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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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Abstract:	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. 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. 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. 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.
Response to Reviewers:	To Dr Barbara D Alexander M.D. Associate Editor Clinical Infectious Diseases Dated: 30th Dec 2018 Dear Dr Alexander Subject: Response to Reviewers Manuscript Title: EBV and Monoclonal Gammopathy of Clinical Significance in Autologous Stem Cell Transplantation for Multiple Sclerosis. 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: 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. 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. Line 212-215. Please include the conversion factor for your assay to IU/mI in the methods section i.e 10 EBV DNA copies/mI=10 IU/mI

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/cyclopsorin, 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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Click here to access/download;Cover Letter;EBV MS CID Editor revised Cover Letter.docx

> King's College Hospital NHS Foundation Trust



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: Dr Varun Mehra, MRCP(UK), FRCPath Department of Haematological Medicine Kings College Hospital, London, UK Varun.Mehra@nhs.net ; Ph-004478865087013

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- 2 Autologous Stem Cell Transplantation for Multiple Sclerosis.
- 3 Varun Mehra*, Elijah Rhone*, Stefani Widya, Mark Zuckerman, Victoria Potter, Kavita Raj,
- 4 Austin Kulasekararaj, Donal McLornan, Hugues de Lavallade, Nana Benson-Quarm, Christina
- 5 Lim, Sarah Ware, Malur Sudhanva, Omar Malik, Richard Nicholas, Paolo A Muraro, Judith
- 6 Marsh, Ghulam J Mufti, Eli Silber, Antonio Pagliuca and Majid A. Kazmi
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62	Running Title: EBV complications in Auto-HSCT for MS
63	
64 65 66 67	Summary: 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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81 Abstract

82 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.

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-
- Barr Virus Infection; Monoclonal Gammopathy; Post-transplant LymphoproliferativeDisorder
- 113
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120 **INTRODUCTION:**

121 Multiple sclerosis (MS) is a chronic autoimmune, inflammatory, demyelinating disease of the central nervous system[1]-[2], with a relapsing-remitting (RRMS) presentation in the 122 majority of patients at diagnosis. Recovery from relapses may be complete or partial[3] [4]. 123 124 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 inflammatory activity on MRI, younger age, a shorter disease duration, low to moderate 135 136 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 MIST study[12] found AHSCT to be superior to standard disease modifying therapy (DMT) 139

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

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142 of subsequent opportunistic infections following However, risk rise in such 143 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of 144 145 immunosuppressive rabbit anti-thymocyte globulin (rATG) to their conditioning regimen may confer a higher risk of viral reactivation in these patients. The number of AHSCTs 146 performed for MS is rising significantly in Europe[14] and as more centres perform AHSCT 147 148 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**

Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT between February 2012 and February 2017 at Kings College Hospital, London. Peripheral blood stem cells were collected following standard mobilisation strategy consisting of cyclophosphamide 4g/m² over 2 days and granulocyte colony-stimulating factor for 7 days. 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 infusion. conditioned by stem cell One patient was 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 x10^6/kg (range 4.0-17.1x10^6/kg).

165

166 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen; EBV DNA load monitoring was performed on whole blood samples by 167 VCA IqG). 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 guantification of 10 copies/ml considered equivalent to 10 IU/ml DNA reported with the WHO reference method[16]. EBV-R was 172 173 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.

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184 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).

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193 **RESULTS**

194 Baseline characteristics are presented in Table 1. Most MS patients (88.9%) had RRMS 195 phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients 196 had prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients 197 received both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pretreatment, indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. 198 199 Seven MS patients were lost to long-term follow up for EBV monitoring. The median time to 200 first EBV DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels 201 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 202 203 (range 14-404 days) to lymphocyte recovery (defined by total lymphocyte count 204 >1.0x10⁶/ml) following AHSCT (See Figure 1). A high proportion (86%; n-25/29) of the MS 205 patients in active follow-up recovered lymphocyte counts around D56 with a median 206 lymphocyte count of 1.56 (10⁶ cells/ml); Four patients remained lymphopenic at last follow
207 up.

