Analysis of the differential expression of microRNAs in response to single-stranded RNA virus infections in Huh7 cells

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Abstract

The expression of microRNAs (miRNAs) in cells and their translational repression of targeted genes can be severely altered in response to RNA virus infections, potentially leading to positively or negatively regulating viral replication, activating pro-inflammatory pathways or promoting tumorigenesis such as in hepatocellular carcinoma (HCC). Here, we analysed miRNA-seq data of infected and non-infected Huh7 cells from separate experiments involving Zaire Ebola virus (ZIKV), Hepatitis C virus (HCV) and Hepatitis E virus (HEV) to determine which miRNAs were differentially expressed in response to infection and identify similarities and variations between different viruses, then predicted their target genes to suggest their function. We showed the overall miRNA expression profiles in cells varied more due to the experimental conditions rather than their infection status, and only a few miRNA expression profiles matched those found in previous studies, including miR-146a-5p, which is upregulated in all infected cells and was implicated in regulating NF-kB signalling and miR-122-5p, which was downregulated in HCV and HEV infections and is believed to be implicated in viral replication and tumorigenesis. Our results suggested that the use of miRNAs as biomarkers for diagnoses or therapeutic targets for treatments should only be used in conjunction with other methods due to the potential variation in expression levels between patients.

1. Introduction

MicroRNAs (miRNAs) are short noncoding RNAs typically 20-23 nucleotides long involved in the post-transcriptional regulation of gene expression in animals and plants through Watson-Crick base pairing to complementary sequences at the 3’ untranslated regions (UTRs) of mRNA transcripts. With miRNAs being believed to regulate over 60% of genes in mammals [1], they are suggested to be implicated in almost all cellular functions and their dysregulation has been associated with various diseases including cancers [2], genetic disorders, lifestyle ailments and pathogenic infections [3].

The biogenesis of miRNAs in animals involves cytoplasmic transcription from RNA polymerase II, resulting in a pri-miRNA stem-loop transcript that is subsequently cut by the RNase III Drosha and DGCR8 protein into a pre-miRNA molecule that is exported to the nucleus. The RNase III Dicer then cleaves the hairpn to form a mature miRNA:miRNA duplex derived from the 5’ and 3’ arm of the pre-miRNA stem loop . One strand of the duplex is incorporated into the RISC, where the miRNA sequence acts as a guide to bind the complex to targeted mRNA 3’ UTRs, while the RISC-bound protein Argonaute (Ago) cleaves the target if there is perfect complementarity between the miRNA and mRNA sequences, with imperfect binding merely supressing the translation of the transcript [3, 4].

One strand from the mature miRNA:miRNA* duplex may direct mRNA repression, where usually the more functional guide sequence of the 5’ and 3’ derived strands is preferentially selected to be incorporated into RISC, which is believed to result from a higher thermodynamic instability at the 5’-end or by the base composition of the preferred strand [5]. Though the less preferred strands (miRNA*) are more likely to be degraded, recent studies have shown they can be co-expressed with the preferred strand [6] and 5’ and 3’ derived species of the same miRNA can both function by targeting separate mRNAs [7].

The increased use of deep next-generation sequencing (NGS) of small RNAs has led to a large number of publicly available datasets providing an insight into the expression of miRNAs and their biogenesis across various organisms, from which the public repository miRBase (http://www.mirbase.org/) has identified and named thousands of miRNA hairpin and mature sequences [8]. The current nomenclature used by miRBase distinguishes different species of the same mature miRNAs by the arm they are derived from, such as miR-xx-5p (5') or miR-xx-3p (3') [9].

While some animal miRNAs can bind to targeted transcripts with perfect complementarity and induce mRNA cleavage, such as miRNA-196a-5p which can target and degrade human *HOXB8* transcripts [10], most animal miRNAs suppress the translation of mRNAs that are not entirely complementary. Complementarity between targeted transcripts and a region at nucleotides 2-8 from the miRNA 5' end known as the "seed" site is believed be the leading determinant in the efficacy of translational repression by miRNAs, as the expression of targeted genes are usually only significantly downregulated when miRNA:mRNA paring involves a perfectly complementary seed sequence [11].

