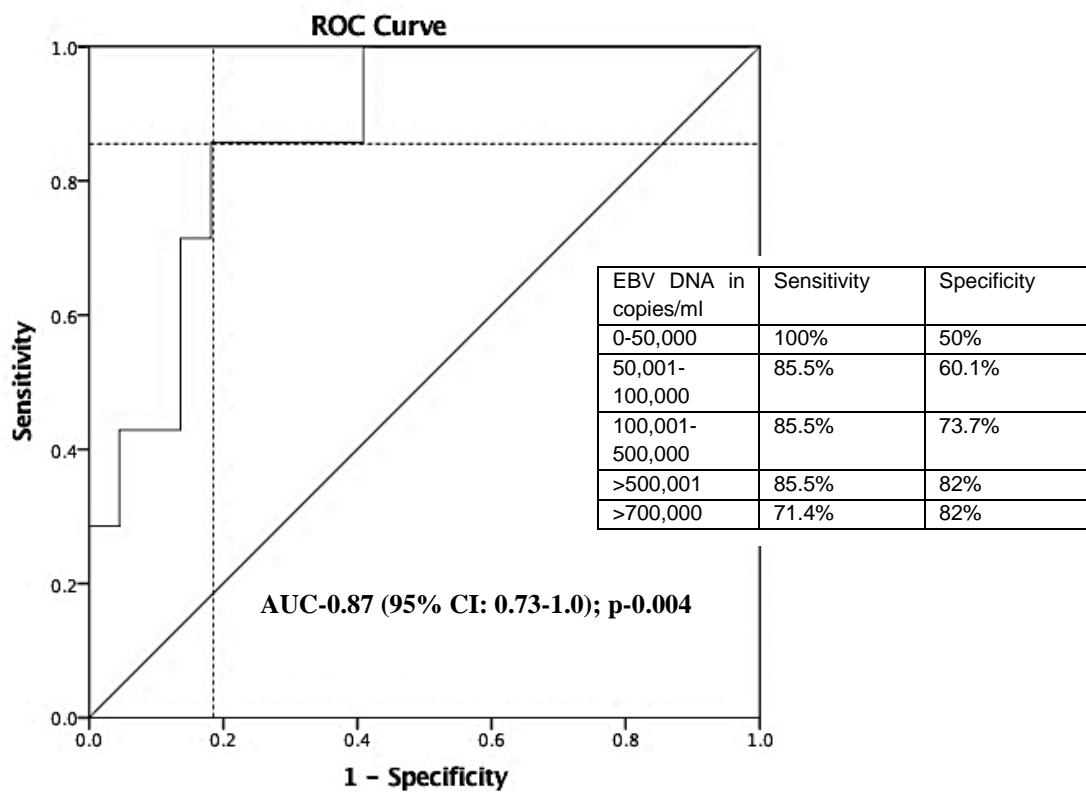
Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT



Abbreviations:

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.



Abbreviations:

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis.

Running Title: *EBV complications in Auto-HSCT for MS*

Varun Mehra*, Elijah Rhone*, Stefani Widya, Mark Zuckerman, Victoria Potter, Kavita Raj, Austin Kulasekararaj, Donal McLornan, Hugues de Lavallade, Nana Benson-Quarm, Christina Lim, Sarah Ware, Malur Sudhanva, Omar Malik, Richard Nicholas, Paolo A Muraro, Judith Marsh, Ghulam J Mufti, Eli Silber, Antonio Pagliuca and Majid A. Kazmi

***These authors contributed equally to this work as 1st Authors.**

Key Points:

EBV reactivation is common post-transplant with ATG for multiple sclerosis (MS), with significant lymphoproliferative & neurological sequelae associated with rising M-protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as is pre-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.

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Key Words:

Multiple Sclerosis; Autologous Hematopoietic Stem Cell Transplantation, Epstein-Barr Virus Infection; Monoclonal Gammopathy; Post-transplant Lymphoproliferative Disorder

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- 79 1. **Varun Mehra-** no competing financial interests
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- 81 3. **Stefani Widya-** no competing financial interests
- 82 4. **Mark Zuckerman-** no competing financial interests
- 83 5. **Victoria Potter-** no competing financial interests
- 84 6. **Kavita Raj-** no competing financial interests
- 85 7. **Austin Kulasekararaj-** no competing financial interests
- 86 8. **Donal McLornan-** no competing financial interests
- 87 9. **Hugues de Lavallade-** no competing financial interests
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- 97 19. **Eli Silber-** no competing financial interests
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111 **Abstract**

112 **Introduction**

113 Autologous haematopoietic stem cell transplantation (AHSCT) with anti-thymocyte globulin
114 (ATG) conditioning as treatment of active multiple sclerosis (MS) is rapidly increasing across
115 Europe (EBMT registry data 2017). Clinically significant Epstein Barr virus reactivation (EBV-
116 R) following AHSCT with ATG for severe autoimmune conditions is an under-recognised
117 complication relative to T-cell deplete transplants performed for haematological diseases.
118 This retrospective study reports EBV-R associated significant clinical sequelae in MS patients
119 undergoing AHSCT with rabbit ATG.

120 **Methods**

121 Retrospective data was analysed for 36 consecutive MS-AHSCT patients at Kings College
122 Hospital, London. All patients routinely underwent weekly EBV DNA PCR monitoring and
123 serum electrophoresis for monoclonal gammopathy (MG or M-protein). EBV-R with rising
124 EBV viral load, M-protein and associated clinical sequelae were captured from clinical
125 records.

