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

--Manuscript Draft--

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Corresponding Author:	Varun Mehra, MRCP (UK), FRCPath King's College Hospital London, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	King's College Hospital
Corresponding Author's Secondary Institution:	
First Author:	Varun Mehra, MRCP (UK), FRCPath
First Author Secondary Information:	
Order of Authors:	Varun Mehra, MRCP (UK), FRCPath
	Elijah Rhone
	Stefani Widya
	Mark Zuckerman
	Victoria Potter
	Kavita Raj
	Austin Kulasekararaj
	Donal McLornan
	Hugues de Lavallade
	Nana Benson-Quarm
	Christina Lim
	Sarah Ware
	Malur Sudhanva
	Omar Malik
	Richard Nicholas
	Paolo A Muraro
	Judith Marsh
	Ghulam J Mufti
	Eli Silber
	Antonio Pagliuca
	Majid A. Kazmi
Order of Authors Secondary Information:	

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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/ml in the methods section i.e 10 EBV DNA copies/ml=10 IU/ml

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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:

Dr Varun Mehra, MRCP(UK), FRCPath Dr Majid Kazmi; FRCP(UK) FRCPath
Department of Haematological Medicine Chief of Cancer Division & Consultant Haematologist
Kings College Hospital, London, UK Kings, Guy’s & St Thomas’ Hospital, London, UK
Varun.Mehra@nhs.net ; Ph-004478865087013Majidkazmi@nhs.net; PH-004478027617716

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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

Dr Majid Kazmi; FRCP(UK) FRCPath
Chief of Cancer Division & Consultant Haematologist
Kings, Guy's & St Thomas' Hospital, London, UK
Majidkazmi@nhs.net; PH-004478027617716

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

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

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

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

***Key Points**:*

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

Corresponding authors:

1. Dr Varun Mehra*;* Varun.Mehra@nhs.net; +442032995378

Alternate Corresponding Author:

2. Dr Majid Kazmi; majidkazmi@nhs.net ; +442071882757

Key Words:

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

Author Affiliations:

1. **Dr Varun Mehra***; Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
2. **Dr Elijah Rhone***: Department of Neurology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
3. **Stefani Widya:** GKT School of Medical Education, Kings College London University, London
4. **Dr Mark Zuckerman:** Department of Virology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
5. **Dr Victoria Potter:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
6. **Dr Kavita Raj:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom **AND** Department of Haematology, Guy's and St. Thomas' NHS Foundation Trust, London, United Kingdom
7. **Dr Austin Kulasekararaj:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
8. **Dr Donal McLornan**: Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom **AND** Department of Haematology, Guy's and St. Thomas' NHS Foundation Trust, London, United Kingdom
9. **Dr Hugues de Lavallade**: Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
10. **Nana Benson-Quarm:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
11. **Christina Lim:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
12. **Sarah Ware:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
13. **Dr Malur Sudhanva:** Department of Virology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
14. **Dr Omar Malik:** Department of Neurology, Imperial College Healthcare, London, United Kingdom
15. **Dr Richard Nicholas:** Department of Neurology, Imperial College Healthcare, London, United Kingdom
16. **Professor Paolo A. Muraro:** Department of Neurology, Imperial College Healthcare, London, United Kingdom **AND** Department of Neuroimmunology, Imperial College London, London, United Kingdom
17. **Professor Judith Marsh:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
18. **Professor Ghulam J. Mufti:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
19. **Dr Eli Silber:** Department of Neurology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
20. **Professor Antonio Pagliuca:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
21. **Dr Majid Kazmi:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom **AND** Department of Haematology, Guy's and St. Thomas' NHS Foundation Trust, London, United Kingdom

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

1. **Varun Mehra-** no competing financial interests
2. **Elijah Rhone-** no competing financial interests
3. **Stefani Widya-** no competing financial interests
4. **Mark Zuckerman-** no competing financial interests
5. **Victoria Potter-** no competing financial interests
6. **Kavita Raj-** no competing financial interests
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12. **Sarah Ware-** no competing financial interests
13. **Malur Sudhanva-** no competing financial interests
14. **Omar Malik-** no competing financial interests
15. **Richard Nicholas-** no competing financial interests
16. **Paolo A Muraro-** no competing financial interests
17. **Judith Marsh-** no competing financial interests
18. **Ghulam J Mufti-** no competing financial interests
19. **Eli Silber-** no competing financial interests
20. **Antonio Pagliuca-** no competing financial interests
21. **Majid A. Kazmi-** no competing financial interests

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 in MS patients in the first 3 months post AHSCT

INTRODUCTION:

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 majority of patients at diagnosis. Recovery from relapses may be complete or partial[3]-[4]. After a variable period of time, people with RRMS may develop a more progressive disability accumulation with or without superimposed relapses; termed secondary progressive multiple sclerosis (SPMS). A minority experience progressive disability from the onset of disease, termed primary progressive multiple sclerosis (PPMS)[4]**.** A number of immunomodulatory disease modifying therapies (DMTs) are currently licensed for treatment of RRMS with an aim of reducing number of relapses and accrual of disability, although with variable efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation (AHSCT) has been a novel approach for MS management, using immunoablation followed by immunomodulation mechanisms, with evidence of significant suppression of inflammatory activity and qualitative changes in the reconstituted immune system (immune 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 disability levels (Expanded Disability Status Scale [EDSS] <6 or up to 6.5 if recent progression) and failure of at least 1 highly active DMT (natalizumab or alemtuzumab) with 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) for RRMS with respect to both

171 treatment failure and disability progression.

172

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

183

184 **METHODS**

185 **Patients and procedures**

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

(50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion followed by stem cell infusion. One patient was conditioned with carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned was 7.17 x10^6/kg (range 4.0-17.1x10^6/kg).