Many methods have been developed to predict the targets of miRNAs *in silico* based on the complementarity between their mature sequences and those of mRNA sequences [12]. TargetScan (v7.2, http://www.targetscan.org/) is a target prediction method that primarily focuses on potential binding sites for the miRNA seed region in the 3' UTRs of the gene transcripts, thus providing an indication of the functional roles for miRNAs when targeting and supressing annotated genes [13].

The identification of miRNAs and the prediction of their gene targets in small RNA-seq datasets of viral infected cells has shown viruses can greatly alter cellular miRNA expression which may dysregulate gene expression and positively or negatively impact viral replication [14]. Numerous infectious diseases in humans result from RNA viruses, such as Zika virus (ZIKV), Dengue virus (DENV), West Nile Virus (WNV), Zaire ebola virus (EBOV), Hepatitis C virus (HCV) and Hepatitis E virus (HEV) [15-20], which unlike DNA viruses, are predominantly reliant on the host's cellular functions for their replication due to their small genome size [21], suggesting miRNAs may play a critical role in the pathogenies of RNA viruses.

Some RNA viruses lead to the global repression of miRNAs in cells, as studies in ZIKV infections of astrocytes have shown almost all miRNAs are downregulated after 72 hours of exposure [15], leading to the dysregulation of numerous genes involved in normal physiological functioning, which is believed to result from disruption to the expression of genes encoding Drosha, DGCR8 and Dicer responsible for miRNAs biogenesis [22]. The inhibition of components of the miRNA biogenesis pathway through downregulation of gene expression appears to be conserved in infections by flaviviruses, as it occurs in Dengue virus (DENV) [16] and Yellow Fever virus (YFV) infection [23] or alternatively by producing viral derived pre-miRNA-like molecules that compete with host pre-miRNAs in West Nile Virus (WNV) infections [17].

Infections by other RNA viruses typically result in a small number of miRNAs that are significantly upregulated such as miR-215, which is highly expressed in the DNA virus HBV [24] and HCV infections [19], and has been suggested to inhibit the expression of *TRIM22,* leading to the positive regulation of NF-κB activity in the immune response [25]. The upregulation of miR-21-5p has also been shown in EBOV [18], HEV [20] and HCV infections, which is believed to repress interferon-α (IFN-α) signalling to evade the host immune response, thus promoting viral replication [26].

The downregulation of specific miRNAs is less common in RNA virus infections, but some may play a key role for viral replication, such as the downregulation of miR-432 in Japanese encephalitis virus (JEV), which supresses the expression of genes encoding the regulation of cytokine signalling in the immune response [27]. It is currently believed miR-122-5p expression is reduced in HCV infected hepatocytes, though this has previously been contested [28] and the mechanisms of its downregulation are unclear [29].

Host derived miRNAs have also been suggested to regulate viral replication following infection by directly targeting the viral genome, as an analysis of the HBV genome indicated potential binding sites for miR-199a-3p and miR-210, the upregulation of which were shown to supress HBV replication proteins in infected hepatomas [30]. Conversely, some miRNAs, such as miR-122-5p, have been shown to positively regulate viral replication by binding to 5' sites in the genomes of HCV and HEV [31-32]. In Ebola virus (EBOV), miR-145-3p has been shown to target the 5' UTRs of the EBOV genome, though its impact on viral replication and expression profile in EBOV infection is not yet characterised [33].

Some differentially expressed miRNAs effect genes with no obvious function regarding viral infection, with ZIKV being shown to upregulate multiple miRNAs leading to the suppression of *MCPH* genes implicated in foetal brain development, resulting in microcephaly [34]. Many miRNAs are tumour suppressors, such as miR-122-5p [35] or tumorigenic such as miR-146a-5p [36], suggesting their dysregulated expression can result in the development of carcinomas, such as hepatocellular carcinoma (HCC) in HCV infections.

