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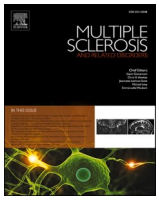

Ocrelizumab B cell depletion has no effect on HERV RNA expression in PBMC in MS patients

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ABSTRACT

Background: Epstein barr virus (EBV) infection of B cells is now understood to be one of the triggering events for the development of Multiple Sclerosis (MS), a progressive immune-mediated disease of the central nervous system. EBV infection is also linked to expression of human endogenous retroviruses (HERVs) of the HERV-W group, a further risk factor for the development of MS. Ocrelizumab is a high-potency disease-modifying treatment (DMT) for MS, which depletes B cells by targeting CD20.
Objectives: We studied the effects of ocrelizumab on gene expression in peripheral blood mononuclear cells (PBMC) from paired samples from 20 patients taken prior to and 6 months after beginning ocrelizumab therapy. We hypothesised that EBV and HERV-W loads would be lower in post-treatment samples.
Methods: Samples were collected in Paxgene tubes, subject to RNA extraction and Illumina paired end short read mRNA sequencing with mapping of sequence reads to the human genome using Salmon and differential gene expression compared with DeSeq2. Mapping was also performed separately to the HERV-D database of HERV sequences and the EBV reference sequence.
Results: Patient samples were more strongly clustered by individual rather than disease type (relapsing/remitting or primary progressive), treatment (pre and post), age, or sex. Fourteen genes, all clearly linked to B cell function were significantly down regulated in the post treatment samples. Interestingly only one pre-treatment sample had detectable EBV RNA and there were no significant differences in HERV expression (of any group) between pre- and post-treatment samples.
Conclusions: While EBV and HERV expression are clearly linked to triggering MS pathogenesis, it does not appear that high level expression of these viruses is a part of the ongoing disease process or that changes in virus load are associated with ocrelizumab treatment.

1. Introduction

Multiple sclerosis (MS) is a disabling immune-mediated, inflammatory disease of the central nervous system (CNS) (Reich et al., 2018). Pathological damage is directed against CNS myelin and axons, with clinical manifestations that are characterised by relapses and / or progression of neurological deficits. Most patients present with a relapsing-remitting clinical course (RRMS) characterised by partial recovery between bouts of inflammation, followed by secondary progressive disease (SPMS) in which gradual neurological decline is independent of relapses. A smaller number of patients have progressive disease from the start (primary progressive course, PPMS). In spite of variations in clinical presentation, MS is considered a single disease. It is likely that different pathological processes underlie relapses and progression. Numerous immunotherapies have been approved for the treatment of relapsing-remitting disease and only two for progressive disease. There is general consensus that early treatment of MS is advantageous (Forster et al., 2019; Kuhlmann et al., 2023).

While not solely responsible for MS pathogenesis, viral infections are a known risk factor for the development of the disease and indeed the first established effective therapy for MS was interferon beta, a cytokine with a central role in antiviral immune responses (Dumitrescu et al., 2018). In particular, post pubertal infection with Epstein Barr Virus (EBV), normally a common childhood infection, is strongly linked to

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disease risk and exposure to the virus appears to be required for disease development (Afrasiabi et al., 2019). Recent large clinical studies including one of repeated long term blood samples from over 10 million US military personnel have demonstrated this epidemiological link beyond doubt (Bjornevik et al., 2022; Loosen et al., 2022). Over-expression of human endogenous retroviruses (HERVs) of the HERV-W family is also associated with MS disease risk (Morandi et al., 2017). These HERVs are copies of ancestral viral infections that have become integrated into the host's genome and now perform essential host functions (one HERV-W protein is an essential component of the human placenta) (Morandi et al., 2017).

There is a rapidly increasing body of evidence linking EBV and HERV (particularly the HERV-W family) proteins with MS pathogenesis. EBV replicates primarily in B cells, (Ricigliano et al., 2015) where it establishes latency, associated with the viral protein Epstein Barr nuclear antigen 2 (EBNA2). EBNA2 binds to genetic loci associated with MS risk competing for transcription binding sites with Vitamin D, high levels of which are protective against MS risk and inhibit B cell proliferation (Ricigliano et al., 2015). HERV-W env is expressed specifically in monocytes, T and B lymphocytes and NK cells and is particularly associated with activation of the non-classical monocyte class ($CD14^{low}CD16^{+}$) that are upregulated in MS (Garcia-Montojo et al., 2020; Gjelstrup et al., 2018; Carstensen et al., 2020).