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 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-Gene™ (Qiagen) assay of EBV BZLF1 DNA. This assay was adapted from our published assay using LightCycler (Roche)[15] and since been validated against the recently published WHO standard, with our lab's EBV DNA quantification of 10 copies/ml considered equivalent to 10 IU/ml DNA reported with the WHO reference method[16]. EBV-R was defined as rising EBV DNA load of >10 copies/millilitre (ml) detected on two consecutive tests based on our assay sensitivity. Symptomatic EBV-R was captured by peak blood EBV DNA load, presence of B symptoms (defined by presence of either unexplained weight loss, recurrent fever, night sweats); which was in-turn defined by clinical, radiological and/or histological evidence based 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 rising EBV viraemia in MS patients. Serum protein electrophoresis was routinely tested around 3 months post HSCT as part of our institutional practice, with immunoglobulin subclasses identified by immunofixation electrophoresis. Patient outcomes were assessed at last follow up as of April 2017.

Statistics

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

RESULTS

Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment, indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS patients were lost to long-term follow up for EBV monitoring. The median time to first EBV DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days) to lymphocyte recovery (defined by total lymphocyte count >$1.0x10^6$/ml) following AHSCT (**See Figure 1**). A high proportion (86%; n-25/29) of the MS patients in active follow-up

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

All patients were stratified into following 3 groups according to peak rise/burden of EBV DNA-aemia (copies/ml): <100,000 (<100k) copies/ml, 100,001-500,00 (100k-500k) copies/ml and >500,000 (>500k) copies/ml to identify any specific thresholds for clinically significant events related to rising EBV-R (Table 1). The majority of patients (76%) with rising EBV viral load >100k copies/ml were routinely screened by computed tomographic (CT) scans to assess for evidence of LPD, in line with our institutional policy. One third (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 and/or B symptoms. Of these 8 patients, only 1 (12.5%) had a peak EBV viraemia <100kDNA copies/ml with the remaining 7 (87.5%) patients having a peak EBV viraemia of >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 diagnosis. Three MS patients had worsening neurological symptoms concurrent with rising EBV viraemia >500k copies/ml and clonal gammopathy, as described below.

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

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 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking 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 supplementary case vignettes**). **Figure 2** highlights the association of neurological symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts (x 10^6/ml) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient developed 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 evidence of MS related new disease activity.

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 load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis (**Figure 3**) confirmed EBV viraemia of >500k copies/ml correlated with high sensitivity (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 treatment with rituximab. The sensitivity dropped significantly on lower 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.

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].

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
297 & T cell lymphodepletion. In contrast, ATG primarily affects the T-cell repertoire with delayed
298 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections
299 including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease
300 and systemic sclerosis is increasingly recognised, but the development of lymphoproliferative
301 disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32] concerningly reported
302 2 deaths (1 MS and 1 systemic sclerosis patient) from EBV related LPD in 56 ATG
303 conditioned AHSCT for autoimmune diseases. Additionally, EBV associated
304 haemophagocytosis in ATG-AHSCT for ADs have also been reported[34], with one resulting
305 in death of the patient[35].
306
307 Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher
308 than published reports with allo-HSCTs (4.5-7%)[36,37] and our own centre's unpublished T-
309 cell depleted allo-HSCT experience (6.5%); possibly a reflection of underlying
310 immunopathological state of MS itself[38]. This is further corroborated by the fact that similar
311 LPD risk has not been observed in other ADs, e.g. Crohn's disease, treated with ATG -
312 AHSCT in this centre. Another example from our centre's experience of severe aplastic
313 anaemia (n-40) treated with ATG/ciclosporin, only 52% (n-21/40) developed EBV-R
314 (unpublished data) and none had LPD or required any treatment, suggesting that the problem
315 may not be ATG specific.

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

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

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

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

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

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

Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender 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)

Abbreviations:

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

Figure Legends

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

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

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

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

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

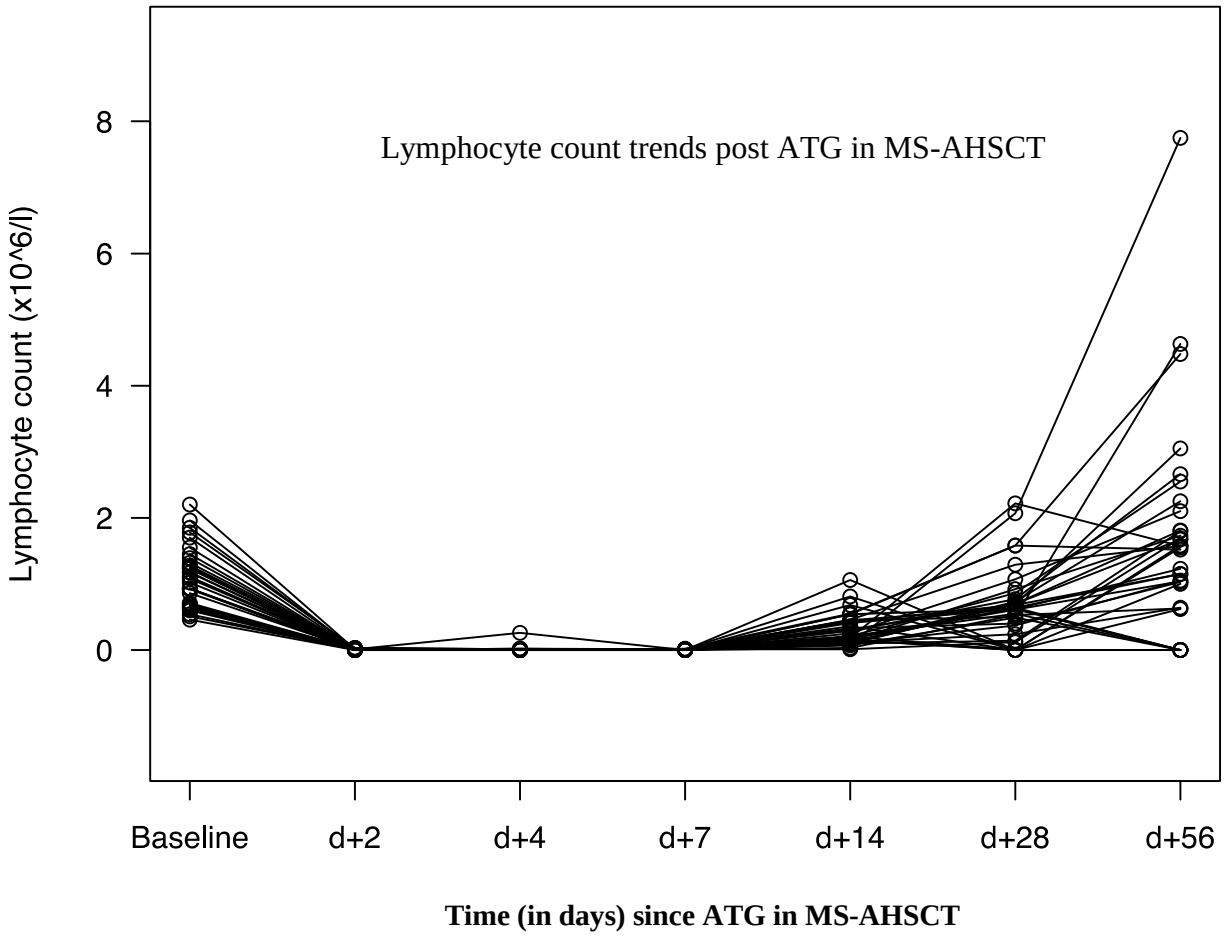
D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

