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## **Plasma EBV microRNAs in paediatric renal transplant recipients.**

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Informed consent: Informed consent was obtained from all individual participants (or parents) included in the study.

**Plasma EBV microRNAs in paediatric renal transplant recipients.**

## ABBREVIATIONS

BART, BamH1A region rightward transcript.

BHRF1, BamH1 fragment H rightward open reading frame 1.

EBV, Epstein Barr virus.

miRNA, microRNA.

## ABSTRACT

**Background:** Epstein-Barr virus (EBV) was the first human virus identified to express miRNAs. To date, 44 mature miRNAs are encoded for within the EBV genome. EBV miRNAs have not been profiled in paediatric renal transplant recipients. In this study circulating EBV miRNA profiles were investigated as novel biomarkers in paediatric renal transplant patients.

**Methods:** Forty two microRNAs encoded within 2 EBV open reading frames (BART and BHRF) were examined in renal transplant recipients who resolved EBV infection (REI), patients who maintained chronic high viral loads (CHL) and patients with acute infectious mononucleosis (IM).

**Results:** Plasma EBV-miR-BART2-5p was present in higher numbers of IM (7/8) and CHL (7/10) compared to REI (7/12) patients. A trend was observed between the numbers of plasma EBV miRNAs expressed and EBV viral load ( $p < 0.07$ ). Several EBV-miRs including BART7-3p, 15, 9-3p, 11-3p, 1-3p and 3-3p were detected in IM and CHL patients only. The lytic EBV-miRs, BHRF1-2-3p and 1-1, indicating active viral replication, were detected in IM patients only. One CHL patient developed PTLN after several years and analysis of 10 samples over a 30 month period showed an average 24 fold change in plasma EBV-miR-BART2-5p compared to the CHL group and 110 fold change compared to the REI group.

**Conclusions:** Our results suggest that EBV-miR-BART2-5p which targets the stress-induced immune ligand MICB to escape recognition and elimination by NK cells may have a role in sustaining high EBV viral loads in CHL paediatric kidney transplant recipients.

**Keywords** EBV miRNA, renal transplant recipients, plasma EBV-miR-BART2-5p, EBV

viral load

## Introduction:

EBV infection is the causative agent of infectious mononucleosis and is associated with post-transplant lymphoproliferative disease (PTLD) in renal transplant recipients. Current strategies to identify patients at risk of EBV complications utilizes surveillance by EBV viral load monitoring. Significant changes in EBV DNA load over time are used to assess the efficacy of antiviral therapy and is also used widely to predict or to detect EBV-associated disorders [1-5]. A critical limitation is that it is impossible to distinguish EBV DNA from the latent EBV reservoir from virions being produced actively by infected cells. Hence, quantitation of EBV DNA from whole blood alone may be viewed as having a limited role in informing on patient management in different clinical contexts [6]. This is highlighted by the observation that renal transplant recipients both with chronically high EBV viral loads and those who resolve EBV infection can remain clinically well [7].

MicroRNAs (miRNAs) are a subset of 18-25 nucleotides of non-coding RNAs which negatively regulate gene expression by targeting complementary sequences in messenger RNAs [8]. They have been demonstrated to play key roles in growth and development, regulation of immunity, cell differentiation, metabolism, apoptosis, cell proliferation, and malignant transformation and their function has been linked to certain human diseases [9-11].

EBV was the first human virus identified to express miRNAs [12]. To date, 44 mature miRNAs are encoded for within the EBV genome, of which 4 miRNAs are derived from the *Bam*H1 fragment H rightward open reading frame 1 (BHRF1), and the remaining 40 are encoded by the *Bam*H1A region rightward transcript (BART) [13]. The potential for EBV miRNAs as biomarkers for monitoring nasopharyngeal carcinoma[14,15] , in pediatric liver transplant recipients [16] and chronic active EBV infection [17] has recently been published. However, EBV miRNAs have not been profiled in paediatric renal transplant recipients. In

1 this study, the profiles of 42 EBV miRNAs were investigated and compared in paediatric  
2 renal transplant patients between those who resolve EBV infection and those patients with  
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4 chronically high EBV viral loads. Differential plasma EBV miRNA expression in healthy  
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6 individuals with infectious mononucleosis is also studied.  
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## Materials and Methods:

### Patients and clinical samples

Paediatric transplant recipients who underwent renal transplantation at the Children's University Hospital Temple Street, Dublin between 2007 and 2010 were included in the study (n=22). Briefly, the primary diseases were renal dysplasia, juvenile nephronophthisis, branchiotorenal syndrome, posterior urethral valves, cystinosis, mitochondrial cytopathy, membranoproliferative glomerulonephritis, focal segmental glomerular sclerosis, anephrosis, mainzer-saldino syndrome and primary oxalosis. One patient was undiagnosed. Ethical approval for the study was obtained from the Research Ethics Committees at Children's University Hospital Temple Street, Dublin 1 and University College Dublin (LS-E-14-65-Dean-Hassan).

Samples were collected prospectively post-transplant according to the protocol set out by Children's University Hospital Temple Street. Samples were taken for EBV viral load measurements weekly for the first 3 months, then fortnightly for the next 3 months and then monthly for the following 6 months and then at annual review clinics. If a patient became symptomatic for EBV infection, irrespective of their follow-up schedule, samples were taken weekly until EBV DNA became undetectable in two consecutive samples. EBV viral load was determined in whole blood using quantitative real-time polymerase chain reaction (qPCR).

CHL patients (n=10) were defined as those who maintained EBV loads >5000 copies/mL in whole blood for a minimum period of 6 months post primary infection or reactivation of infection. REI patients were classified as those who experienced primary infection post-transplant or a reactivation of an existing infection but who resolved infection, and did not have a detectable viral load for more than 6 months (n=12). Demographic characteristics of

the patient groups including age at transplant and EBV serostatus pre-transplant have been previously published [7].

The standard immunosuppressive regimen consisted of basiliximab induction, tacrolimus, prednisolone, azathioprine or mycophenolate. Target tacrolimus trough levels in plasma were 12-15ng/mL for the first 8 weeks after transplant, 8-10ng/mL for the first year and <6ng/mL after the first year. Mycophenolate and azathioprine were stopped when a patient who had detectable EBV DNA developed clinical symptoms or the viral load increased to more than  $\log_{10}^5$  copies/ml. Thereafter there was a marked improvement in the majority of patients.

A cohort of healthy individuals with infectious mononucleosis (IM, n=8) was included in the study.

### **Nucleic acid extraction.**

For the isolation of EBV DNA, commercially available MagNA Pure 96 DNA and Viral nucleic acids (NA) Small Volume kits were used in combination with the MagNA Pure 96 System (Roche, Mannheim, Germany) for the isolation of EBV DNA from 200  $\mu$ l whole blood or plasma for *in vitro* diagnostic purposes. The nucleic acid isolation procedure was performed according to the manufacturer's instructions. Briefly, the sample is lysed and the nucleic acids which are released are denatured. The nucleic acids bind to the silica surface of the added MagNA Pure Magnetic Glass Particles (MGPs) and are magnetically separated from the residual lysed sample and unbound substances. The purified nucleic acids are then eluted in a volume of 100 $\mu$ l.

### **Quantification of EBV DNA.**

1 The Artus ® EBV TM PCR Kit which is ready-to-use system for the *in vitro* quantitation of  
2 Epstein Barr Virus (EBV) DNA (Qiagen GmbH, Hilden, Germany) using the *ABI Prism*  
3 (Taqman) 7500 Analyser was used in accordance with manufacturer's instruction. This kit  
4 contains reagents and enzymes for the specific amplification of a 97 bp region of the EBV  
5 genome and provides 4 standards and an internal Quality Control which is used to prevent  
6 misinterpretation of false negative results caused by inhibition of the amplification process or  
7 an ineffective nucleic acid extraction. Base matrix is used as the negative control. For  
8 standardization of quantitative EBV DNA detection, a positive control containing EBV DNA  
9 at 17,780 copies/ml (Acrometrix, Thermo Fisher Scientific Ireland), is included in every run.  
10 EBV DNA is reported as copies/ml and in log values and the lower limit of detection of the  
11 assay is 500 copies/ml. External monitoring of EBV DNA quantitation is through  
12 participation in the Quality Control for Molecular Diagnostics scheme.  
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### 30 **miRNA isolation.**