EBV replication in B cells triggers HERV-W and HERV-K expression (Morandi et al., 2019; Irizar et al., 2014; Mameli et al., 2012; Wieland et al., 2022) initiating a cascade of stimulation of inflammation (Rolland et al., 2006; Duperray et al., 2015; Perron et al., 2013) and cross reactivity with myelin oligodendrocyte protein (MOG) (de Luca et al., 2019). HERV-W and EBV expression levels are also associated and EBV and HERV-W loads are correlated in MS patients undergoing therapy (Pérez-Pérez et al., 2022). An additional line of evidence linking HERV-W proteins to MS pathogenesis has shown HERV-W expression in microglia (brain-resident myeloid cells) associates with axons inducing a degenerative phenotype resulting in damage to myelinated axons (Kremer et al., 2019). HERV-W has also been shown to inhibit oligodendrocyte precursor cell formation and remyelination, an effect that can be blocked by the anti-HERV monoclonal antibody GNbAC1 (Kremer et al., 2014; Göttle et al., 2019).

Both Epstein Barr nuclear antigen 1 (EBNA1) and HERV-W env demonstrate binding to the HLADR2 allele that is the strongest genetic predisposition to MS (DRB1(*) 15:01) (Hedström et al., 2019; Ramasamy et al., 2017; Ramasamy et al., 2020; Menegatti et al., 2021; do Olival et al., 2013) and exhibit cross reactivity with myelin components (Ramasamy et al., 2020; Lünemann et al., 2008). EBV specific HLA1 responses are more likely in MS patients and these patients also have EBV-specific memory T cells in their cerebrospinal fluid (Schneider-Hohendorf et al., 2022). BCR sequencing of the B cell compartment combined with screening of the sequenced antibodies against EBV and CNS proteins of MS patient and controls has demonstrated clonal amplification of EBNA1 and GlialCAM (a protein and chloride channel regulator in glial cells important in CNS repair mechanisms) cross reactive B cells in the PMBC and CNS of MS patients (Lanz et al., 2022). A similar study demonstrated antibodies cross reactive to both EBV and alpha-crystallin B (CRYAB), a molecular chaperone protein involved in glial responses to injury) enriched in MS patients compared with healthy controls (Thomas et al., 2023). Genome wide association studies (GWAS) and transcriptome studies of MS patients have repeatedly indicated antiviral proteins as risk factors in disease occurrence and progression (Afrasiabi et al., 2019; Umeton et al., 2022; International Multiple Sclerosis Genetics Consortium., MultipleMS Consortium Locus for severity implicates CNS resilience in progression of multiple sclerosis, 2023). Clinical trials of T cell therapy specifically targeting EBV have even begun (Smith and Khanna, 2023) with promising early results for both clinical improvement and decrease in EBV antibody titre.

It is unclear whether these viruses initiate a triggering event creating an aberrant immune response or B cell type that perpetuates itself in the absence of the viral trigger or whether chronic or high viral loads are part of the underlying pathology. There are a range of studies demonstrating that antibody and T cell responses to EBV are consistently higher in MS patients than controls and that these are elevated during relapsing phases of RRMS (Hedström et al., 2019; Lünemann et al., 2008; Banwell et al., 2007; Kvistad et al., 2014; Yea et al., 2013; Makhani et al., 2016; Langer-Gould et al., 2017; Giess et al., 2017; Czarnowska et al., 2018; Jakimovski et al., 2019; Persson Berg et al., 2022; Comabella et al., 2023). However, EBV nucleic acid in the blood or shed in saliva is usually not associated with MS (Yea et al., 2013; Giess et al., 2017; Lindsey et al., 2009; Cocuzza et al., 2014; Mostafa et al., 2017; Holden et al., 2018; Pereira et al., 2023) though it can be detected in CNS/Brain samples (Hassani et al., 2018).