126 **Results**

127 All patients had evidence of rising EBV DNA-emia, including 7 who were lost to long term
128 follow-up, with a number of them developing high EBV viral load & associated
129 lymphoproliferative disorder (LPD). Nearly 72% (n-18/29) developed de-novo MG, some with
130 significant neurological consequences with high M-protein and EBV-R. Six patients required
131 anti-CD20 therapy (rituximab) with complete resolution of EBV related symptoms. Receiver
132 operating characteristics (ROC) estimated a peak EBV viraemia of >500,000 DNA copies/ml

133 correlated with high sensitivity (85.5%) & specificity (82.5%) (AUC-0.87; p-0.004) in
134 predicting EBV-R related significant clinical events.

135 **Conclusion**

136 Symptomatic EBV reactivation increases risk of neurological sequelae and LPD in MS-
137 AHSCT. We recommend regular monitoring for EBV and serum electrophoresis for MG in
138 MS patients in the first 3 months post AHSCT

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151 INTRODUCTION:

152 Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, demyelinating disease of the
153 central nervous system[1][2], with a relapsing-remitting (RRMS) presentation in the majority
154 of patients at diagnosis. Recovery from relapses may be complete or partial[3][4]. After a
155 variable period of time, people with RRMS may develop a more progressive disability
156 accumulation with or without superimposed relapses; termed secondary progressive multiple
157 sclerosis (SPMS). A minority experience progressive disability from the onset of disease,
158 termed primary progressive multiple sclerosis (PPMS)[4]. A number of immunomodulatory
159 disease modifying therapies (DMTs) are currently licensed for treatment of RRMS with an
160 aim of reducing number of relapses and accrual of disability, although with variable
161 efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation (AHSCT) has
162 been a novel approach for MS management, using immunoablation followed by
163 immunomodulation mechanisms, with evidence of significant suppression of inflammatory
164 activity and qualitative changes in the reconstituted immune system (immune reset theory)[6–
165 8]. AHSCT appears most effective for MS patients with evidence of inflammatory activity on
166 MRI, younger age, a shorter disease duration, low to moderate disability levels (Expanded
167 Disability Status Scale [EDSS] <6 or up to 6.5 if recent progression) and failure of at least 1
168 highly active DMT (natalizumab or alemtuzumab) with no significant comorbidities[9–11].
169 Recently reported preliminary results of randomised MIST study[12] found AHSCT to be
170 superior to standard disease modifying therapy (DMT) for RRMS with respect to both

171 treatment failure and disability progression.

172

173 However, risk of subsequent rise in opportunistic infections following such
174 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing
175 AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of
176 immunosuppressive rabbit anti-thymocyte globulin (rATG) to their conditioning regimen may
177 confer a higher risk of viral reactivation in these patients. The number of AHSCTs performed
178 for MS is rising significantly in Europe[14] and as more centres perform AHSCT for this
179 indication, it is increasingly important to recognise the unique problems faced by these
180 patients post AHSCT. This retrospective study reports for the first time, EBV-R associated
181 neurological sequelae and lymphoproliferative disorder (LPD) in MS patients undergoing
182 rATG conditioned AHSCT in our centre.

183

184 **METHODS**

185 **Patients and procedures**

186 Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT
187 between February 2012 and February 2017 at Kings College Hospital, London. Peripheral
188 blood stem cells were collected following standard mobilisation strategy consisting of
189 cyclophosphamide 4g/m² over 2 days and granulocyte colony-stimulating factor for 7 days.
190 Thirty-five (97%) patients were conditioned using standard protocol of cyclophosphamide

191 (50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion
192 followed by stem cell infusion. One patient was conditioned with
193 carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG
194 (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned was 7.17
195 $\times 10^6/\text{kg}$ (range 4.0-17.1 $\times 10^6/\text{kg}$).

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197 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen; VCA
198 IgG). EBV DNA load monitoring was performed on whole blood samples by standardised
199 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-GeneTM (Qiagen)
200 assay of EBV BZLF1 DNA. This assay was adapted from our published assay using
201 LightCycler (Roche)[15] and since been validated against the recently published WHO
202 standard, with our lab's EBV DNA quantification of 10 copies/ml considered equivalent to 10
203 IU/ml DNA reported with the WHO reference method[16]. EBV-R was defined as rising EBV
204 DNA load of >10 copies/millilitre (ml) detected on two consecutive tests based on our assay
205 sensitivity. Symptomatic EBV-R was captured by peak blood EBV DNA load, presence of B
206 symptoms (defined by presence of either unexplained weight loss, recurrent fever, night
207 sweats); which was in-turn defined by clinical, radiological and/or histological evidence based
208 on recent ECIL-6 guidelines[17]. In addition, significant 'clinical events' were also defined as
209 new & persistent organ dysfunction (e.g. neurological events) temporally associated with
210 rising EBV viraemia in MS patients. Serum protein electrophoresis was routinely tested
211 around 3 months post HSCT as part of our institutional practice, with immunoglobulin
212 subclasses identified by immunofixation electrophoresis. Patient outcomes were assessed at
213 last follow up as of April 2017.

214

215 **Statistics**

216 The database of transplants and outcomes was built in Microsoft Excel 2016 and statistical
217 analyses were performed using IBM SPSS Statistics version 24.0. Patient characteristics are
218 presented as medians (with inter-quartile ranges; IQR) for data with non-normal distribution.
219 Comparisons of baseline characteristics used Mann-Whitney U test, Fisher's exact test, or
220 Chi-squared test for trend as appropriate. Receiver Operating characteristics (ROC) curve
221 was obtained correlating LPD and clonal gammopathy associated clinical events with rising
222 EBV viraemia (copies/ml).

223

224 **RESULTS**

225 Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS
226 phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had
227 prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received
228 both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment,
229 indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS
230 patients were lost to long-term follow up for EBV monitoring. The median time to first EBV
231 DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a
232 median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline
233 lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days)
234 to lymphocyte recovery (defined by total lymphocyte count $>1.0 \times 10^6/\text{ml}$) following AHSCT
235 (**See Figure 1**). A high proportion (86%; n=25/29) of the MS patients in active follow-up

236 recovered lymphocyte counts around D56 with a median lymphocyte count of 1.56×10^6
237 cells/ml); Four patients remained lymphopenic at last follow up.