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34 Total RNA was extracted from plasma using the miRCURY™ RNA isolation kit – biofluids  
35 (Exiqon, Vedbaek, Denmark). Plasma was thawed on ice and centrifuged at 3000 x g for 5  
36 min in a 4°C microcentrifuge. An aliquot of 200 µL of plasma per sample was transferred to a  
37 new microcentrifuge tube and 60 µl of Lysis solution BF containing 1µg carrier-RNA per  
38 60µl Lysis Solution BF and RNA spike-in template mixture (UniSp2 and UniSp4) was added  
39 to the sample. The tube was vortexed and incubated for 3 min at room temperature, followed  
40 by addition of 20 µL Protein Precipitation solution BF. The tube was vortexed, incubated for  
41 1 min at room temperature and centrifuged at 11,000 x g for 3 min. The clear supernatant was  
42 transferred to a new collection tube, and 270 µL isopropanol was added. The solutions were  
43 vortexed and transfer to a binding column. The column was incubated for 2 min at room  
44 temperature, and emptied using a vacuum-manifold. 100 µL wash solution 1 BF was added to  
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1 the columns. The liquid was removed using a vacuum-manifold, and 700  $\mu$ L wash solution 2  
2 BF was added. The liquid was removed using a vacuum-manifold. 250 $\mu$ L wash solution was  
3 added and the column was spun at 11.000 x g to dry the columns entirely. The dry columns  
4 were transferred to a new collection tube and 50  $\mu$ L RNase free H<sub>2</sub>O was added directly on  
5 the membrane of the spin column. The column was incubated for 1 min at room temperature  
6 prior to centrifugation at 11,000 x g. The RNA was stored in a -80°C freezer.  
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### 16 **cDNA synthesis and real-time qPCR**

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19 Expression levels of 42 EBV miRNAs with 95% coverage of the EBV genome, encoded  
20 within 2 EBV open reading frames (BART and BHRF) and 4 human miRNAs (hsa-miR-16-  
21 5p, hsa-miR-103a-3p, hsa-miR-423-5p and hsa-miR-30c-5p) were determined using  
22 miRCURY LNA<sup>TM</sup> Universal reverse transcription microRNA PCR analysis (Exiqon,  
23 Denmark). Briefly, 2  $\mu$ L RNA was reverse transcribed in 10  $\mu$ L reactions using the miRCURY  
24 LNA<sup>TM</sup> Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon).  
25 Each RT was performed in duplicates, including an artificial RNA spike-in (UniSp6). cDNA  
26 was diluted 50 x and assayed in 10  $\mu$ L PCR reactions according to the protocol for miRCURY  
27 LNA<sup>TM</sup> Universal RT microRNA PCR; each microRNA was assayed once by qPCR using  
28 assays for miR-23a, miR-30c, miR-103, miR-142-3p, and miR-451. In addition to these  
29 miRNA assays, the RNA spike-ins were assayed. Negative controls excluding template from  
30 the reverse transcription reaction was performed and profiled like the samples. The  
31 amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche,  
32 Germany) in 384 well plates. The amplification curves were analyzed using the Roche LC  
33 software, both for determination of C<sub>p</sub> (by the 2nd derivative method) and for melting curve  
34 analysis.  
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### 60 **Data analysis**

The raw data was extracted from the Lightcycler 480 software. An average Cp was calculated for the duplicate RT's, and evaluation of expression levels was performed based on raw Cp-values. The average expression level of the above 4 human miRNAs which were identified in all plasma samples, was used to normalize the remaining data.

### **Statistical Analysis**

Differential expression of miRNAs was compared using a Student's t-test with Benjamini-Hochberg correction. Mann-Whitney U test was used to compare patient cohorts. The association between 2 variables was assessed by Spearman rank correlation coefficient (r).  $P < 0.05$  was considered statistically significant.