EBV establishes life-long latency in a subpopulation of memory B cells and there are strong indications that aberrant latency programming in EBV infected cells, indicated by the presence of the EBNA2 protein may be an important factor in the development of MS (Keane et al., 2021). B cell depletion therapies that broadly target B cell such as cladribine, anti CD-52 antibodies (alemtuzumab) and anti CD-20 antibodies (ocrelizumab, rituxumab) have proven effective in control of clinical disease in MS (Furman et al., 2023; Dyer et al., 2023; Gensicke et al., 2012; Ho et al., 2023). In some cases these therapies also result in decreased EBV antibody titre and cellular immune responses (Persson Berg et al., 2022; Pham et al., 2021; Pham et al., 2023; Domínguez-Mozo et al., 2022; Zivadinov et al., 2022). Those therapies that specifically target naïve and plasma B cells (atacicept) or boost memory T cells such as infliximab (anti TNF- alpha antibody) or lenercept on the other hand enhance disease (Furman et al., 2023; Gensicke et al., 2012; Kappos et al., 2014).

Generation of spontaneous lymphoblastoid cell lines (transformed EBV infected B cells) is more common in MS patients (and in other autoimmune diseases) than in healthy controls (Soldan et al., 2023; Fraser et al., 1979) and genetic variation in EBV latency associated proteins (Varvatsi et al., 2021) in MS patients has been demonstrated. Expression of the latency-associated protein, EBNA1, is enhanced in B cells from younger patients (Wieland et al., 2022) while "age-associated" B cells (which are expanded in older patients) are also expanded in MS patients and altered based on herpesvirus status (Mouat et al., 2022). This B cell subset are T-bet/CXCR3 + memory B cells that skew immune responses to a Th1 (viral and intracellular pathogen) cellular immune response. They are neuroinvasive and are associated with EBV reactivation (Leffler et al., 2022). This subset of cells can be induced by an atypical latency programme in EBV infected B cells (SoRelle et al., 2022) and are currently a key suspect in the cellular triggers of MS.

This study sought to address whether targeting the primary site of viral antigen production for EBV and HERV-W proteins (peripheral B cells) by depletion with the monoclonal antibody ocrelizumab, which specifically targets the B cell surface protein CD-20 (Forster et al., 2019) reduces viral load and is thereby associated with a reduction in MS pathology and clinical disease. As most prior studies of HERV expression in MS have used qPCR based differential expression on whole blood or PBMC samples (Morandi et al., 2017; Pérez-Pérez et al., 2022; Nali et al., 2022) rather than individual cell fractions we followed a format of mRNAseq from whole blood to allow comparison with published work.

2. Materials and methods

Ethics approval was granted by the University of Nottingham, Faculty of Medicine and Health Sciences Research and Ethical Committee number: MREC 08/H0408/167.

Twenty patients with RRMS or PPMS were recruited prior to beginning ocrelizumab therapy (Table 1 and Supplementary Information). Patients were recruited from those undergoing routine therapy for MS through the Nottingham University Hospitals NHS trust, including routine clinical assessment of clinical activity (and usually one MRI brain scan a year). Recruitment criteria aimed to be as even as possible

Table 1
Summary of Patient Demographics.

Characteristic	Number of Patients
Type of Disease	
Relapsing Remitting MS	15
Primary Progressive MS	5
Sex	
F	15
M	5
Age Bracket	
20–29	5
30–49	3
40–59	9
50–65	3
Time since initial diagnosis	
<1 year	15
<2 years	1
7–10 years	2
Unknown	2
Time since onset of symptoms	
<1 year	5
<2 years	3
<3 years	2
3–7 years	3
8–18 years	3
Unknown	4
Total	**20**

while remaining representative of typical MS patients treated with ocrelizumab within the timeframe of the study. Patients were between 18 and 65 years old, 75 % female and 25 % male, 75 % with RRMS and 25 % with PPMS. Patients had no history of other disease modifying therapies (DMTs) (19 patients) or no DMT during the previous 3 months and no treatment with high-dose steroids for MS relapse within the last 30 days (1 patient). This patient had one dose of Copaxone (glatiramer acetate) several years previously. Seventy five percent of the patients were recently diagnosed (<1 year). A minimum sample size of 13 (pre and post treatment) was estimated for demonstrating significant differences in transcriptomic studies with an FDR of 0.05 and an expected fold change of 4 (RnaSeqSampleSize, 2023).