238

239 All patients were stratified into following 3 groups according to peak rise/burden of EBV DNA-
240 aemia (copies/ml): <100,000 (<100k) copies/ml, 100,001-500,00 (100k-500k) copies/ml and
241 >500,000 (>500k) copies/ml to identify any specific thresholds for clinically significant events
242 related to rising EBV-R (Table 1). The majority of patients (76%) with rising EBV viral load
243 >100k copies/ml were routinely screened by computed tomographic (CT) scans to assess for
244 evidence of LPD, in line with our institutional policy. One third (34.5%) of patients developed
245 peak EBV viraemia of >500k copies/ml. Eight patients (27.6%) developed symptomatic EBV-
246 R; defined as persistent fever, lymphadenopathy and/or B symptoms. Of these 8 patients,
247 only 1 (12.5%) had a peak EBV viraemia <100kDNA copies/ml with the remaining 7 (87.5%)
248 patients having a peak EBV viraemia of >500k copies/ml. Three patients with rising EBV
249 viraemia >500k copies/ml had findings consistent with probable LPD on CT imaging;
250 however, none had definitive histological diagnosis. Three MS patients had worsening
251 neurological symptoms concurrent with rising EBV viraemia >500k copies/ml and clonal
252 gammopathy, as described below.

253

254 Interestingly, we also observed frequent de novo monoclonal gammopathy (MG or M-protein)
255 in 18 MS patients (62.4%) following rising EBV viraemia; the majority (n-16) of whom

256 developed IgG subtype and the remaining 2 developed IgA and IgM M-protein. Concerningly
257 two of these patients developed clinically significant M-Protein burden; one patient with IgG
258 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking
259 MS relapse, requiring plasma exchange. Another patient developed significant lumbosacral
260 radiculopathy with rising EBV-R and high IgM lambda M-protein (IgM 48.5g/L) (**see**
261 **supplementary case vignettes**). **Figure 2** highlights the association of neurological
262 symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts (\times
263 $10^6/\text{ml}$) with significant rise in M-protein (gm/l) levels post AHSCT. A third patient developed
264 painful lower limb paraesthesia following rising EBV viraemia $>500\text{k}$ copies/ml, although did
265 not have any M-protein detected. Their symptoms persisted at last follow up despite no
266 evidence of MS related new disease activity.

267

268 Six patients were treated with anti-CD20 antibody, rituximab (375mg/m² weekly up to 4
269 weeks), due to clinical severity of EBV reactivations and leading to reduction in EBV viral
270 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis
271 (**Figure 3**) confirmed EBV viraemia of $>500\text{k}$ copies/ml correlated with high sensitivity
272 (85.5%) and specificity (82.5%) (AUC-0.87; 95%CI-0.73-1.0; p-0.004) in predicting significant
273 EBV related clinical events (evidence of LPD and/or neurological symptoms) that may require
274 treatment with rituximab. The sensitivity dropped significantly on lower estimates for events
275 below 500k copies/ml.

276

277 The median time to resolution of EBV viraemia post-rituximab was 21 days (IQR 19-124 days)
278 in 5 patients with >500k copies/ml (one patient was treated for late onset persistent
279 symptomatic clonal gammopathy, despite fall in EBV levels). No significant adverse events
280 were noted in the treated group. Nine patients had a persistent low level EBV viraemia
281 detectable at last follow up. All patients who underwent AHSCT were alive as of April 2017.

282

283 **DISCUSSION:**

284 MS as an autoimmune disorder (AD) is theorised to have generally similar underlying
285 pathophysiological immune dysregulation mechanisms[18–22] relative to other chronic
286 autoimmune conditions. Epstein-Barr virus (EBV) is increasingly implicated in the
287 pathogenesis of MS by virtue of epidemiological and serological evidence, impaired CD8+ T
288 cell immune responses to EBV and possible underlying genetic susceptibility for
289 autoimmunity (with EBV encoded protein interactions), as recently described by Harley et al
290 and others[1,23–25].

291

292 Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid
293 organ transplants treated with immunosuppressive therapy, often with a significant impact
294 on organ function and overall survival[26–30]. It is observed that reduced intensity allo-HSCT
295 for malignant haematological conditions using alemtuzumab have a relatively lower overall

296 risk of LPD compared to ATG based treatments, possibly mediated by more effective pan-B
297 & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell repertoire with delayed
298 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections
299 including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease
300 and systemic sclerosis is increasingly recognised, but the development of lymphoproliferative
301 disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32] concerningly reported
302 2 deaths (1 MS and 1 systemic sclerosis patient) from EBV related LPD in 56 ATG
303 conditioned AHSCT for autoimmune diseases. Additionally, EBV associated
304 haemophagocytosis in ATG-AHSCT for ADs have also been reported[34], with one resulting
305 in death of the patient[35].

306

307 Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher
308 than published reports with allo-HSCTs (4.5-7%)[36,37] and our own centre's unpublished T-
309 cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying
310 immunopathological state of MS itself[38]. This is further corroborated by the fact that similar
311 LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with ATG -
312 AHSCT in this centre. Another example from our centre's experience of severe aplastic
313 anaemia (n=40) treated with ATG/ciclosporin, only 52% (n=21/40) developed EBV-R
314 (unpublished data) and none had LPD or required any treatment, suggesting that the problem
315 may not be ATG specific.