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

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

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

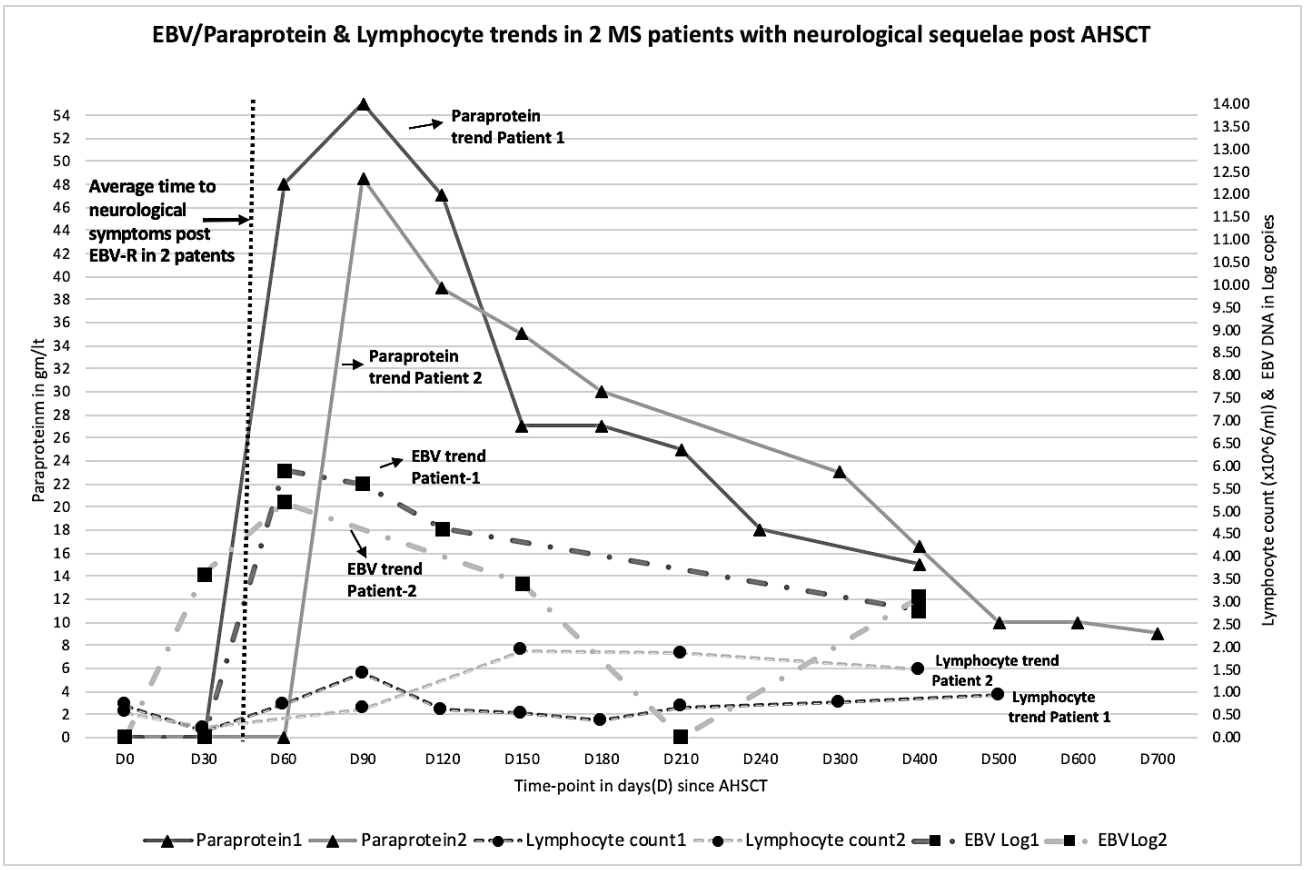
Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.



Abbreviations:

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

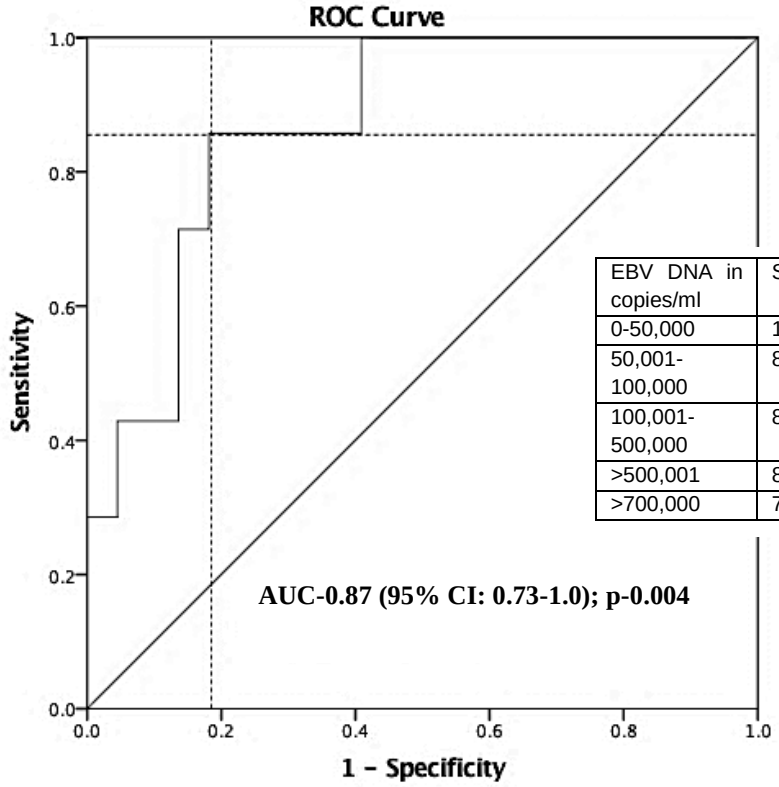
Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT



Abbreviations:

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

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



EBV DNA in copies/ml	Sensitivity	Specificity
0-50,000	100%	50%
50,001-100,000	85.5%	60.1%
100,001-500,000	85.5%	73.7%
>500,001	85.5%	82%
>700,000	71.4%	82%

Abbreviations:

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

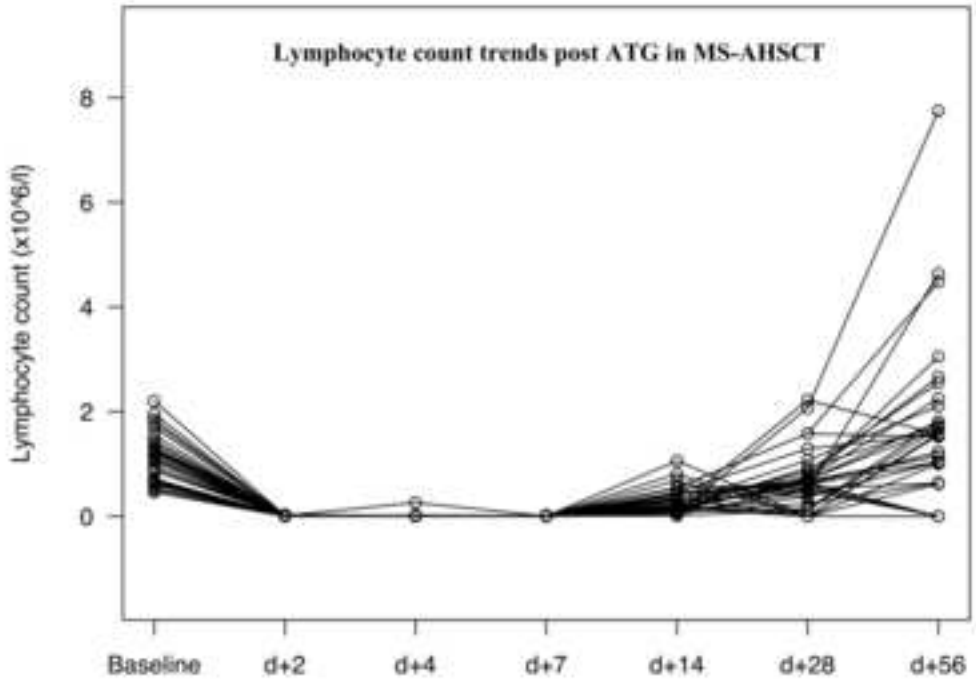
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No. of patients with detectable EBV post AHSCT (n; %)	29 (80.5%)	Confirmed EBV resolution at last follow up (n)	7	2	7
No. of patients lost to long term follow up (n; %)	7 (19.5%)	Detectable EBV DNA at last follow up (n)	9	1	3
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Median 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)

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

Click here to access/download;Figure (.tif and .eps files only);Figure 1.1 EBV MS tif.tiff

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

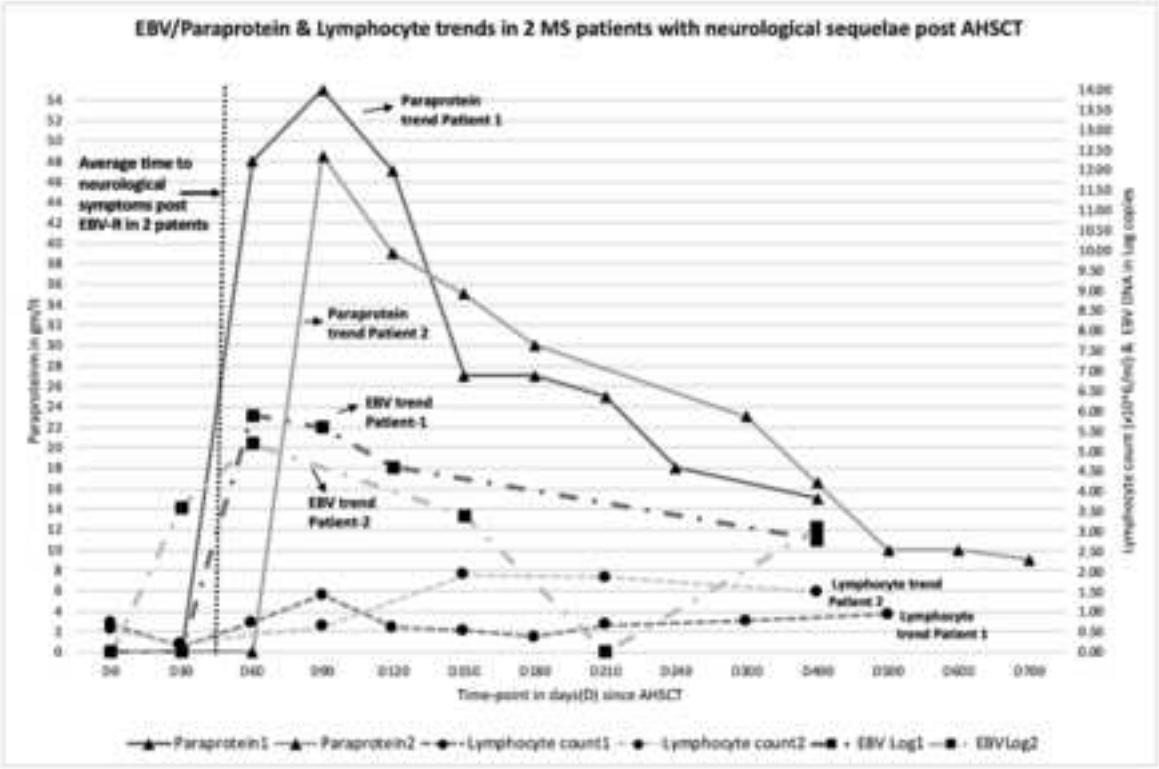


Abbreviations:
AHSCT: Autologous Haematopoietic Stem Cell Transplantation; ATG: Anti-Thymocyte Globulin; d+: Days post AHSCT; MS: Multiple Sclerosis.