Two and half ml of blood was collected into PaxGene tubes (Qiagen). A follow up blood sample was taken 5 months later, when patients had received their first 2 infusions and were reviewed by the MS Team in preparation for the third infusion at 6 months. Repeat sample timing at this point was selected both to minimise patient visits and samples (and retain participants) and because patient clinical parameters and response to treatment are more stable at this time point than earlier in treatment. Blood samples were stored at −20 °C until RNA extraction. RNA extraction was performed with a Paxgene blood RNA kit (Qiagen) as per manufacturer's protocol. Illumina NovaSeq RNA sequencing with polyA library preparation (150 base pair, paired end reads) was performed by Novogene UK.

The resulting data files were trimmed (quality score 30, min length 150 and adapters removed) with FASTP (Chen et al., 2018). Mapped to the unmasked ensembl version of the human genome (GrCh38) with Salmon (Patro et al., 2017). Additional mapping was performed to the EBV reference genome (NC 007605) and a custom Human endogenous retrovirus databases (HERVd) (Paces et al., 2002). Differential gene expression analysis was performed with the DESeq2 (Love et al., 2014) pipeline implemented in iDEP (Ge et al., 2018) with the parameters: false discovery rate 0.1, min fold change 2, model: treatment and patient. Hierarchical clustering and heat maps were also generated in iDEP.

3. Results

205,132 transcripts had detectable expression. The complete set of transcripts with detectable expression was taken forward into different gene expression analysis.

Hierarchical clustering of sequencing data demonstrated very clear clustering by patient ID (Fig. 1). This was a much stronger effect than any other factor in the study (including pre or post treatment, age, sex, or type of disease). This informed DESeq2 model selection with patient effects included as a correcting factor alongside the treatment effects we were interested in.

Fourteen genes, of which 13 were clearly linked to B cell function, were downregulated in the post treatment samples; no genes were upregulated. Downregulated genes were: IgG chains (IGHM, 2 variants of IGHD, IGHG2), CD79A and CD79B (part of the B cell receptor complex), CD75 (part of the MHC class II antigen presentation complex), BLK (B lymphocyte tyrosine kinase), MS4A1 (B lymphocyte surface molecule involved in B cell differentiation), VPREB3 (pre assembly of B cell receptor), TCL1A (T cell receptor activator, FCER2 (immunoglobulin E receptor – B cell growth factor) and an unknown transcript (ENSG00000288133) (Table 2).

Only one patient had detectable EBV reads (in a pre-treatment sample). Reads for many different HERV groups were detected but there was no differential expression of HERVs in any grouping of patient samples.

4. Discussion

MS is a complex disease with the triggering events possibly occurring many years before clinical disease onset and no single antigen target identified (Gåsland et al., 2023). Between-patient variation is large, necessitating very large epidemiological studies to pin down causal associations like the role of EBV in disease development (Bjornevik et al., 2022). This is reflected in this study where the strongest factor apparent in hierarchical clustering was the individual patient with pre and post treatment samples from the same patient clustering closely (Fig. 1). Other factors such as age, sex, type of disease, time since diagnosis or onset of disease had no statistically evident effect on gene expression in this cohort.

With individual patients controlled for in the differential expression model applied to the transcriptomics data the effects of ocrelizumab B cell depletion were remarkably targeted to a small number of genes (14) with clear B cell associated functions. There have been a small number of other studies using comparable methods and patient cohorts. Fong et al. (2023) looked at pre and post treatment gene expression in PBMC from 15 ocrelizumab treated MS patients at 2 weeks and 6 months post therapy compared with 10 healthy controls, 10 untreated MS patients and 9 MS patients treated with Interferon beta, using microarrays. Perhaps unsurprisingly with a more complicated and less controlled cohort and statistical analysis a much larger number of differentially expressed genes (413 decreased and 184 increased) were identified in their study. Similar to our study however the pathways identified were primarily B cell related and 6 of the same genes were identified, namely CD79A, CD22, CD79B, MS4A1 and two IGHD variants. CD22, CD79A and CD79B can be downregulated by EBNA2 and EBNA3 proteins and this affects BCR signalling (Khasnis et al., 2022). This was suggested to be an additional way through which EBV maintains viral latency and controls the survival of infected B cells (Khasnis et al., 2022).