316

317 Our study's observation of significant persistent neurological events (with no evidence of new
318 MS disease activity) associated with clonal gammopathy suggest a potentially new clinical
319 syndrome, described for the first time in ATG conditioned AHSCTs in MS and possibly
320 induced by clonal B cell dysregulation following EBV-R. It could be hypothesised that any
321 remaining EBV infected latent B cells, surviving despite high doses of cyclophosphamide
322 (given with mobilisation and conditioning in MS ASHCT)[8] and compounded by depletion of
323 CD8+ T cells by ATG, may serve as potential source for EBV escape while interacting
324 abnormally within the host immune micro-environment[39] and leading to rise in M-protein,
325 LPD and neuro-inflammatory insults in some of the MS patients post AHSCT. Reports of
326 lower incidence of EBV-R and LPD in another commonly used protocol for MS-AHSCT using
327 BEAM-ATG (Ricardo Saccardi, Carregi University Hospital, Florence; personal
328 communication) may reflect the greater myeloablative effect of BEAM chemotherapy which
329 could further deplete the residual B cell pool and thus lower potential for EBV proliferation. It
330 is plausible that dose of rATG is critical, given we have not seen similar reports from other
331 centres where less rATG doses were given for MS-AHSCT (range between 5.0-6.5 mg/Kg;
332 personal communication) but there seems to be some variability in prospective serial EBV
333 monitoring in these patients.

334

335 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is
336 widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is
337 significantly associated with probable LPD and neurological events in MS patients with high
338 sensitivity (85.5%) and specificity (82.5%) (p=0.004) **(Fig 3, ROC curve)**. Our ROC curve
339 estimates are potentially limited by the relatively small number of events analysed but this
340 has consistently been useful in our MS-AHSCT experience for predicting clinical events with
341 high EBV load. Our EBV PCR assay has been validated against the recently defined standard
342 WHO reference method (i.e. 10 copies/ml=10 IU/ml EBV DNA) [16] and thus this EBV
343 threshold for pre-emptive treatment with Rituximab, can potentially be applied in relevant
344 clinical context in other centres using similar validated essays. Rituximab treatment delivered
345 good overall response in our symptomatic patients, with resolution of EBV related clinical
346 symptoms and no subsequent viral or bacterial infections at last follow up. The role of
347 prophylactic or pre-transplant rituximab in MS-AHSCT is also a potential area of interest in
348 reducing risk of EBV-LPD in stem cell transplants as observed by Burns et al[36] and future
349 randomised studies are required to investigate its potential benefit.

350

351 Our study limitations include its retrospective nature and that no suspected LPD patients had
352 histological confirmation, mainly related to patient refusal or technical difficulties. Seven MS
353 patients were lost to follow up for EBV monitoring following discharge, which limits the

354 findings of this study. Additionally, our numbers were too small to identify any association of
355 EBV related clinical events with previous DMT exposure in MS patients.

356

357 In conclusion, symptomatic EBV reactivation increases risk of neurological sequelae and
358 LPD in MS-AHSCT. Regular monitoring for rising EBV viraemia, as recommended by
359 Snowden et al [10] and Muraro et al [11], and serum electrophoresis for M-protein should be
360 considered in the first 3 months post-AHSCT for MS. We recommend persistent high EBV
361 viraemia > 500k DNA copies/ml as potential trigger for consideration of pre-emptive anti-
362 CD20 therapy and potentially reduce associated morbidity.

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369 **Acknowledgements:** To our patients and their families and carers in supporting this study.

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375 REFERENCES

376

- 377 1. Pender MP, Burrows SR. Epstein–Barr virus and multiple sclerosis: potential opportunities for
378 immunotherapy. *Clinical & Translational Immunology* **2014**; 3:e27. Available at:
379 <http://doi.wiley.com/10.1038/cti.2014.25>.
- 380 2. Reich DS, Lucchinetti CF, Calabresi PA. Multiple Sclerosis. *New England Journal of Medicine* **2018**;
381 378:169–180. Available at: <http://www.nejm.org/doi/10.1056/NEJMra1401483>.
- 382 3. Compston A, Coles A. Multiple sclerosis. *The Lancet* **2008**; 372:1502–1517. Available at:
383 <http://linkinghub.elsevier.com/retrieve/pii/S0140673608616207>.
- 384 4. Lublin FD, Reingold SC, Cohen JA, et al. Defining the clinical course of multiple sclerosis: The 2013
385 revisions. *Neurology* **2014**; 83:278–286. Available at:
386 <http://www.neurology.org/cgi/doi/10.1212/WNL.0000000000000560>.
- 387 5. Comi G, Radaelli M, Soelberg Sørensen P. Evolving concepts in the treatment of relapsing multiple
388 sclerosis. *The Lancet* **2017**; 389:1347–1356. Available at:
389 <https://linkinghub.elsevier.com/retrieve/pii/S0140673616323881>.
- 390 6. Muraro PA, Douek DC, Packer A, et al. Thymic output generates a new and diverse TCR repertoire
391 after autologous stem cell transplantation in multiple sclerosis patients. *The Journal of Experimental*
392 *Medicine* **2005**; 201:805–816. Available at: <http://www.jem.org/lookup/doi/10.1084/jem.20041679>.
- 393 7. Muraro PA, Robins H, Malhotra S, et al. T cell repertoire following autologous stem cell transplantation
394 for multiple sclerosis. *J Clin Invest* **2014**; 124:1168–1172. Available at:
395 <http://www.ncbi.nlm.nih.gov/pubmed/24531550>.
- 396 8. Cull G, Hall D, Fabis-Pedrini M, et al. Lymphocyte reconstitution following autologous stem cell
397 transplantation for progressive MS. *Multiple Sclerosis Journal – Experimental, Translational and*
398 *Clinical* **2017**; 3:205521731770016. Available at: <https://doi.org/10.1177/2055217317700167>.
- 399 9. Sormani MP, Muraro PA, Schiavetti I, et al. Autologous hematopoietic stem cell transplantation in
400 multiple sclerosis. *Neurology* **2017**; 88:2115–2122. Available at:
401 <http://www.neurology.org/lookup/doi/10.1212/WNL.0000000000003987>.
- 402 10. Snowden JA, Saccardi R, Allez M, et al. Haematopoietic SCT in severe autoimmune diseases:
403 updated guidelines of the European Group for Blood and Marrow Transplantation. *Bone Marrow*
404 *Transplantation* **2012**; 47:770–790. Available at: <http://www.nature.com/articles/bmt2011185>.
- 405 11. Muraro PA, Pasquini M, Atkins HL, et al. Long-term Outcomes After Autologous Hematopoietic Stem
406 Cell Transplantation for Multiple Sclerosis. *JAMA Neurology* **2017**; 74:459. Available at:
407 <http://archneur.jamanetwork.com/article.aspx?doi=10.1001/jamaneurol.2016.5867>.
- 408 12. Burt RK, Balabanov R, Snowden JA, Sharrack B, Oliveira MC, Burman J. Non-myeloablative
409 hematopoietic stem cell transplantation (HSCT) is superior to disease modifying drug (DMD) treatment
410 in highly active Relapsing Remitting Multiple Sclerosis (RRMS): interim results of the Multiple Sclerosis