Analysing the expression of miRNAs has been considered for diagnosing the nature and severity of virally infectious diseases, with high levels of serum miR-21-5p being a biomarker for acute liver failure in HEV infections [20], while low levels of miR-122-5p may be used as a biomarker for the development of HCC in HCV infected patients [37]. It has also lead to development of potential treatments for infections through targeting and inhibiting miRNAs implicated in viral replication [38].

A comparison between miRNA-seq data in different RNA viral infectious would be expected to identify expression profiles that are shared or distinct, thus possibly suggesting new targets for therapeutic treatments or biomarkers for diagnoses of diseases caused by viral infection. Here, miRNA-seq datasets from multiple studies using Huh7 cells were analysed to determine and compare the differential expression of miRNAs in hepatitis C virus, hepatitis E virus, Dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV) and Ebola (EBOV). We further predicted target genes of differentially expressed miRNAs, and suggest their function through GO term enrichment.

2. Methods

2.1 Processing of miRNA-Seq datasets

The study analysed small RNA-seq datasets from 5 independent experiments that used control and viral infected Huh7 or Huh7.5.1 cells (Table 1), three of which were obtained from The European Bioinformatics institute (EMBL-EBI) [39] (https://www.ebi.ac.uk/) database in FASTQ format, including investigations on HEV, HCV, DENV and WNV infection. FastQC [40] was used to identify adapter sequences that were subsequently trimmed using Cutadapt [41] then converted to FASTA format (Appendix 1A). Reads over 30 nt or under 15 nt were filtered, after which the abundance of each sequence was counted in all datasets using a custom Python [42] script (Appendix 1B). Two additional small RNA-seq datasets were obtained from a collaborator of Moxon at the Boston University, including investigations in EBOV and ZIKV infection, the reads of which were already trimmed and quantified.

Table 1. miRNA-seq datasets used in this investigation

Virus	Cell line	Infected replicates	Control replicates	Accession	Reference
ZIKV	Huh7	2	2	-	*Boston University*
EBOV	Huh7	4	4	-	*Boston University*
HEV	Huh7	2	2	PRJNA381374	*Paliwal et al, 2017 [43]*
HCV	Huh7.5.1	2	2	PRJNA298741	*Bandiera et al, 2016 [36]*
WNV	Huh7	1	0	PRJNA245819	*Bogerd et al, 2014 [44]*
DENV	Huh7	1	0	PRJNA245819	*Bogerd et al, 2014 [44]*

Reads in each dataset were mapped to all known miRNAs in miRBase (http://www.mirbase.org/) using the PatMaN alignment tool [45] set to include no gaps or mismatches (2A), following which a custom Python script filtered the reads remove non-human miRNAs (Appendix 2B). The human miRNAs and their counts in each datasets were compiled into a dictionary (Appendix 3A), where they were written into a single CSV file (Appendix 3B).

2.2 Differential miRNA expression analysis

Differential expression analysis of the quantified miRNA counts was performed using RStudio [46] with the packages *edgeR* [47] and *limma* [48], where miRNA counts in replicates from the same experiment were grouped together, with subgroups distinguishing infected cells from controls.

To remove low expressed miRNAs that are unlikely to be statistically significant, miRNA counts were first normalised to counts per million (CPM), which avoids a bias towards miRNAs in larger libraries. Libraries were filtered so only miRNAs in which 2 or more replicates that had a CPM above 1 were included (Appendix 4), which was chosen as a high cut-off point as the miRNA library sizes were relatively small (< 10 million), with at least 2 libraries being required because there was a minimum of 2 replicates for each subgroup.

The miRNA libraries were normalised for composition bias, which is important to reduce the effect of a small number of highly abundant miRNAs dominating the sequencing, thus leading to smaller counts for other miRNAs. The normalisation factors for each replicate were calculated using a trimmed mean of M-values (TMM) between each pair of replicates, resulting in the counts for each library being scaled up or down [49].