208

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

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

- 9 -

226 whom developed IgG subtype and the remaining 2 developed IgA and IgM M-protein. Concerningly two of these patients developed clinically significant M-Protein burden; one 227 patient with IgG Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological 228 229 symptoms mimicking MS relapse, requiring plasma exchange. Another patient developed significant lumbosacral radiculopathy with rising EBV-R and high IgM lambda M-protein 230 (IgM 48.5g/L) (see supplementary case vignettes). Figure 2 highlights the association of 231 232 neurological symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts (x 10⁶/ml) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient 233 234 developed painful lower limb paraesthesia following rising EBV viraemia >500k 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.

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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 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis 240 (Figure 3) confirmed EBV viraemia of >500k copies/ml correlated with high sensitivity 241 242 (85.5%) and specificity (82.5%) (AUC-0.87; 95%CI-0.73-1.0; p-0.004) in predicting significant EBV related clinical events (evidence of LPD and/or neurological symptoms) that 243 244 may require treatment with rituximab. The sensitivity dropped significantly on lower 245 estimates for events below 500k copies/ml.

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

252

253 **DISCUSSION:**

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

261

Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid organ transplants treated with immunosuppressive therapy, often with a significant impact on organ function and overall survival[26–30]. It is observed that reduced intensity allo-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 effective pan-B & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell 267 repertoire with delayed EBV specific CD8+ T cell recovery[31]. Clinically significant 268 endogenous viral infections including EBV following ATG conditioned AHSCT for severe 269 270 ADs such as Crohn's disease and systemic sclerosis is increasingly recognised, but the development of lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33]. 271 272 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 T-cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying 279 immunopathological state of MS itself[38]. This is further corroborated by the fact that 280 similar LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with 281 282 ATG -AHSCT in this centre. Another example from our centre's experience of severe aplastic anaemia (n-40) treated with ATG/ciclosporin, only 52% (n-21/40) developed EBV-R 283 284 (unpublished data) and none had LPD or required any treatment, suggesting that the 285 problem may not be ATG specific.

286

Our study's observation of significant persistent neurological events (with no evidence of 287 new MS disease activity) associated with clonal gammopathy suggest a potentially new 288 289 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 that any remaining EBV infected latent B cells, surviving despite high doses of 291 292 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 for EBV proliferation. It is plausible that dose of rATG is critical, given we have not seen 300 301 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 widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is 306 significantly associated with probable LPD and neurological events in MS patients with high 307 sensitivity (85.5%) and specificity (82.5%) (p-0.004) (Fig 3, ROC curve). Our ROC curve 308 estimates are potentially limited by the relatively small number of events analysed but this 309 310 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 future randomised studies are required to investigate its potential benefit. 319

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.

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.
333	

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341 **Conflict of interest:** The authors declare no competing financial interests as below:

- 342 1. Varun Mehra- no competing financial interests 343 2. Elijah Rhone- no competing financial interests 344 3. Stefani Widya- no competing financial interests 345 4. Mark Zuckerman- no competing financial interests 346 5. Victoria Potter- no competing financial interests 347 6. Kavita Raj- no competing financial interests 348 7. Austin Kulasekararaj- no competing financial interests 349 8. Donal McLornan- no competing financial interests 350 9. Hugues de Lavallade- no competing financial interests 351 10. Nana Benson-Quarm- no competing financial interests 352 11. Christina Lim- no competing financial interests 353 12. Sarah Ware- no competing financial interests 354 13. Malur Sudhanva- no competing financial interests 355 14. Omar Malik- no competing financial interests 356 15. Richard Nicholas- no competing financial interests 357 16. Paolo A Muraro- no competing financial interests 358 17. Judith Marsh- no competing financial interests 359 18. Ghulam J Mufti- no competing financial interests 360 19. Eli Silber- no competing financial interests 361 20. Antonio Pagliuca- no competing financial interests 362 21. Majid A. Kazmi- no competing financial interests 363 364 365 366
- 367
- 368

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520 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**

521 EBV DNA-aemia burden.

522

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 Male Female	19 (52.8%) 17 (47.2%)	M-Protein (n)	11	0	7
Disease Type (n; %) Relapsing Remitting MS Secondary Progressive MS Primary Progressive MS	22 (61.1%) 10 (27.8%) 4 (11.1%)	Median EBV DNA log value at peak (IQR)	4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n) Natalizumab Alemtuzumab Both	22 8 6	Symptomatic EBV (n)	1	0	7
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

529 Figure Legends

530

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

532

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.