- International Stem cell Transp. Neurology **2018**; 90. Available at:
http://n.neurology.org/content/90/15_Supplement/S36.004.abstract.
13. Daikeler T, Tichelli A, Passweg J. Complications of autologous hematopoietic stem cell transplantation for patients with autoimmune diseases. *Pediatric Research* **2012**; 71:439–444. Available at:
<http://www.nature.com/doi/10.1038/pr.2011.57>.
 14. Snowden JA, Badoglio M, Labopin M, et al. Evolution, trends, outcomes, and economics of hematopoietic stem cell transplantation in severe autoimmune diseases. *Blood Advances* **2017**; 1:2742–2755. Available at:
<http://www.bloodadvances.org/lookup/doi/10.1182/bloodadvances.2017010041>.
 15. Patel S, Zuckerman M, Smith M. Real-time quantitative PCR of Epstein–Barr virus BZLF1 DNA using the LightCycler. *Journal of Virological Methods* **2003**; 109:227–233. Available at:
<http://linkinghub.elsevier.com/retrieve/pii/S0166093403000764>.
 16. Semenova T, Lupo J, Alain S, et al. Multicenter Evaluation of Whole-Blood Epstein-Barr Viral Load Standardization Using the WHO International Standard. *Journal of Clinical Microbiology* **2016**; 54:1746 LP-1750. Available at: <http://jcm.asm.org/content/54/7/1746.abstract>.
 17. Styczynski J, van der Velden W, Fox CP, et al. Management of Epstein-Barr Virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: Sixth European Conference on Infections in Leukemia (ECIL-6) guidelines. *Haematologica* **2016**; 101:803–811. Available at:
<http://www.haematologica.org/cgi/doi/10.3324/haematol.2016.144428>.
 18. Dejaco C, Duftner C, Grubeck-Loebenstien B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* **2006**; 117:289–300. Available at:
<http://doi.wiley.com/10.1111/j.1365-2567.2005.02317.x>.
 19. Arellano G, Acuña E, Reyes LI, et al. Th1 and Th17 Cells and Associated Cytokines Discriminate among Clinically Isolated Syndrome and Multiple Sclerosis Phenotypes. *Frontiers in immunology* **2017**; 8:753. Available at: <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00753/full>.
 20. Jha S, Srivastava SY, Brickey WJ, et al. The Inflammasome Sensor, NLRP3, Regulates CNS Inflammation and Demyelination via Caspase-1 and Interleukin-18. *Journal of Neuroscience* **2010**; 30:15811–15820. Available at: <http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.4088-10.2010>.
 21. Beynon V, Quintana FJ, Weiner HL. Activated Human CD4+CD45RO+ Memory T-Cells Indirectly Inhibit NLRP3 Inflammasome Activation through Downregulation of P2X7R Signalling. *PLoS ONE* **2012**; 7:e39576. Available at: <https://dx.plos.org/10.1371/journal.pone.0039576>.
 22. Fernández-Menéndez S, Fernández-Morán M, Fernández-Vega I, Pérez-Álvarez A, Villafani-Echazú J. Epstein–Barr virus and multiple sclerosis. From evidence to therapeutic strategies. *Journal of Neurological Sciences* **2016**; 361:213–219. Available at:
https://www.clinicalkey.com.ezsecureaccess.balamand.edu.lb/service/content/pdf/watermarked/1-s2.0-S0022510X16300132.pdf?locale=en_US.