Using *limma*, we performed a voom transformation (Appendix 5), in which miRNA counts were converted to log2-CPM (logCPM) and the mean-variance relationship was estimated to determine a precision weight for each observation for linear modelling in the differential expression analysis. We used the voom method as it has been shown to have the lowest false discovery rate (FDR) and most powerful detection for differentially expressed sequences compared to other packages, especially in data such as ours with unequal library sizes [50].

The count data was used to produce a multidimensional scaling plot (MDS), a form of principle component analysis (PCA) which determines the largest sources of variation in the data. The leading log2-fold change (logFC), which represents the root-mean-square of the largest 500 logFC between samples, was used to determine the variation between replicates. The relationship between replicates was also analysed through hierarchical clustering, using the *gplots* package [51] to produce a heatmap in the top 500 variable miRNAs (Appendix 6).

The mean logCPMs in the control and infected subgroups were estimated alongside the mean-wise variances, after which a pairwise comparison was performed between the subgroups in each experimental group. An empirical Bayes function was used to reduce the variability across miRNAs and perform moderated t-statistics to determine significance (Appendix 7), after which the significantly up- or downregulated miRNAs ($Q < 0.05$) were compared across experimental groups to determine which were shared between different viral infections (Appendix 8).

2.3 Target prediction of differentially expressed miRNAs

The gene targets for significantly up- or downregulated miRNAs ($Q < 0.05$) were predicted using TargetScan [13] (v7.2, http://www.targetscan.org/) which searches for sites in human mRNA transcript 3' UTRs that are complementary to the miRNA seed region. Gene ontology (GO) enrichment analysis was performed on the top 50 most significant targets for differential miRNAs using g:Profiler [52] (https://biit.cs.ut.ee/gprofiler/gost) to determine the function of said targets, where only significant GO terms (adjusted P-value < 0.05) were selected.

2.4 Statistical analysis

An empirical Bayes function was used to estimate the moderated t-statistics and the resulting p-values, from which the FDR was controlled using the Benjamini-Hochberg correction to determine the adjusted p-value (Q-value). This multiple-comparison procedure is essential to reduce the high rate of false positives that arise in single-inference procedures and involves the multiplication of the p-value by the number of miRNAs and dividing by the p-value rank [53]. Up- or downregulated miRNAs were only considered significantly differentially expressed if the adjusted p-value, $Q < 0.05$.

As GO terms are hierarchically related and intersecting, the Benjamini-Hochberg correction is unsuitable for correcting the P-value in GO analyses because it would consider terms to be independent from one another. For this reason, G:Profiler uses a custom algorithm to correct the P-value, taking into account the fact that genes associated to a specific term will also be associated to a more general term [52]. Terms for miRNA target genes were only considered significant with an adjusted P-value < 0.05.

3. Results

3.1 Variation between experimental groups and control/infected subgroups

In the analyses of the 22 miRNA-seq datasets of Huh7 or Huh7.5.1 cells, replicates derived from the investigation in ZIKV infected cells included a large number of reads that mapped to non-human miRNAs suggestive of contamination, resulting in the exclusion of all datasets from the ZIKV experiment in the differential analysis, while datasets derived from the investigation in DENV and WNV infection were also discarded as they contained relatively low miRNA library sizes compared to those from other investigations. The remaining 16 datasets were derived from 3 experiments investigating EBOV, HCV and HEV infection.