538

539 AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte 540 Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

541 542

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

546 Legend: This figure demonstrates trends of EBV copies (log), paraprotein levels (g/lt) and 547 Lymphocyte levels (counts x10^6/ml) in two MS patients with significant neurological 548 symptoms following EBV reactivation. Both patients had significant EBV viraemia (log>5.2 549 or >500,000 copy number) and developed significant paraproteinaemia, which was only 550 noted after persistent unexplained neurological symptoms. The trend reversed following 551 anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.

552

553 D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; 554 AHSCT: Autologous Haematopoietic Stem Cell Transplants.

555 556

557 Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical 558 events in MS post AHSCT.

559

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).

563

564 EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis 565 patients treated with autologous haematopoietic stem cell transplants; ROC: receiver 566 operating characteristics

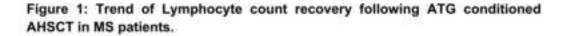
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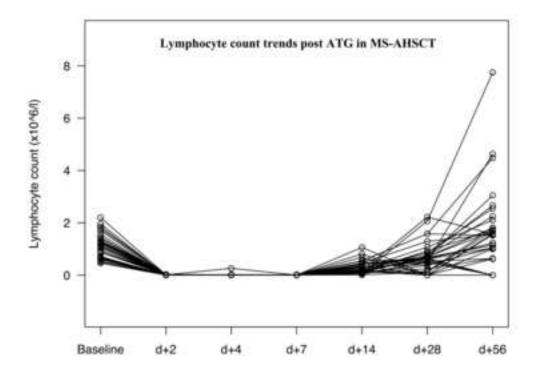
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Median follow up post AHSCT in days (range)	436 (188 – 785)	Neuro/autoimmune complications (n)	0	0	3
No. of patients with prior EBV exposure (n; %)	36 (100%)	Treated with Rituximab (n)	0	0	6
No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
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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)

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

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





Abbreviations:

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

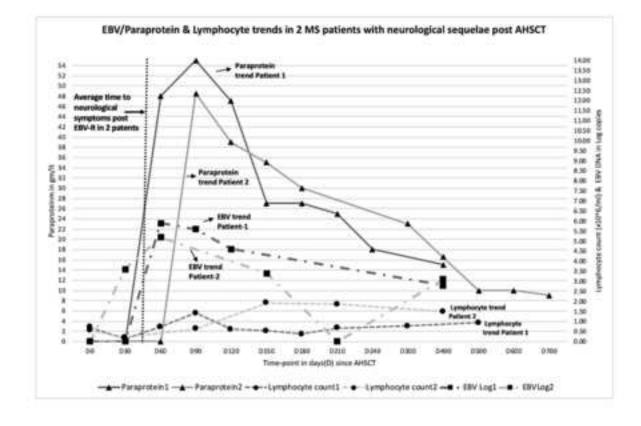


Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT

Abbreviations:

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

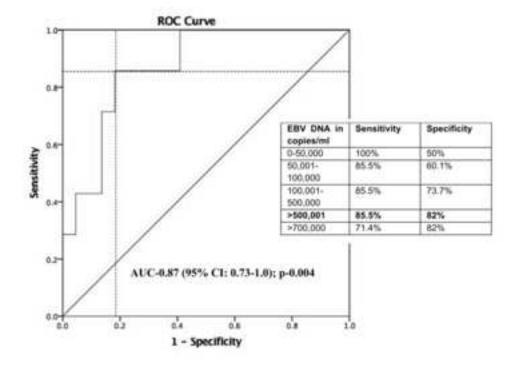


Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical events in MS post AHSCT.

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 Haematopoeitic Stem cell transplant (AHSCT) procedure and was discharged on day 15 posttransplant. 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 posttransplant 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

- 2 Stem Cell Transplantation for Multiple Sclerosis.
- 3 Running Title: EBV complications in Auto-HSCT for MS

4	Varun Mehra*, Elijah Rhone*, Stefani Widya, Mark Zuckerman, Victoria Potter, Kavita Raj,
5	Austin Kulasekararaj, Donal McLornan, Hugues de Lavallade, Nana Benson-Quarm, Christina
6	Lim, Sarah Ware, Malur Sudhanva, Omar Malik, Richard Nicholas, Paolo A Muraro, Judith
7	Marsh, Ghulam J Mufti, Eli Silber, Antonio Pagliuca and Majid A. Kazmi
8	
9	*These authors contributed equally to this work as 1 st Authors.
10	
11	Key Points:
12	EBV reactivation is common post-transplant with ATG for multiple sclerosis (MS),
13	with significant lymphoproliferative & neurological sequelae associated with rising
14	M-protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as
15	is pre-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.
16	
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111 Abstract

112 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.