## Results:

Considerable variation was observed in both the number of EBV miRNAs detected (ranging from 0 to 17) and in their average relative expression levels as shown in the heatmaps in Figure 1. In the three study cohorts examined, most EBV miRNAs were detected in the IM group (7.25 on average), followed by the CHL (3.7) and REI (1.9) groups. EBV-miR-BART2-5p and EBV-miR-BHRF1-2-5p were detected most frequently in the patient cohorts. Plasma EBV-miR-BART2-5p was present in higher numbers of IM (7/8) and CHL (7/10) patients compared to REI (7/12). However, there is no significant difference in the relative levels of expression of either EBV miRNA, when comparing REI with CHL, or either transplant group with the IM cohort. Only four other EBV miRNAs were detected in any of the REI samples (BART5-5p, 6-3p, 6-5p and 12). Of note, 3 EBV miRNAs (BART5-5p, 6-5p and 12) were observed in the REI and CHL cohorts only. In contrast, both the IM and CHL groups showed expression of a much broader range of miRNAs, with: 25 in total, with 9 of which were common to both (BART1-3p, 3-3p, 6-3p, 7-3p, 9-3p, 11-3p, 13-3p, 15, and 19-3p). Plasma EBV-miR-BHRF1-2-5p was present in higher numbers of REI (11/12) and IM (6/8) when compared to CHL (5/10) patients.

1 Interestingly, the lytic EBV-miR, BHRF1-2-3p and 1-1, indicating active viral replication,  
2 were detected in IM patients only. A trend albeit not significant ( $p<0.07$ ) was observed  
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4 between EBV viral load and the number of EBV miRNAs expressed in 15 plasma samples  
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6 (Figure 2). Viral loads were not available in the remaining samples.  
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10 Seventeen distinct EBV miRNAs were detected during the analysis of 10 samples over a 30  
11 month period from a patient whom PTLD was clinically diagnosed and subsequently  
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13 clinically improved (Figure 3). The levels and numbers of EBV miRNAs peaked at the time  
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15 of PTLD diagnosis. BHRF1-2-5p appeared, albeit at low levels, 5 months prior to  
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17 development of PTLD and remained detectable for 9 months post-PTLD diagnosis. Several  
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19 other miRNAs including BART14-3p, 17-3p, 3-3p, 5-3p, 6-5p, 8-3p, 6-3p, 21-5p, 9-3p and 7-  
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21 3p were detectable at the time of PTLD diagnosis. Although not significant, a trend was  
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23 observed between EBV viral load and the number of EBV miRNAs expressed in this patient  
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25 over the 10 time points studied ( $p=0.058$ ).  
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34 As with the other cohorts studied, the BART2-5p and BHRF1-2-5p miRNAs were most  
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36 commonly detected, and represented the highest and lowest relative expression levels,  
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38 respectively. Comparing these longitudinal samples with the other study cohort samples, the  
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40 patient who developed PTLD demonstrated an average 24-fold higher level of plasma EBV-  
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42 miR-BART2-5p compared to the CHL group ( $p<0.003$ ), 38-fold higher compared to the IM  
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44 group ( $p<0.02$ ) and 110-fold higher, compared to the REI group ( $p<0.001$ ).  
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50 Principal Component Analysis (PCA) was performed by including the top 7 microRNAs that  
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52 had the largest variation across all samples. An overview of how the samples cluster based on  
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54 this variance is shown in Figure 4. Apart from the time series samples (TP01-TP10), the data  
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56 show no clear clustering of groups and no outliers were detected.  
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## Discussion:

Epstein Barr Virus (EBV) is associated with significant complications in the post-transplant setting due to the administration of immunosuppressive therapy to prevent rejection. As a result the patient's immune response is unable to control virus infection and replication. The greatest risk associated with EBV infection is the development of post-transplant lymphoproliferative disease (PTLD), which is associated with a high risk of morbidity and mortality [18]. Biomarkers utilised to diagnose PTLD and predicting long-term outcomes are needed to improve clinical decision-making following kidney transplantation. To date, monitoring of EBV DNA loads in whole blood, plasma and peripheral blood mononuclear cells from this cohort has been routinely employed to facilitate the diagnosis and management of EBV infection and EBV associated PTLD. However, recent studies from our laboratory and others have demonstrated a number of transplant recipients with chronically high EBV viral loads, thereby calling into question the utility of monitoring EBV viral loads<sup>7</sup>.