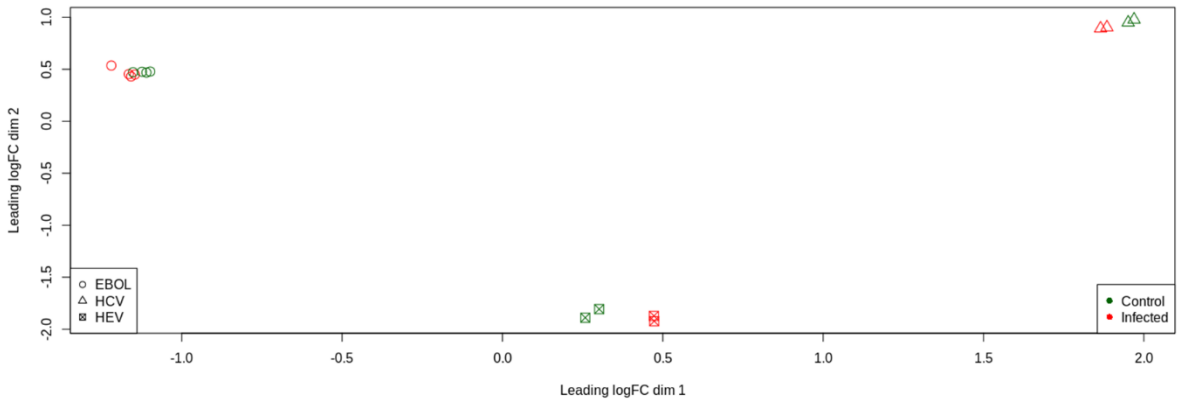

Figure 1. Multidimensional scaling (MDS) analysis of the leading log-2 fold change (logFC) in control (green) and infected (red) cells for experimental groups including those involving EBOV (circle), HEV (square) and HCV (triangle) infections. Leading logFC dim 1 represents the variation between miRNA expression profiles, while leading logFC dim 2 represents differences between library sizes. Cells within experimental groups clustered together, with a leading fold change of around 8-fold between EBOV-HCV, 2.5-fold between EBOV-HEV and 2.5-fold between HEV-HCV.

The MDS analysis (Figure 1) on these datasets showed the miRNA expression profile of cells derived from the same experiments in viral infection clustered closely together with a large separation between other experimental groups, with small separations between respective control and infected cells, suggesting the variation in miRNA expression was greater between different experiments than between control and infected cells. The greatest variation appeared between EBOV and HCV, experimental groups, with the smallest between EBOV and HEV groups.

Within each experimental group, the largest variation in miRNA expression profile was found in HEV infections, where infected cells show a greater overall miRNA expression than in controls. The lowest variation was found in EBOV infections, which alongside HCV, had a smaller miRNA expression in infected cells than in controls.