Figure 2

Click here to access/download;Figure (.tif and .eps files only);Figure 2.1 EBV MS tif.tiff

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

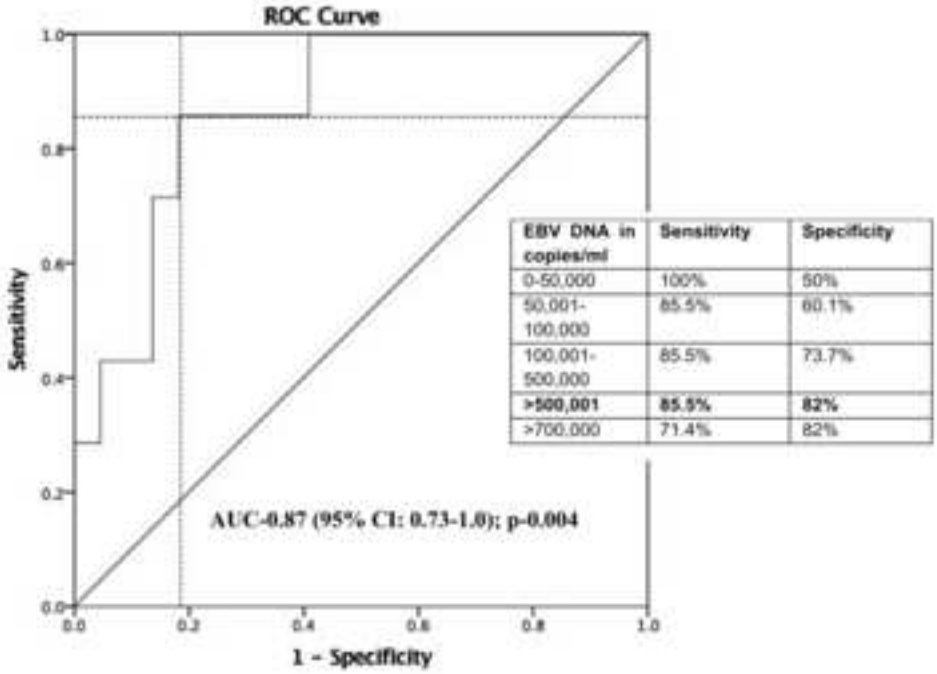


Abbreviations:

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

Click here to access/download;Figure (.tif and .eps files only);Figure 3.1 EBV MS tif.tiff

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



EBV DNA in copies/ml	Sensitivity	Specificity
0-50,000	100%	50%
50,001-100,000	85.5%	60.1%
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>500,001	**85.5%**	**82%**
>700,000	71.4%	82%

Abbreviations:

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

Supplemental -Case Vignettes
Click here to access/download;Supplemental data/Appendix - published online only;SUPPLEMENTARY-MS EBV updated

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 post-transplant. A blood test on day 26 demonstrated Epstein-Barr Virus (EBV) reactivation (155,845 copies/ml). A repeat test on day 34 showed an increase in copy number to 638,634 copies/ml. She was asymptomatic, and the plan was to monitor this closely. On day 37 post-transplant she developed a significant deterioration in strength in the right lower limb and on day 42 she developed pyrexia and was admitted to a local hospital. She was found to have CMV reactivation which was treated with IV ganciclovir as well as ongoing EBV reactivation and she remained an inpatient for 4 weeks. She did not receive rituximab at the local centre but on repeat testing at day 145 the copy number was vastly reduced at 2,355 copies/ml. A high IgM paraproteinaemia was first detected at day 92 post-transplant (48.58g/L). This had not been routinely monitored previously. This paraproteinaemia was initially felt to be asymptomatic and was monitored closely, slowly improving over time. A CT scan was performed which demonstrated a single 1.7cm right hilar lymph node requiring observation.

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

The EBV reactivation initially settled at 6 months post-transplant. At one-year post transplant she had a persistent IgM paraprotein (23g/L) and her right leg weakness had continued to progress with her EDSS now at 5.0. There was also a mild recurrence of EBV (DNA at 1,335 copies/ml). It was considered that as the onset of the right leg weakness had coincided with the high level of EBV reactivation and paraproteinaemia that these factors may have driven a peripheral neuropathy. She was treated with rituximab 375mg/m^2 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/m^2 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 vireamia 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.

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

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

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

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

***Key Points**:*

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

Corresponding authors:

1. Dr Varun Mehra*;* Varun.Mehra@nhs.net; +442032995378

Alternate Corresponding Author:

2. Dr Majid Kazmi; majidkazmi@nhs.net ; +442071882757

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Author Affiliations:

1. **Dr Varun Mehra***; Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
2. **Dr Elijah Rhone***: Department of Neurology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
3. **Stefani Widya:** GKT School of Medical Education, Kings College London University, London
4. **Dr Mark Zuckerman:** Department of Virology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
5. **Dr Victoria Potter:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
6. **Dr Kavita Raj:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom **AND** Department of Haematology, Guy's and St. Thomas' NHS Foundation Trust, London, United Kingdom
7. **Dr Austin Kulasekararaj:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
8. **Dr Donal McLornan**: Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom **AND** Department of Haematology, Guy's and St. Thomas' NHS Foundation Trust, London, United Kingdom
9. **Dr Hugues de Lavallade**: Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
10. **Nana Benson-Quarm:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
11. **Christina Lim:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
12. **Sarah Ware:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom.
13. **Dr Malur Sudhanva:** Department of Virology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
14. **Dr Omar Malik:** Department of Neurology, Imperial College Healthcare, London, United Kingdom
15. **Dr Richard Nicholas:** Department of Neurology, Imperial College Healthcare, London, United Kingdom
16. **Professor Paolo A. Muraro:** Department of Neurology, Imperial College Healthcare, London, United Kingdom **AND** Department of Neuroimmunology, Imperial College London, London, United Kingdom
17. **Professor Judith Marsh:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
18. **Professor Ghulam J. Mufti:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
19. **Dr Eli Silber:** Department of Neurology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
20. **Professor Antonio Pagliuca:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom
21. **Dr Majid Kazmi:** Department of Haematology, King's College Hospital NHS Foundation Trust, Denmark Hill, London, United Kingdom **AND** Department of Haematology, Guy's and St. Thomas' NHS Foundation Trust, London, United Kingdom