23. Pender MP. The Essential Role of Epstein-Barr Virus in the Pathogenesis of Multiple Sclerosis. *The Neuroscientist* **2011**; 17:351–367. Available at:
<http://journals.sagepub.com/doi/10.1177/1073858410381531>.
24. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Annals of Neurology* **2007**; 61:288–299. Available at: <http://doi.wiley.com/10.1002/ana.21117>.
25. Harley JB, Chen X, Pujato M, et al. Transcription factors operate across disease loci, with EBNA2 implicated in autoimmunity. *Nature Genetics* **2018**; 50:699–707. Available at:
<http://www.nature.com/articles/s41588-018-0102-3>.
26. Loren AW, Porter DL, Stadtmauer EA, Tsai DE. Post-transplant lymphoproliferative disorder: a review. *Bone Marrow Transplantation* **2003**; 31:145–155. Available at:
<http://www.nature.com/articles/1703806>.
27. Dotti G, Fiocchi R, Motta T, et al. Lymphomas occurring late after solid-organ transplantation: influence of treatment on the clinical outcome. *Transplantation* **2002**; 74:1095–102. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/12438953>.
28. Nagle SJ, Reshef R, Tsai DE. Posttransplant Lymphoproliferative Disorder in Solid Organ and Hematopoietic Stem Cell Transplantation. *Clinics in Chest Medicine* **2017**; 38:771–783. Available at:
<https://linkinghub.elsevier.com/retrieve/pii/S0272523117300874>.
29. Meijer E, Dekker AW, Weersink AJL, Rozenberg-Arska M, Verdonck LF. Prevention and treatment of epstein–barr virus-associated lymphoproliferative disorders in recipients of bone marrow and solid organ transplants. *British Journal of Haematology* **2002**; 119:596–607. Available at:
<http://dx.doi.org/10.1046/j.1365-2141.2002.03887.x>.
30. Dierickx D, Habermann TM. Post-Transplantation Lymphoproliferative Disorders in Adults. *New England Journal of Medicine* **2018**; 378:549–562. Available at:
<http://dx.doi.org/10.1056/NEJMr1702693>.
31. Meij P. Impaired recovery of Epstein-Barr virus (EBV)--specific CD8+ T lymphocytes after partially T-depleted allogeneic stem cell transplantation may identify patients at very high risk for progressive EBV reactivation and lymphoproliferative disease. *Blood* **2003**; 101:4290–4297. Available at:
<http://www.bloodjournal.org/cgi/doi/10.1182/blood-2002-10-3001>.
32. Nash R a, Dansey R, Storek J, et al. Epstein-Barr virus-associated posttransplantation lymphoproliferative disorder after high-dose immunosuppressive therapy and autologous CD34-selected hematopoietic stem cell transplantation for severe autoimmune diseases. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* **2003**; 9:583–91. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14506660>.
33. Daikeler T, Tzankov A, Hoenger G, et al. Minimal T-cell requirements for triggering haemophagocytosis associated with Epstein–Barr virus-driven B-cell proliferation: a clinical case study. *Annals of the Rheumatic Diseases* **2011**; 70:1338 LP-1339. Available at:
<http://ard.bmj.com/content/70/7/1338.abstract>.

- 485 34. Daikeler T, Tzankov A, Hoenger G, et al. Minimal T-cell requirements for triggering
486 haemophagocytosis associated with Epstein-Barr virus-driven B-cell proliferation: a clinical case study.
487 *Annals of the Rheumatic Diseases* **2011**; 70:1338–1339. Available at:
488 <http://ard.bmj.com/cgi/doi/10.1136/ard.2010.139246>.
- 489 35. Brinkman DMC, de Kleer IM, ten Cate R, et al. Autologous stem cell transplantation in children with
490 severe progressive systemic or polyarticular juvenile idiopathic arthritis: Long-term followup of a
491 prospective clinical trial. *Arthritis & Rheumatism* **2007**; 56:2410–2421. Available at:
492 <http://doi.wiley.com/10.1002/art.22656>.
- 493 36. Burns DM, Rana S, Martin E, et al. Greatly reduced risk of EBV reactivation in rituximab-experienced
494 recipients of alemtuzumab-conditioned allogeneic HSCT. *Bone Marrow Transplantation* **2016**; 51:825–
495 832. Available at: <http://www.nature.com/articles/bmt201619>.
- 496 37. van Esser JWJ. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell
497 transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-
498 depleted SCT. *Blood* **2001**; 98:972–978. Available at:
499 <http://www.bloodjournal.org/cgi/doi/10.1182/blood.V98.4.972>.
- 500 38. Tørring C, Andreassen C, Gehr N, Bjerg L, Petersen T, Höllsberg P. Higher incidence of Epstein-Barr
501 virus-induced lymphocyte transformation in multiple sclerosis. *Acta Neurologica Scandinavica* **2014**;
502 130:90–96. Available at: <http://doi.wiley.com/10.1111/ane.12249>.
- 503 39. Martinez OM, Krams SM. The Immune Response to Epstein Barr Virus and Implications for
504 Posttransplant Lymphoproliferative Disorder. *Transplantation* **2017**; 101:2009–2016. Available at:
505 <http://insights.ovid.com/crossref?an=00007890-201709000-00018>.

526 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**
527 **EBV DNA-aemia burden.**
528

Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender		M-Protein (n)	11	0	7
Male	19 (52.8%)				
Female	17 (47.2%)				
Disease Type (n; %)		Median EBV DNA log value at peak (IQR)			
Relapsing Remitting MS	22 (61.1%)		4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Secondary Progressive MS	10 (27.8%)				
Primary Progressive MS	4 (11.1%)				
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n)		Symptomatic EBV (n)			
Natalizumab	22		1	0	7
Alemtuzumab	8				
Both	6				
Median EDSS (range)	6.0 (2.5 – 8.0)	LPD diagnosis (CT/Biopsy) (n)	0	0	3 by CT alone
Median follow up post AHSCT in days (range)	436 (188 – 785)	Neuro/autoimmune complications (n)	0	0	3
No. of patients with prior EBV exposure (n; %)	36 (100%)	Treated with Rituximab (n)	0	0	6
No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
No. of patients lost to long term follow up (n; %)	7 (19.5%)	Detectable EBV DNA at last follow up (n)	9	1	3
Median Time to EBV detection post AHSCT in days (IQR)	30 (23-46)	Median time for EBV resolution (IQR in days)	67 days (44-155)	47 days (N/A)	63 days (45 - 170)
Median Time to peak EBV DNA levels in days (IQR)	32 (31-53)	Median Time to peak EBV DNA levels in days (IQR)	40 days (25-85)	30 days (N/A)	39 days (32-43)

529

530 **Abbreviations:**

531 AHSCT- Autologous Haematopoietic Stem cell transplant; CT- computed tomography; DMT-
532 Disease modifying therapy; EBV- Epstein Barr virus; EDSS- Kurtzke Expanded Disability
533 Status Scale; IQR- Interquartile range; LPD- Lymphoproliferative disease; M-Protein:
534 Monoclonal paraprotein or gammopathy; MS- Multiple Sclerosis

Figure Legends

Figure 1: Trend of Lymphocyte count recovery following ATG in MS patients.