Measuring differential expression of HERVs in RNAseq data is not straightforward due to the repetitive nature of transposons, making them not readily distinguishable in some mapping algorithms (Schwarz et al., 2022). Mapping success is also heavily dependent on the database chosen as the reference sequence. Schwarz et al. (Schwarz et al., 2022) compared the existing algorithms used for this kind of work, TEtranscripts (Jin et al., 2015), SalmonTE (Jeong et al., 2018), Telescope (Bendall et al., 2019), SQuiRE (Yang et al., 2019) and TEtools (Lerat et al., 2017) against a known test dataset and found that SalmonTE and Telescope performed reliably. As the original SalmonTE programme is not currently curated, we recreated its functionality using the Salmon mapping algorithm (Patro et al., 2017) and the HERVd (Paces et al., 2002) database of human ERVs, currently the most comprehensive

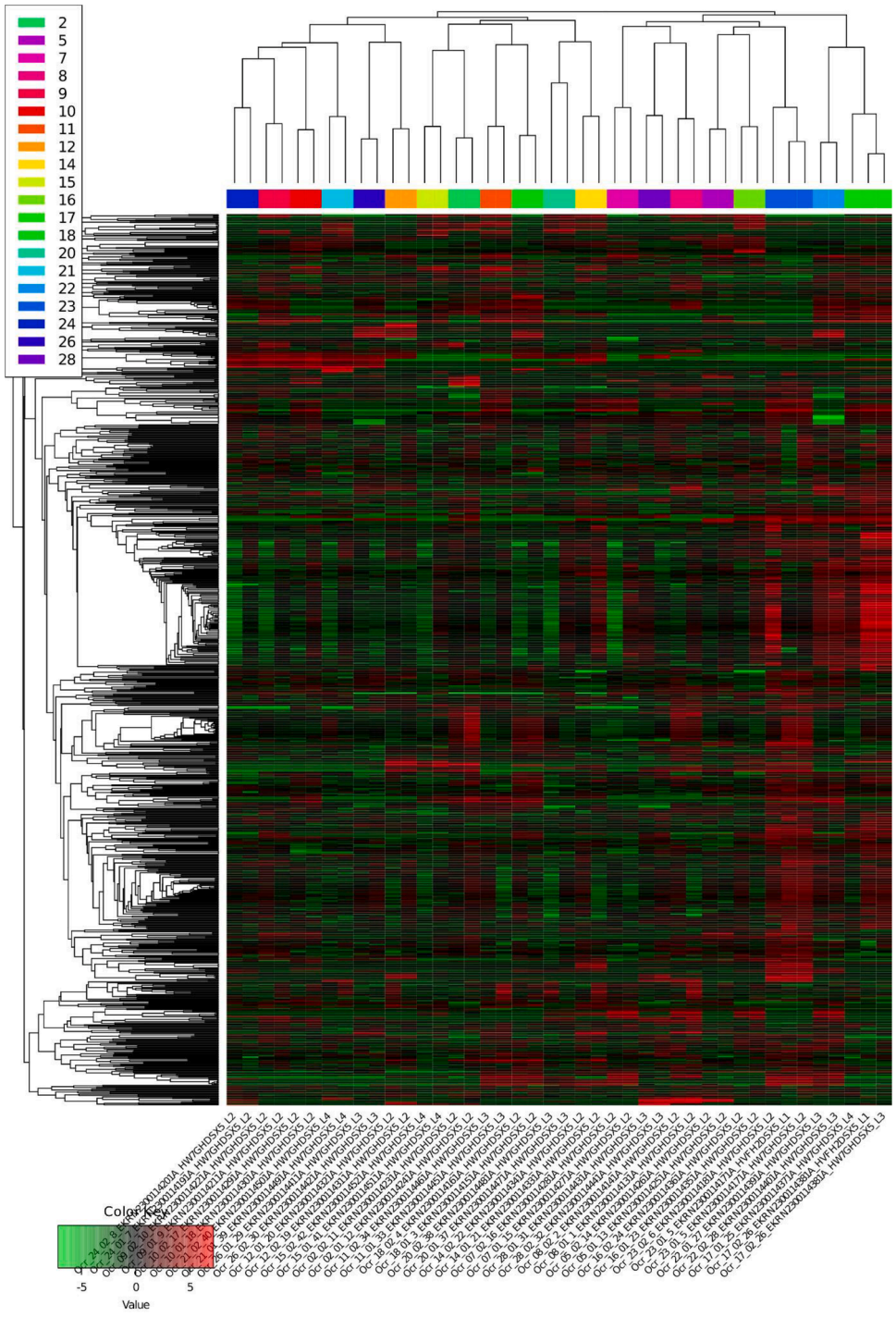

Fig. 1. Heat map and Dendogram of hierarchical clustering of patient samples, samples are coloured by patient number (dendrogram) and named by Ocr_patient number_sample number (first or second visit) unique sample ID (two samples were run twice)_unique run ID. Green=lower expression, red=higher expression.

curated database of HERVs. The same approach was taken to EBV mapping. Nali et al. (Nali et al., 2022) looked at differential expression of HERVs in 7 MS patients with secondary progressive MS and 3 healthy control PBMCs with Illumina RNAseq and a very similar bio-informatics pipeline but had quite different results to us. HERV-W and 18 additional HERV families were upregulated in MS patients. These differences however probably reflect the different disease stage, smaller number of samples and choice of control in their study (pre-treatment from the same patient in ours vs healthy control in Nali et al. (2022)). Similar results to ours indicating a lack of differential expression of HERV transcripts (by RNAseq) in the brains of MS patients and controls were reported by Ekljaer et al. 2021 (Elkjaer et al., 2021).