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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

INTRODUCTION:

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 majority of patients at diagnosis. Recovery from relapses may be complete or partial[3]·[4]. After a variable period of time, people with RRMS may develop a more progressive disability accumulation with or without superimposed relapses; termed secondary progressive multiple sclerosis (SPMS). A minority experience progressive disability from the onset of disease, termed primary progressive multiple sclerosis (PPMS)[4]**.** A number of immunomodulatory disease modifying therapies (DMTs) are currently licensed for treatment of RRMS with an aim of reducing number of relapses and accrual of disability, although with variable efficacy[5]. Since 1996, Autologous Hematopoietic Stem Cell Transplantation (AHSCT) has been a novel approach for MS management, using immunoablation followed by immunomodulation mechanisms, with evidence of significant suppression of inflammatory activity and qualitative changes in the reconstituted immune system (immune 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 disability levels (Expanded Disability Status Scale [EDSS] <6 or up to 6.5 if recent progression) and failure of at least 1 highly active DMT (natalizumab or alemtuzumab) with 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) for RRMS with respect to both

171 treatment failure and disability progression.

172

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

183

184 **METHODS**

185 **Patients and procedures**

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

(50mg/kg for 4 days) and rATG (2.5mg/kg/day for 3 days) for in-vivo lymphodepletion, followed by stem cell infusion. One patient was conditioned with carmustine/etoposide/cytarabine/melphalan regimen along with an equivalent dose of rATG (BEAM-ATG) prior to stem cell infusion. The median CD34 stem cell dose returned was 7.17 x10^6/kg (range 4.0-17.1x10^6/kg).

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 quantitative real-time polymerase chain reaction (RT-PCR) using Rotor-Gene™ (Qiagen) assay of EBV BZLF1 DNA. This assay was, adapted from our published assay using LightCycler (Roche)[15] and since been validated against the recently published WHO standard, with our lab's EBV DNA quantification of 10 copies/ml considered equivalent to 10 IU/ml DNA reported with the WHO reference method[16]. EBV-R was defined as rising EBV DNA load of >10 copies/millilitre (ml) detected on two consecutive tests based on our assay sensitivity. Symptomatic EBV-R was captured by peak blood EBV DNA load, presence of B symptoms (defined by presence of either unexplained weight loss, recurrent fever, night sweats); which was in-turn defined by clinical, radiological and/or histological evidence based 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 rising EBV viraemia in MS patients. Serum protein electrophoresis was routinely tested around 3 months post HSCT, as part of our institutional practice, with immunoglobulin subclasses identified by immunofixation electrophoresis. Patient outcomes were assessed at last follow up as of April 2017.

Statistics

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

RESULTS

Baseline characteristics are presented in **Table 1**. Most MS patients (88.9%) had RRMS phenotype with median of 2 (range 0-3) DMTs prior to AHSCT. Twenty-two MS patients had prior exposure to natalizumab and 7 were treated with Alemtuzumab (6 patients received both). All 36 (100%) MS patients were serologically positive for EBV VCA IgG pre-treatment, indicating prior EBV exposure and had detectable EBV DNA post-AHSCT. Seven MS patients were lost to long-term follow up for EBV monitoring. The median time to first EBV DNA detection post-transplant was 30 days (IQR 23-46 days). EBV DNA levels peaked at a median of 32 days post-transplant (IQR 31-53 days). All MS patients had normal baseline lymphocyte counts (median) pre-HSCT with a median time of 46 days (range 14-404 days) to lymphocyte recovery (defined by total lymphocyte count >$1.0x10^6$/ml) following AHSCT (**See Figure 1**). A high proportion (86%; n-25/29) of the MS patients in active follow-up

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

All patients were stratified into following 3 groups according to peak rise/burden of EBV DNA-aemia (copies/ml): <100,000 (<100k) copies/ml, 100,001-500,00 (100k-500k) copies/ml and >500,000 (>500k) copies/ml to identify any specific thresholds for clinically significant events related to rising EBV-R (Table 1). The majority of patients (76%) with rising EBV viral load >100k copies/ml were routinely screened by computed tomographic (CT) scans to assess for evidence of LPD, in line with our institutional policy. One third (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 and/or B symptoms. Of these 8 patients, only 1 (12.5%) had a peak EBV viraemia <100kDNA copies/ml with the remaining 7 (87.5%) patients having a peak EBV viraemia of >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 diagnosis. Three MS patients had worsening neurological symptoms concurrent with rising EBV viraemia >500k copies/ml and clonal gammopathy, as described below.

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

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 Kappa M-protein of 45.6g/L developed hyper-viscosity and neurological symptoms mimicking 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 supplementary case vignettes**). **Figure 2** highlights the association of neurological symptom onset following rising EBV viraemia (log copies), falling lymphocyte counts (x 10^6/ml) with significant rise in M-protein (gm/lt) levels post AHSCT. A third patient developed 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 evidence of MS related new disease activity.

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 1reduction in EBV viral load and concurrent improvement/resolution of EBV related symptoms. ROC curve analysis (**Figure 3**) confirmed EBV viraemia of >500k copies/ml correlated with high sensitivity (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 treatment with rituximab. The sensitivity dropped significantly on lower 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 >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.

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].

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

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 EBV specific CD8+ T cell recovery[31]. Clinically significant endogenous viral infections including EBV following ATG conditioned AHSCT for severe ADs such as Crohn's disease and sSystemic sSclerosis is increasingly recognised, but the development of lymphoproliferative disorders (LPD) remains rare in these ADs[13,32,33]. Nash et al[32] concerningly reported 2 deaths (1 MS and 1 systemic sclerosis patient) from EBV related LPD in 56 ATG conditioned AHSCT for autoimmune diseases. Additionally, EBV associated haemophagocytosis in ATG-AHSCT for ADs have also been reported[34], with one resulting in death of the patient[35].