120 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.

126 **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

133	correlated	with	high	sensitivity	(85.5%)	&	specificity	(82.5%)	(AUC-0.87;	p-0.004)	in
134	predicting	EBV-I	R rela	ted significa	ant clinica	lev	vents.				

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 superior to standard disease modifying therapy (DMT) for RRMS with respect to both 170

171 treatment failure and disability progression.

172

173 of subsequent opportunistic infections following However, risk rise in such 174 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of 175 176 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**

Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT between February 2012 and February 2017 at Kings College Hospital, London. Peripheral blood stem cells were collected following standard mobilisation strategy consisting of cyclophosphamide 4g/m² over 2 days and granulocyte colony-stimulating factor for 7 days. 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, followed infusion. conditioned 192 by stem cell One patient was 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 x10^6/kg (range 4.0-17.1x10^6/kg).

196

197 Prior exposure to EBV was assessed by serological evidence (EBV viral capsid antigen; VCA IgG). EBV DNA load monitoring was performed on whole blood samples by standardised 198 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-Gene[™] (Qiagen) 199 200 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 new & persistent organ dysfunction (e.g. neurological events) temporally associated with 209 210 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 subclasses identified by immunofixation electrophoresis. Patient outcomes were assessed at 212 213 last follow up as of April 2017.

214

215 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).

223

224 **RESULTS**

Baseline characteristics are presented in Table 1. Most MS patients (88.9%) had RRMS 225 phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had 226 prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received 227 both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment, 228 indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS 229 patients were lost to long-term follow up for EBV monitoring. The median time to first EBV 230 DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a 231 median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline 232 lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days) 233 234 to lymphocyte recovery (defined by total lymphocyte count >1.0x10⁶/ml) following AHSCT 235 (See Figure 1). A high proportion (86%; n-25/29) of the MS patients in active follow-up

- 9 -

recovered lymphocyte counts around D56 with a median lymphocyte count of 1.56 (10⁶
 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

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

- 10 -

256 developed IgG subtype and the remaining 2 developed IgA and IgM M-protein. Concerningly two of these patients developed clinically significant M-Protein burden; one patient with IgG 257 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking 258 259 MS relapse, requiring plasma exchange. Another patient developed significant lumbosacral radiculopathy with rising EBV-R and high IgM lambda M-protein (IgM 48.5g/L) (see 260 supplementary case vignettes). Figure 2 highlights the association of neurological 261 symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts (x 262 10⁶/ml) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient developed 263 264 painful lower limb paraesthesia following rising EBV viraemia >500k copies/ml, although did not have any M-protein detected. Their symptoms persisted at last follow up despite no 265 266 evidence of MS related new disease activity.

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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 4 reduction in EBV viral load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis 270 271 (Figure 3) confirmed EBV viraemia of >500k 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 EBV related clinical events (evidence of LPD and/or neurological symptoms) that may require 273 274 treatment with rituximab. The sensitivity dropped significantly on lower estimates for events 275 below 500k copies/ml.

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

282

283 **DISCUSSION:**

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

291

Generally, EBV-R related LPD has been reported in both allogeneic (allo-HSCT) and solid organ transplants treated with immunosuppressive therapy, often with a significant impact on organ function and overall survival[26–30]. It is observed that reduced intensity allo-HSCT 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 & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell repertoire with delayed 297 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections 298 including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease 299 300 and seven to seven is increasingly recognised, but the development of lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32] 301 302 concerningly 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 depleted allo-HSCT experience (6.5%); possibly a reflection of underlying cell immunopathological state of MS itself[38]. This is further corroborated by the fact that similar 310 811 LPD risk has not been observed in other ADds, e.g. Crohn's disease, treated with ATG -812 AHSCT in this centre. Another example from ouour centre's experience of in-severe aplastic 813 anaemiaa_(n-40), a type of AD causing severe bone marrow failure(n-40) treated -& treated 814 with ATG/ciclosporin,n; only 52% (n-21/40) -patients developed EBV-R (unpublished data) 815 and -nNone had LPD or required any treatment, supporting the notion that it may not just be ³¹⁶ a specific ATG related problem.suggesting that the problem may not be ATG specific.