Legend: This figure shows trends of lymphocyte count from baseline to recovery post AHSCT for MS patients. Majority of patients had normal baseline lymphocyte counts pre-HSCT and became lymphopenic post ATG with counts recovering towards d+28 and >85% of patients recovered counts by d+56, with some overshooting from their baseline, possibly reflective of EBV related lymphoproliferation in some of these patients.

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

Figure 2: Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT.

Legend: This figure demonstrates trends of EBV copies (log), paraprotein levels (g/l) and Lymphocyte levels (counts $\times 10^6/\text{ml}$) in two MS patients with significant neurological symptoms following EBV reactivation. Both patients had significant EBV viraemia (log>5.2 or >500,000 copy number) and developed significant paraproteinaemia, which was only noted after persistent unexplained neurological symptoms. The trend reversed following anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.

D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

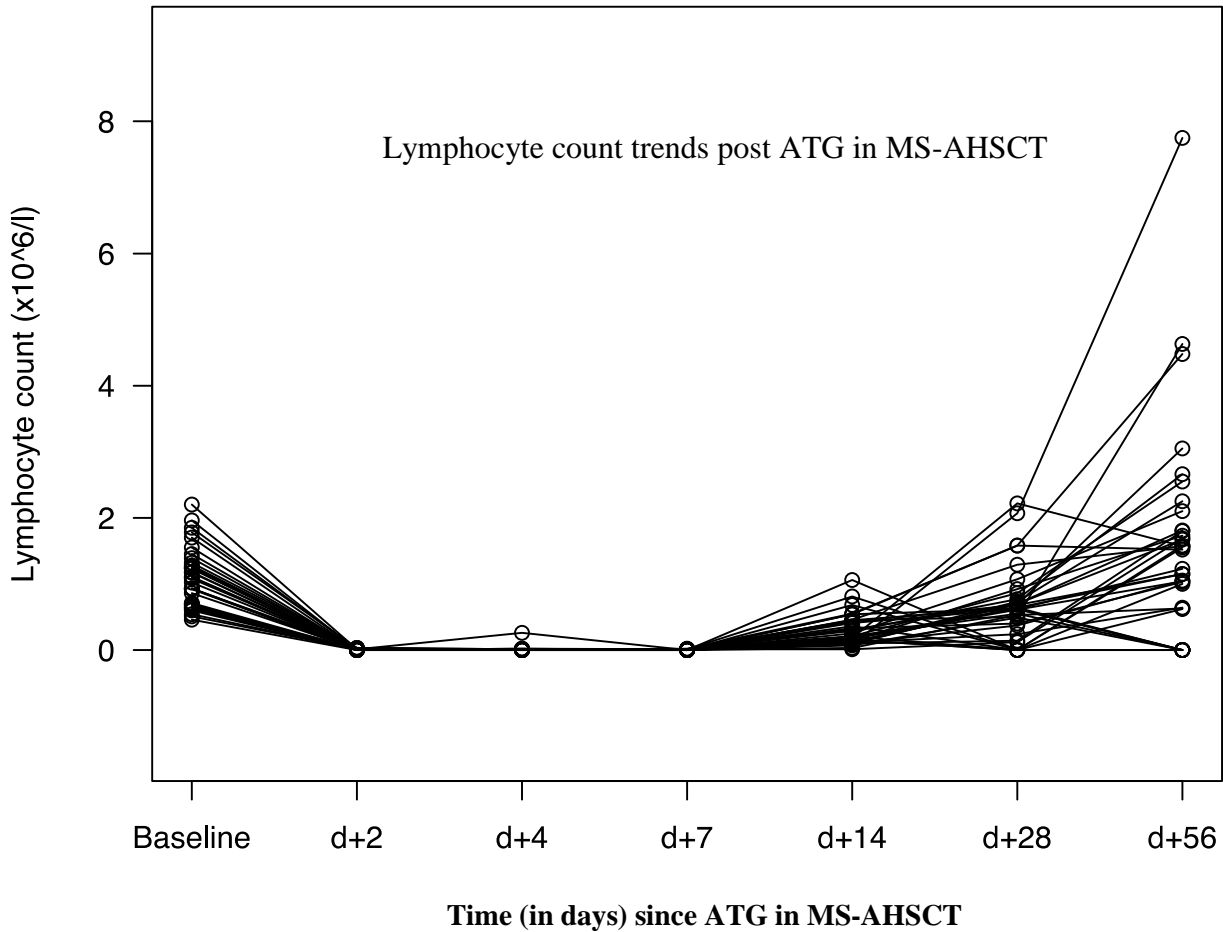
Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.

Legend: ROC curve demonstrating significant correlation between high EBV levels and clinical events (LPD & neurological events) in MS-AHSCT patients, with highest sensitivity and specificity noted with peak EBV viraemia of >500,000 copies/ml (p=0.0004).

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

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Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.