As EBV detection in blood is low in normal adults and MS patients, with EBV infected cells representing <0.1 % of all B cells (Cocuzza et al., 2014; Babcock et al., 1998) it is perhaps not surprising that we only detected this in one pre-treatment patient. A lack of differential expression of any HERV family was however unexpected given our original hypothesis that ocrelizumab depletion of B cells would decrease the opportunity for viral expression in MS patients. This study does however offer support to the body of evidence that suggests that an aberrant cell type or immune response triggered by these viruses, rather than ongoing high viral expression, drives continued pathology in MS.

Table 2
Differentially expressed transcripts, column 1 is the transcript symbol (gene name), column 2 the ensmbl identifier for the specific transcript, Column 3 the log fold change and column 4 the adjusted P value.

Transcript	ensembl_ID	Log fold change	Adjusted P value
IGHM	ENSG00000211899	−2.17454	0.001233
CD79A	ENSG00000105369	−2.09427	0.001233
CD22	ENSG00000012124	−2.65276	0.001233
CD74	ENSG00000019582	−0.40444	0.005122
IGHD	ENSG00000211898	−2.6254	0.007316
IGHD	ENSG00000278801	−2.6254	0.007316
CD79B	ENSG00000007312	−1.10003	0.013484
BLK	ENSG00000136573	−1.77467	0.020507
MS4A1	ENSG00000156738	−2.66567	0.028423
ENSG00000288133	ENSG00000288133	−1.6356	0.028423
IGHG2	ENSG00000211893	−1.99372	0.028423
VPREB3	ENSG00000128218	−2.3964	0.034593
TCL1A	ENSG00000100721	−1.53778	0.046178
FCER2	ENSG00000104921	−1.65823	0.046178

Current evidence points strongly to an aberrant EBV latency programme and resulting in an expanded T-bet/CXCR3 + memory B cell population that is critical in MS pathology (Soldan et al., 2023; SoRelle et al., 2022).

Data availability

RNAseq reads are available at Genbank Bioproject PRJNA1001267 Accession numbers SRR25490470-SRR25490511

Role of authors

RTar and BG conceptualised the study, BG and RTan recruited patients, RTar performed the RNA extraction and bio-informatics analysis and drafted the manuscript, All authors reviewed and edited the manuscript.

Role of funder

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CRediT authorship contribution statement

Rachael Tarlinton: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Radu Tanasescu:** Conceptualization, Data curation, Writing – review & editing. **Claire Shannon-Lowe:** Formal analysis, Writing – original draft, Writing – review & editing. **Bruno Gran:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2024.105597.

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