317

318 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 319 syndrome, described for the first time in ATG conditioned AHSCTs in MS and, possibly 320 321 induced by clonal B cell dysregulation following EBV-R. It could be hypothesised that any 322 remaining EBV infected latent B cells, which may still have survivedsurviving despite high doses of cyclophosphamide (given with mobilisation and conditioning in MS ASHCT)[8] and 323 324 compounded by depletion of CD8+ T cells by ATG, may serve as potential source for EBV 325 escape while interacting abnormally within the host immune micro-environment[39] and 326 leading to rise in M-protein, LPD and neuro-inflammatory insults in some of the MS patients 327 post AHSCT. Reports of lower incidence of EBV-R and LPD in another commonly used 328 protocol for MS-AHSCT using BEAM-ATG (Ricardo Saccardi, Carregi University Hospital, Florence; personal communication) may reflect the greater myeloablative effect of BEAM 329 chemotherapy which could further deplete the residual B cell pool and thus lower potential 330 for EBV proliferation. It is plausible that dose of rATG is critical, given we have not seen 331 332 similar reports from other centres where less rATG doses were given for MS-AHSCT (range 333 between 5.0-6.5 mg/Kg; personal communication), but there seems to be some variability in prospective serial EBV monitoring in these patients. 334

335

336 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is 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 338 339 sensitivity (85.5%) and specificity (82.5%) (p-0.004) (Fig 3, ROC curve). Our ROC curve 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 341 342 high EBV load. Our EBV PCR assay has been validated against the recently defined standard 343 WHO reference method (i.e. 10 copiesy/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 essays. 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 randomised studies are required to investigate its potential benefit. 350

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 Www 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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527 **Table 1: Baseline patient characteristics and EBV related clinical events according to peak**

528 EBV DNA-aemia burden.

529

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 Male Female	19 (52.8%) 17 (47.2%)	M-Protein (n)	11	0	7
Disease Type (n; %) Relapsing Remitting MS Secondary Progressive MS Primary Progressive MS	22 (61.1%) 10 (27.8%) 4 (11.1%)	Median EBV DNA log value at peak (IQR)	4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n) Natalizumab Alemtuzumab Both	22 8 6	Symptomatic EBV (n)	1	0	7
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

536 Figure Legends

537

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

539

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.

- 545
 546 AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte
 547 Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.
- 548 549

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

- 553 Legend: This figure demonstrates trends of EBV copies (log), paraprotein levels (g/lt) and 554 Lymphocyte levels (counts x10^6/ml) in two MS patients with significant neurological 555 symptoms following EBV reactivation. Both patients had significant EBV viraemia (log>5.2 556 or >500,000 copy number) and developed significant paraproteinaemia, which was only 557 noted after persistent unexplained neurological symptoms. The trend reversed following 558 anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.
- 559
- D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis;
 AHSCT: Autologous Haematopoietic Stem Cell Transplants.
- 562 563

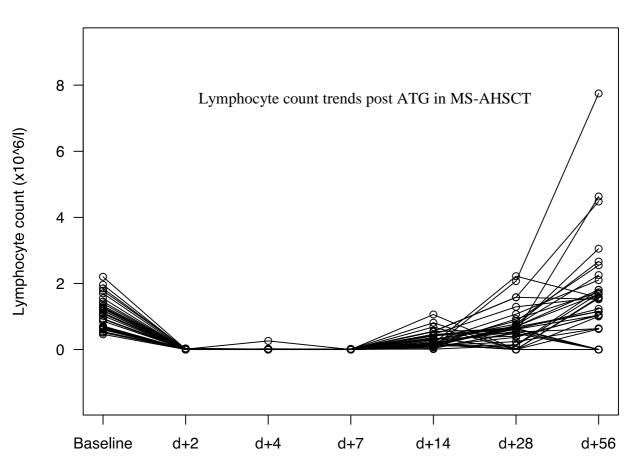
564 Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical 565 events in MS post AHSCT.

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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).

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- 571 EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis 572 patients treated with autologous haematopoietic stem cell transplants; ROC: receiver 573 operating characteristics
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582 583 Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in 584 MS patients.