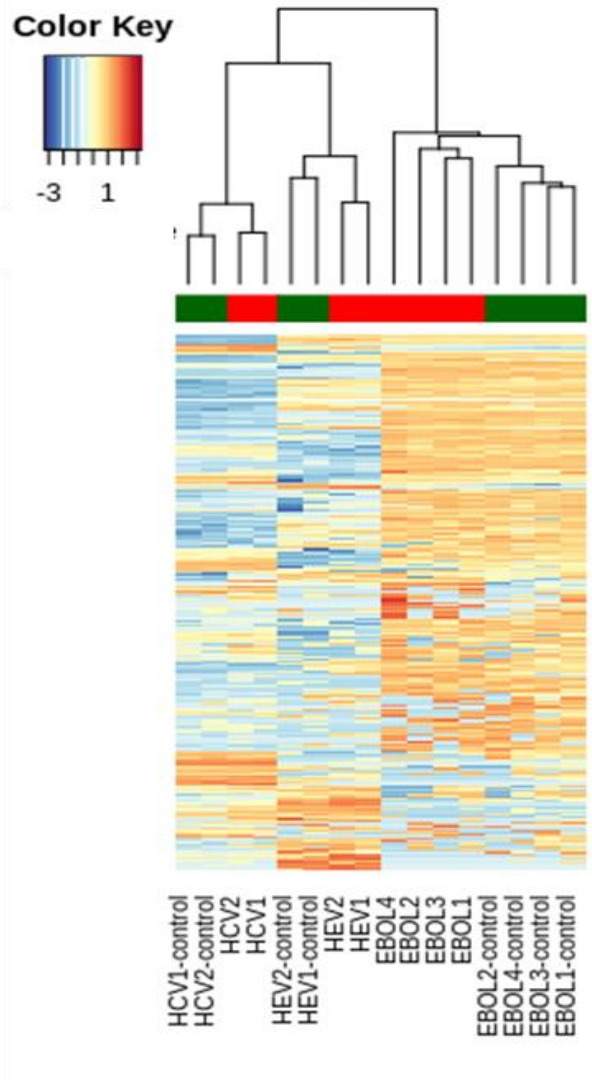

Figure 2. Heatmap of the top 500 most variable miRNAs expressed in control (green) and infected (red) cells in experimental groups including EBOV, HCV and HEV infections. HCV and HEV cluster together first separately from EBOV infected cells, while the second cluster separates all groups and the third separates the control and infected cells.

Hierarchical clustering of the top 500 variable miRNAs shows that in contrast to the MDS, there was a high association of miRNA expression profiles between HEV and HCV experimental groups compared to the EBOV group in the initial cluster tree. Further clustering however was consistent with the MDS in that all cells were separated into their experimental groups with the final cluster separating between control and infected subgroups within experimental groups

3.2 Differentially expressed miRNAs shared between viral infected cells

Using a pairwise comparison between control and infected Huh7/Huh7.5.1 cells in each experimental group to find differentially expressed miRNAs in each viral infection, we selected up- and downregulated miRNAs with a significant adjusted P-value (Q < 0.05) (Appendix 9).

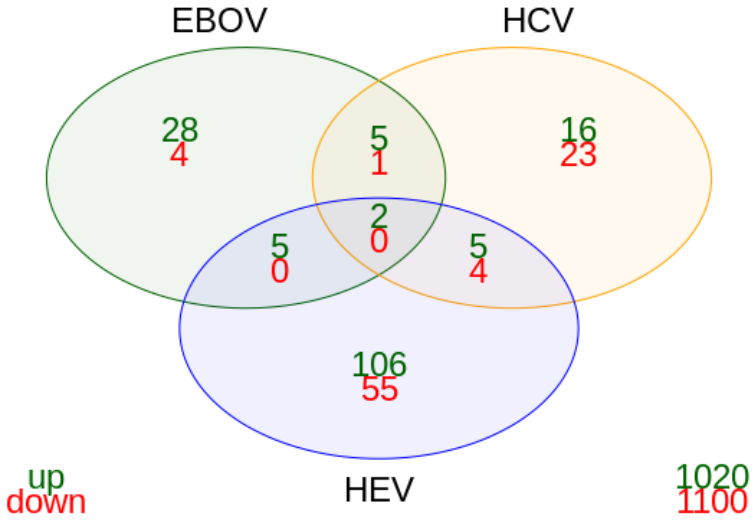

Figure 3. Venn diagram comparing miRNAs that are upregulated (green) or downregulated (red) in EBOV (green circle), HCV (yellow circle) and HEV (blue circle) infections. Differentially expressed miRNAs with a significant adjusted P-value (Q < 0.05) were included in the comparison. The total number of up- and downregulated miRNAs (including non-significant) are found on the bottom-left.

A total of 254 miRNAs were shown to be significantly differentially expressed, of which 167 were upregulated, including 40 in EBOV (Appendix 10A), 28 in HCV (Appendix 11A) and 118 in HEV infected cells (Appendix 12A). Of these miRNAs, 2 were found to be significantly upregulated in all experimental groups, including miR-146a-5p and miR-21-3p (Table 2).