The current study represents the first comprehensive clinical investigation of plasma EBV miRNA expression as potential biomarkers for paediatric renal transplant recipients at risk of developing PTLD. Our findings show that although broad variation can be seen in the profile of EBV miRNA expression, the numbers and levels of expression of many of the 42 EBV miRNAs studied were greater in IM and CHL patients compared to the REI cohort. In 15 plasma samples where EBV viral load was available, a trend although not significant ( $p<0.07$ ) was observed between EBV viral load and the number of EBV miRNAs expressed.

The two most commonly detected EBV miRNAs in our study cohorts were EBV-miR-BART2-5p and BHRF1-2-5p miRNAs. EBV-miR-BART2-5p has been reported to target the lytic viral DNA polymerase encoded by BALF5 and has also been shown to influence the



innate immune response by down-regulating expression of MICB, a natural killer cell ligand which contributes to immune evasion [19, 20]. It is not surprising therefore that BART2-5p was detected in 87.5% of IM, 70% of CHL and 58.3% of REI patients. The dramatically higher relative expression levels of EBV-miR-BART2-5p seen in the renal transplant recipient who developed PTLD may indicate that monitoring BART2-5p expression, alongside other EBV miRNAs and/or “traditional” markers of latent and lytic EBV infection, could provide an important clinical marker in the diagnosis of this disorder. Interestingly, once PTLD developed, expression of BART2-5p disappeared.

Although variable, BHRF1-2-5p was present in 75% of IM, 50% of CHL and 91.7% of REI patients. Of interest, in the patient who developed PTLD, BHRF1-2-5p expression appeared at about 5 months prior to PTLD development and disappeared 6 months post PTLD diagnosis. The BHRF1 cluster is believed to be involved in malignant transformation [21]. Other reports have shown that BHRF1 miRNA facilitates progressive growth, in vitro transformation of infected cells and acute systemic EBV infection [22,23]. Furthermore, BHRF1 has been shown to prevent primary B cells from apoptosis [24]. In agreement with these findings, in the current study, the lytic EBV-miR, BHRF1-2-3p and 1-1, indicating active viral replication, were detected in IM patients only. Of note, a study has reported that while interrogating high confidence targets for BHRF1-1, several cellular 3’UTR clusters could be assigned to both BHRF1-1 and BART4 due to off-set seed homology between these two miRNAs [25]. In the current study, one IM patient expressed BART4-3p and 4-5p supporting the idea that not all EBV miRNAs are expressed throughout all stages of latency; this may be a mechanism which allows EBV to ensure that important cellular transcripts are downregulated [26].

Other studies and our findings highlight the important functional roles of EBV miRNAs during the viral life cycle including immune evasion, cell survival and proliferation as well as control of the latent/lytic switch. Our results suggest that EBV-miR-BART2-5p which targets the stress-induced immune ligand MICB to escape recognition and elimination by NK cells may contribute to the sustained high EBV viral loads in a group of chronic high viral load kidney transplant recipients. Furthermore, the expression of miR-BHRF1-2-5p which correlates with the involvement of BHRF1 mRNAs cluster in cell transformation should be monitored as well. Future studies in a larger cohort of patients is required to provide additional data to assess the role of EBV miRNAs in the development of malignant EBV complications in transplanted patients and to evaluate their detection as biomarkers for latent and lytic EBV infection.

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## Figure captions:

**Fig 1** Heat map illustrating the relative expression levels of EBV miRNAs in 8 patients with infectious mononucleosis (IM), 12 paediatric renal recipients who resolved EBV infection (REI) and 10 renal recipients who were chronic high EBV viral load carriers (CHL). Colours indicate the grading from highest (green) to lowest (red) levels of miRNA expression.

**Fig 2.** Correlation of EBV Viral Load (copies/ml) with the number of EBV miRNAs expressed in 15 plasma samples; CHL (n=8) (■), REI (n=5) (Δ), IM (n=2) (○) gave a value of 0.48,  $p < 0.07$ . Viral loads were not available in the remaining samples.

**Fig 3.** Heat map illustrating the relative expression levels of plasma EBV miRNAs at various time points in a renal transplant recipient who developed PTLT at time denoted by pink line. Colours indicate the grading from highest (green) to lowest (red) levels of miRNA expression.

**Fig 4.** Principal Component Analysis was performed by including the top 7 microRNAs that had the largest variation across all samples; (CHL (n=10), REI (n=12), IM (n=8), Timepoints (TP01-TP10) in one patient who developed PTLT.