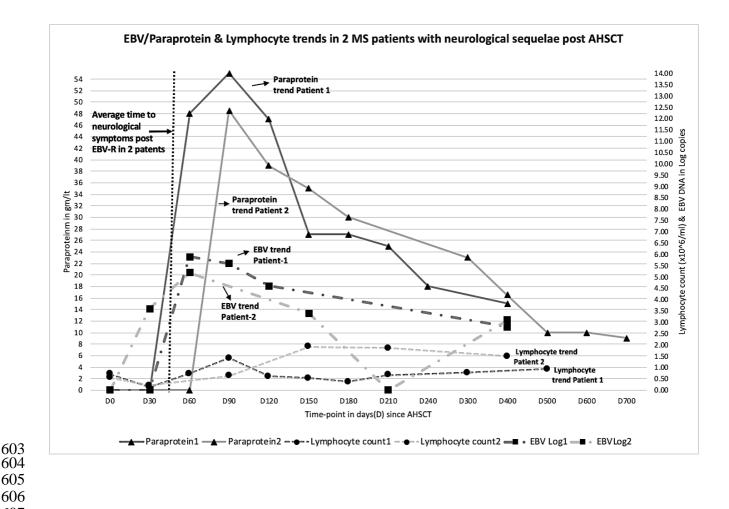


Time (in days) since ATG in MS-AHSCT

589 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

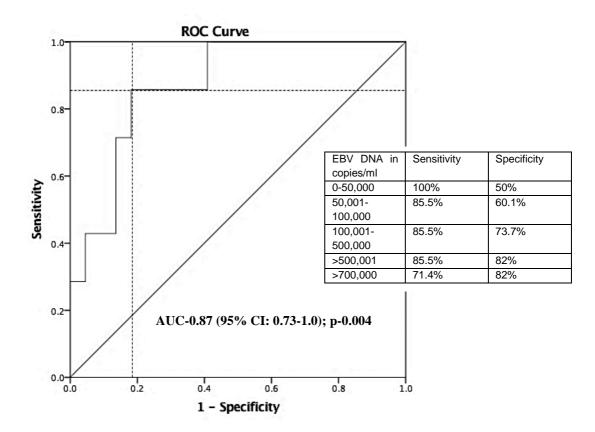


Abbreviations:

611	D: Days post AHS	SCT; EBV-R	: Epstein Barr	Virus reactivation	on; MS: Multiple s	clerosis;

- AHSCT: Autologous Haematopoietic Stem Cell Transplants.

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



628 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

- 2 Stem Cell Transplantation for Multiple Sclerosis.
- 3 Running Title: EBV complications in Auto-HSCT for MS

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5	Austin Kulasekararaj, Donal McLornan, Hugues de Lavallade, Nana Benson-Quarm, Christina
6	Lim, Sarah Ware, Malur Sudhanva, Omar Malik, Richard Nicholas, Paolo A Muraro, Judith
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8	
9	*These authors contributed equally to this work as 1 st Authors.
10	
11	Key Points:
12	EBV reactivation is common post-transplant with ATG for multiple sclerosis (MS),
13	with significant lymphoproliferative & neurological sequelae associated with rising
14	M-protein. Serial monitoring of EBV & M-protein is recommended post-transplant, as
15	is pre-emptive anti-CD20 therapy with EBV DNA >500,000 copies/ml.
16	
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22	
22	Key Words:
23	Multiple Sclerosis; Autologous Hematopoietic Stem Cell Transplantation, Epstein-
24	Barr Virus Infection; Monoclonal Gammopathy; Post-transplant Lymphoproliferative
25	Disorder
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79	1.	Varun Mehra- no competing financial interests
80	2.	Elijah Rhone- no competing financial interests
81	3.	Stefani Widya- no competing financial interests
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83	5.	Victoria Potter- no competing financial interests
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97	19.	Eli Silber- no competing financial interests
98	20.	Antonio Pagliuca- no competing financial interests
99	21.	Majid A. Kazmi- no competing financial interests
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111 Abstract

112 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.

120 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.

126 **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.

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 superior to standard disease modifying therapy (DMT) for RRMS with respect to both 170

171 treatment failure and disability progression.