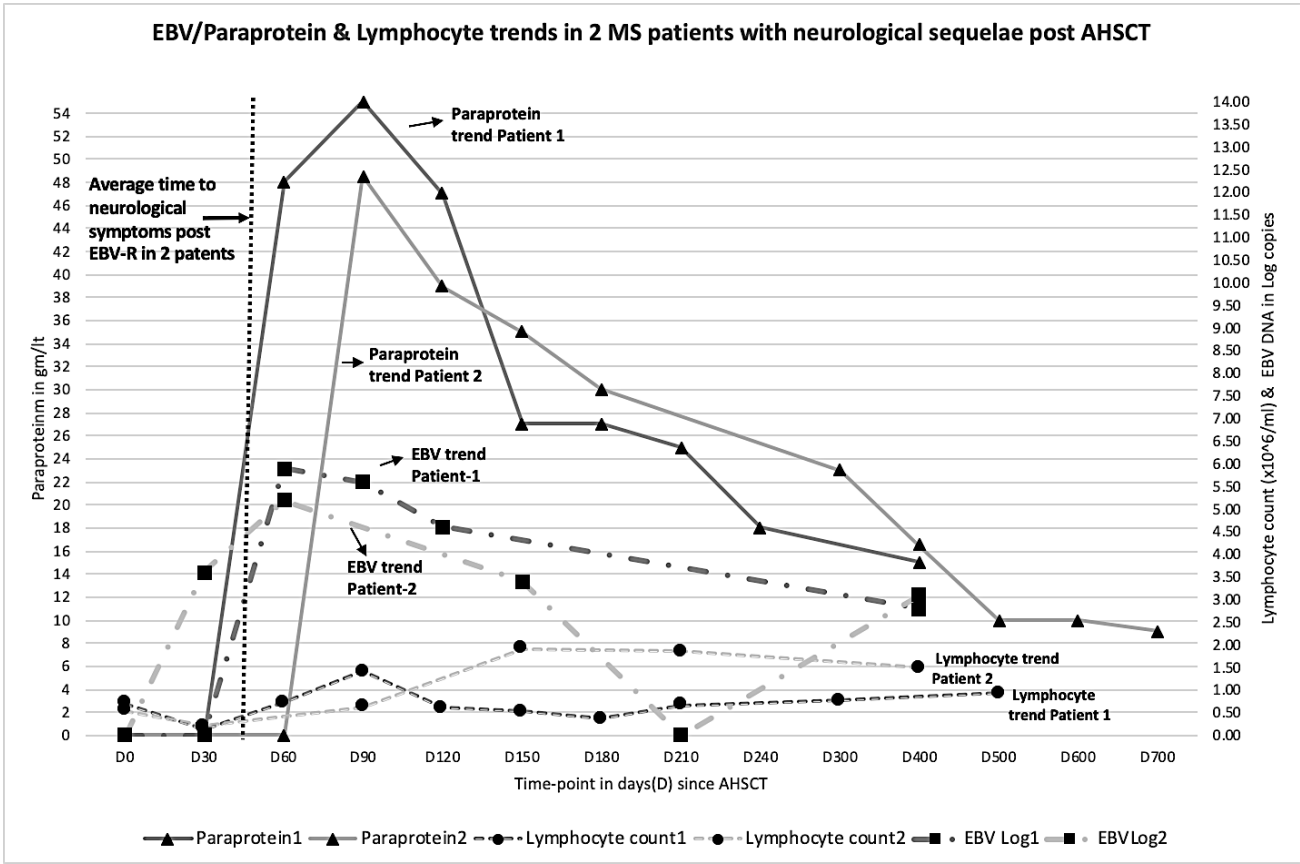


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Abbreviations:

AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

599 **Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant**
600 **neurological sequelae post AHSCT**
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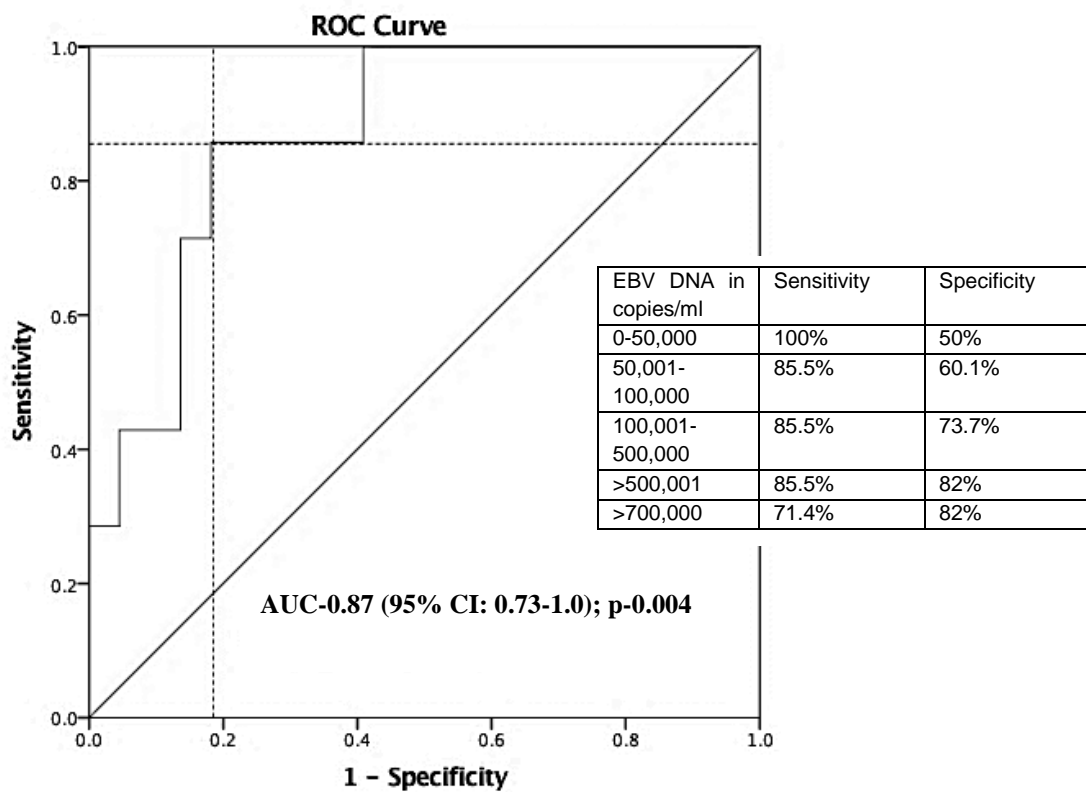
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609 **Abbreviations:**

610 D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis;
611 AHSCT: Autologous Haematopoietic Stem Cell Transplants.

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Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.



Abbreviations:

EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis patients treated with autologous haematopoietic stem cell transplants; ROC: receiver operating characteristics

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