Our report of suspected EBV related LPDs (~10%) in MS-AHSCT group is relatively higher 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 immunopathological state of MS itself[38]. This is further corroborated by the fact that similar LPD risk has not been observed in other ADds, e.g. Crohn's disease,, treated with ATG - AHSCT in this centre. Another example from ouour centre's experience of in severe aplastic anaemiaa ~~(n-40), a type of AD causing severe bone marrow failure~~(n-40) treated ~~& treated~~ with ATG/ciclosporin,n; only 52% (n-21/40) ~~patients~~ developed EBV-R (unpublished data) and . nNone had LPD or required any treatment, ~~supporting the notion that it may not just be~~

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

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

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 significantly associated with probable LPD and neurological events in MS patients with high 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 has consistently been useful in our MS-AHSCT experience for predicting clinical events with high EBV load. Our EBV PCR assay has been validated against the recently defined standard WHO reference method (i.e. 10 copiesy/ml=10 IU/ml EBV DNA) [16] and thus this EBV threshold for pre-emptive treatment with Rituximab, can potentially be applied in relevant clinical context in other centres using similar validated essays. Rituximab treatment delivered good overall response in our symptomatic patients, with resolution of EBV related clinical symptoms and no subsequent viral or bacterial infections at last follow up. The role of prophylactic or pre-transplant rituximab in MS-AHSCT is also a potential area of interest 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.

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

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

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

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

Baseline characteristics (n-36)		Patient Groups according to peak EBV DNA in copies/ml (n-29)	0 - 100,000	100,001 - 500,000	>500,000
Median age at time of AHSCT in years (range)	43.5 (36– 47)	No of patients (%)	16 (55.2)	3 (10.3)	10 (34.5)
Gender 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)

Abbreviations:

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

Figure Legends

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

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

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

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

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

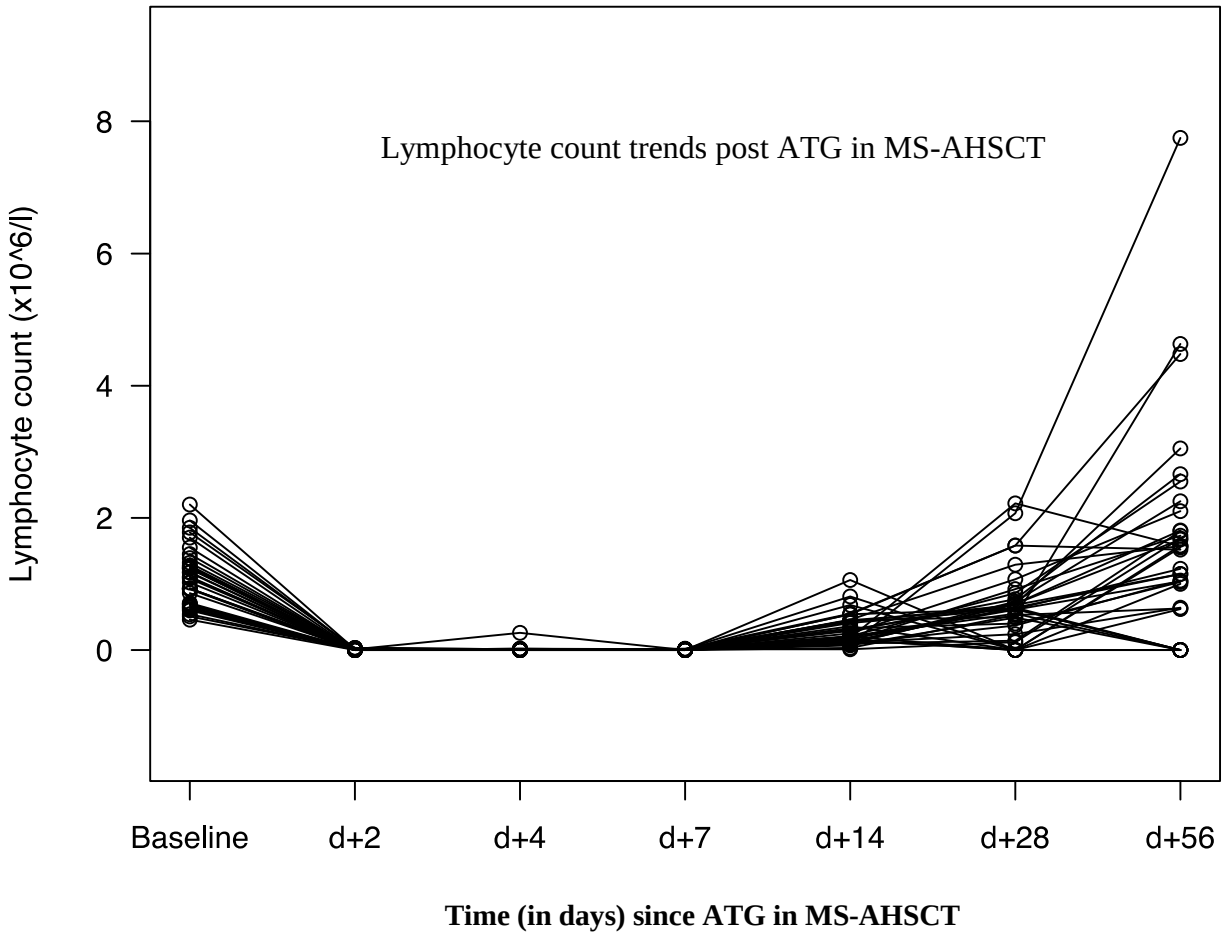
D: Days post AHSCT; EBV-R: Epstein Barr Virus reactivation; MS: Multiple sclerosis; AHSCT: Autologous Haematopoietic Stem Cell Transplants.