172

173 of subsequent opportunistic infections following However, risk rise in such 174 immunosuppressive therapies remain a potential concern[13]. MS patients undergoing AHSCT have often been exposed to a number of immunomodulating DMTs; the addition of 175 176 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**

Data was collected retrospectively on 36 consecutive MS patients undergoing AHSCT between February 2012 and February 2017 at Kings College Hospital, London. Peripheral blood stem cells were collected following standard mobilisation strategy consisting of cyclophosphamide 4g/m² over 2 days and granulocyte colony-stimulating factor for 7 days. 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 infusion. conditioned by stem cell One patient was 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 x10^6/kg (range 4.0-17.1x10^6/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 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-Gene[™] (Qiagen) 199 200 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 IU/mI DNA reported with the WHO reference method[16]. EBV-R was defined as rising EBV 203 204 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 new & persistent organ dysfunction (e.g. neurological events) temporally associated with 209 210 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.

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215 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).

223

224 **RESULTS**

Baseline characteristics are presented in Table 1. Most MS patients (88.9%) had RRMS 225 phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had 226 prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received 227 both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment, 228 indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS 229 patients were lost to long-term follow up for EBV monitoring. The median time to first EBV 230 DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a 231 median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline 232 lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days) 233 234 to lymphocyte recovery (defined by total lymphocyte count >1.0x10⁶/ml) following AHSCT 235 (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 lymphocyte count of 1.56 (10⁶
 cells/ml); Four patients remained lymphopenic at last follow up.

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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

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

- 10 -

256 developed IgG subtype and the remaining 2 developed IgA and IgM M-protein. Concerningly two of these patients developed clinically significant M-Protein burden; one patient with IgG 257 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking 258 259 MS relapse, requiring plasma exchange. Another patient developed significant lumbosacral radiculopathy with rising EBV-R and high IgM lambda M-protein (IgM 48.5g/L) (see 260 supplementary case vignettes). Figure 2 highlights the association of neurological 261 symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts (x 262 10⁶/ml) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient developed 263 264 painful lower limb paraesthesia following rising EBV viraemia >500k copies/ml, although did not have any M-protein detected. Their symptoms persisted at last follow up despite no 265 266 evidence of MS related new disease activity.

267

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

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

282

283 **DISCUSSION:**

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

291

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

- 12 -

296 risk of LPD compared to ATG based treatments, possibly mediated by more effective pan-B & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell repertoire with delayed 297 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections 298 including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease 299 300 and systemic sclerosis is increasingly recognised, but the development of lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32] concerningly reported 301 302 2 deaths (1 MS and 1 systemic sclerosis patient) from EBV related LPD in 56 ATG 303 AHSCT autoimmune conditioned for 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 depleted allo-HSCT experience (6.5%); possibly a reflection of underlying cell immunopathological state of MS itself[38]. This is further corroborated by the fact that similar 310 311 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 anaemia (n-40) treated with ATG/ciclosporin, only 52% (n-21/40) developed EBV-R 313 314 (unpublished data) and none had LPD or required any treatment, suggesting that the problem 315 may not be ATG specific.

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.

335 The clinical threshold for EBV viral load as a significant risk factor for post-transplant LPD is widely debated. This study reports peak EBV viral load >500k copies/ml post AHSCT is 336 significantly associated with probable LPD and neurological events in MS patients with high 337 338 sensitivity (85.5%) and specificity (82.5%) (p-0.004) (Fig 3, ROC curve). Our ROC curve estimates are potentially limited by the relatively small number of events analysed but this 339 has consistently been useful in our MS-AHSCT experience for predicting clinical events with 340 high EBV load. Our EBV PCR assay has been validated against the recently defined standard 341 342 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 randomised studies are required to investigate its potential benefit. 349

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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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 Male Female	19 (52.8%) 17 (47.2%)	M-Protein (n)	11	0	7
Disease Type (n; %) Relapsing Remitting MS Secondary Progressive MS Primary Progressive MS	22 (61.1%) 10 (27.8%) 4 (11.1%)	Median EBV DNA log value at peak (IQR)	4.8 (3.5-4.8)	5.5 (N/A)	6.25 (6.1-6.9)
Median number of previous DMT (range)	2 (0 – 6)	Median number of prior DMTs	2	3	2
Previous use of high efficacy DMT (n) Natalizumab Alemtuzumab Both	22 8 6	Symptomatic EBV (n)	1	0	7
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

535 Figure Legends

536

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

538

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.

545 AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte 546 Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

547 548

544

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

- 552 Legend: This figure demonstrates trends of EBV copies (log), paraprotein levels (g/lt) and 553 Lymphocyte levels (counts x10^6/ml) in two MS patients with significant neurological 554 symptoms following EBV reactivation. Both patients had significant EBV viraemia (log>5.2 555 or >500,000 copy number) and developed significant paraproteinaemia, which was only 556 noted after persistent unexplained neurological symptoms. The trend reversed following 557 anti-CD20 (rituximab) therapy, with limited recovery in neurological symptoms.
- 558

561

559 D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; 560 AHSCT: Autologous Haematopoietic Stem Cell Transplants.

562 563 Figure 3: ROC curve estimates for peak EBV viraemia levels and significant clinical 564 events in MS post AHSCT.

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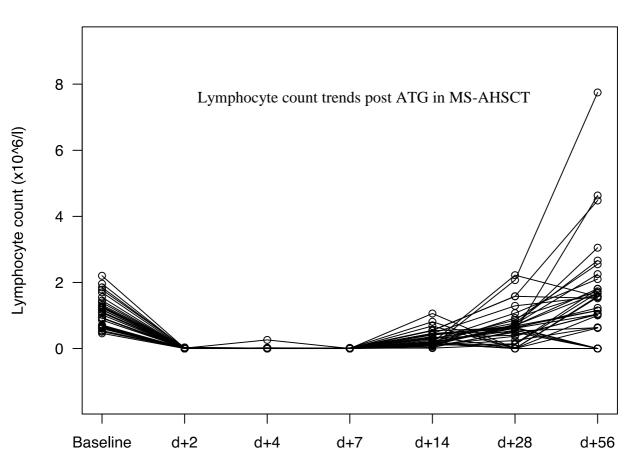
Legend: ROC curve demonstrating significant correlation between high EBV levels and clinical events (LPD & neurological events) in MS-AHSCT patients, with highest sensitivity and specificity noted with peak EBV viraemia of >500,000 copies/ml (p-0.0004).

569

570 EBV: Epstein Barr Virus; LPD- Lymphoproliferative disorder; MS-AHSCT: Multiple Sclerosis 571 patients treated with autologous haematopoietic stem cell transplants; ROC: receiver 572 operating characteristics

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581 582 Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in 583 MS patients.

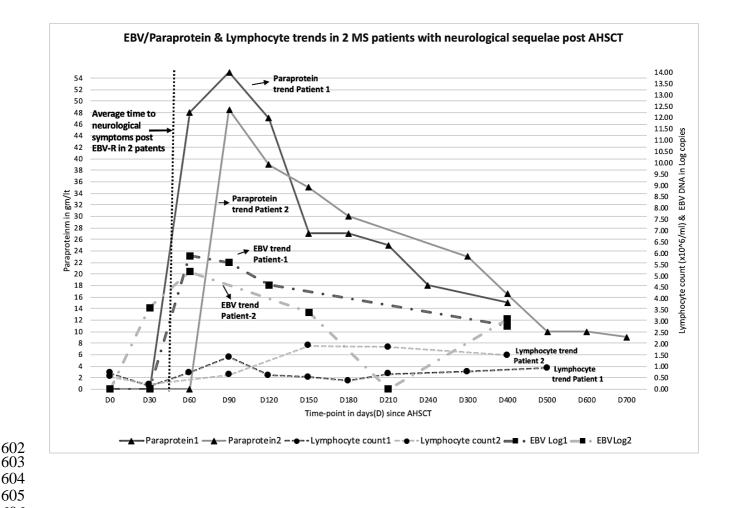


Time (in days) since ATG in MS-AHSCT

588 Abbreviations:

589 AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte 590 Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

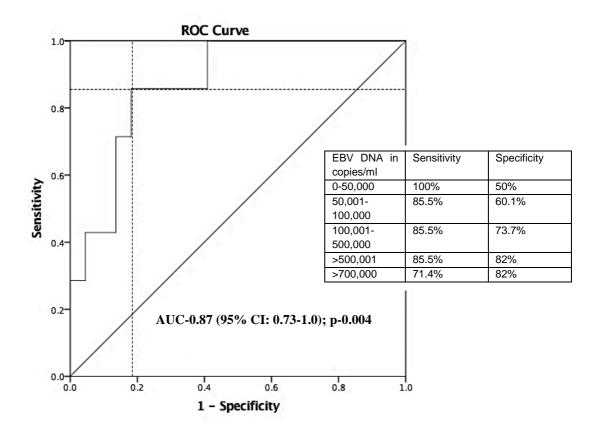
Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT



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

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