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

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

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

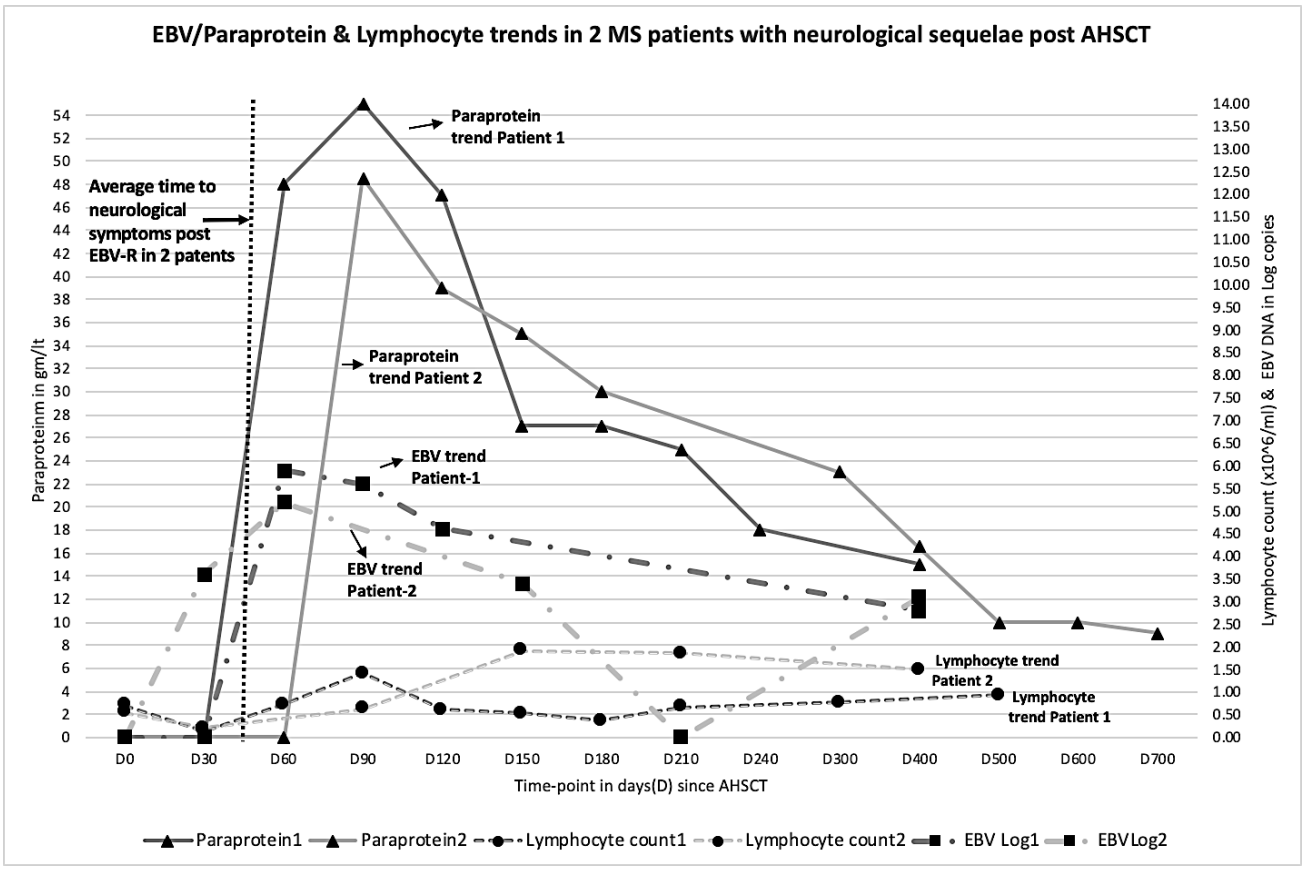
Figure 1: Trend of Lymphocyte count recovery following ATG conditioned AHSCT in MS patients.



Abbreviations:

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

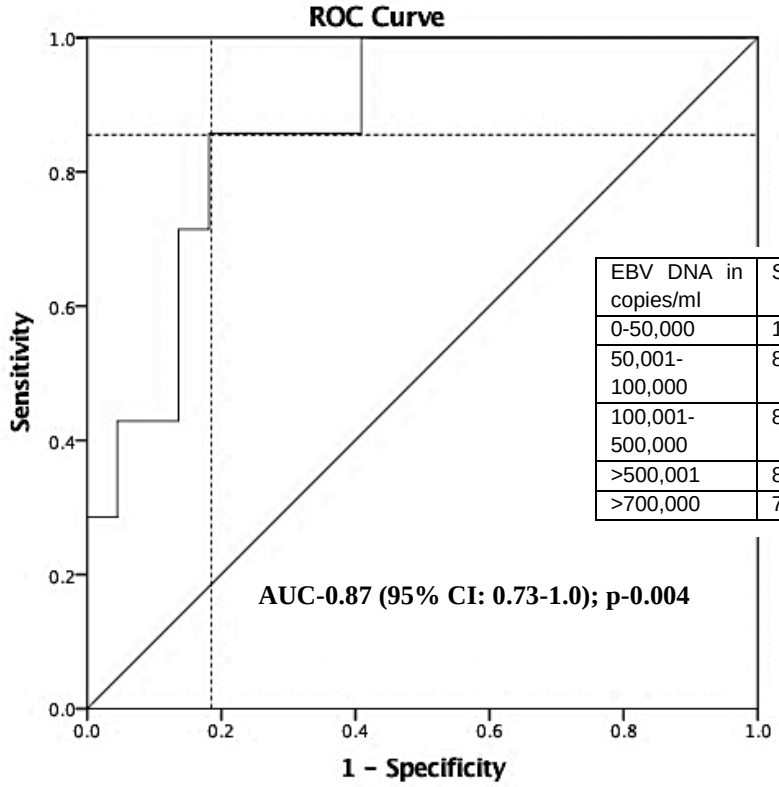
Figure 2. Paraprotein, EBV & Lymphocyte trends in two MS patients with significant neurological sequelae post AHSCT



Abbreviations:

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

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



EBV DNA in copies/ml	Sensitivity	Specificity
0-50,000	100%	50%
50,001-100,000	85.5%	60.1%
100,001-500,000	85.5%	73.7%
>500,001	85.5%	82%
>700,000	71.4%	82%

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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