Table 2. Significantly upregulated miRNAs in all viral infected cells

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
miR-146a-5p	1.111138	0.892156	2.274723	2.17E-08
miR-21-3p	0.884415	1.171939	0.898915	2.62E-06

The data showed 17 upregulated miRNAs were shared between at least two groups, with 5 in EBOV and HCV infections (Table 3) including miR-12136, miR-4516, miR-27a-5p, miR-663a and miR-4284, 5 between EBOV and HEV infections (Table 4) including miR-143-3p, miR-145-5p, miR-143-5p, miR-193b-5p and miR-146a-3p and 5 between HCV and HEV infections (Table 5) including miR-1-3p, miR-486-5p, miR-9985, miR-10a-5p and miR-584-5p.

Table 3. Significantly upregulated miRNAs in EBOV and HCV infected cells

miRNA	LogFC			Q-value
	EBOV	HCV	HEV	
miR-12136	4.893818	1.266745	-1.63695	3.81E-08
miR-4516	3.704678	4.312909	-0.62559	9.75E-07
miR-27a-5p	2.05858	1.367763	0.339352	4.09E-06
miR-663a	3.389883	4.110826	0.154249	7.13E-05
miR-4284	2.88265	3.928663	-0.7014	4.39E-04

Table 4. Significantly upregulated miRNAs in EBOV and HEV infected cells

miRNA	LogFC			Q-value
	EBOV	HCV	HEV	
miR-143-3p	2.525543	1.072625	4.480795	5.01E-09
miR-145-5p	2.24554	0.45765	3.026289	1.54E-07
miR-143-5p	3.400033	0.553259	5.070597	6.78E-07
miR-193b-5p	1.053746	0.711397	1.66041	2.39E-05
miR-146a-3p	1.701549	1.061487	2.778064	1.11E-04

Table 5. Significantly upregulated miRNAs in HCV and HEV infected cells

miRNA	LogFC			Q-value
	EBOV	HCV	HEV	
miR-1-3p	2.196519	7.165276	4.197624	2.62E-08
miR-486-5p	-0.09677	3.587168	1.598476	7.03E-07
miR-9985	1.210287	2.697185	2.077037	2.33E-06
miR-10a-5p	-0.803	1.59663	3.239012	7.22E-05
miR-584-5p	0.501745	1.673188	2.369547	7.22E-05

Downregulated miRNAs, despite comprising the majority without significance testing, made a minority of around a third of the total significant differentially expressed miRNAs, including 87 in all groups, 5 in EBOV (Appendix 10B), 28 in HCV (Appendix 11B) and 59 in HEV infected cells (Appendix 12B). Compared to those that were upregulated, fewer downregulated miRNAs were shared between groups, with none being shared between EBOV and HEV infected cells or in all groups. Between HCV and HEV infections, 4 downregulated miRNAs were found (Table 6) including miR-4454, miR-122-5p, miR-19-3p and miR-20a-5p, while in EBOV and HCV infections, 1 downregulated miRNA was found (Table 7), miR-483-5p.

Table 6. Significantly downregulated miRNAs in HCV and HEV infected cells

miRNA	LogFC			Q-value
	EBOV	HCV	HEV	
miR-4454	0.275907	-2.19656	-0.97664	1.09E-05
miR-122-5p	-0.76975	-1.42995	-1.58345	1.09E-05
miR-19b-3p	-0.52246	-1.13978	-0.70605	2.70E-04
miR-20a-5p	-0.60403	-1.122	-0.92456	3.10E-04

Table 7. Significantly downregulated miRNAs in EBOV and HCV infected cells

miRNA	LogFC			Q-value
	EBOV	HCV	HEV	
miR-483-5p	-1.16498	-1.62891	0.149474	3.83E-05

3.3 Differentially expressed miRNAs exclusive to one viral infection

Our analysis showed 232 miRNAs were significantly differentially expressed ($Q < 0.05$) in only one viral infection, of which 150 were upregulated and 82 downregulated. In EBOV infected cells, 28 miRNAs were exclusively significantly upregulated, the top 10 of which (Table 8) included miR-145-3p, previously shown to target the EBOV genome [33]. EBOV infections also downregulated 4 miRNAs exclusively (Table 9), including miR-122b-5p, which is derived from the same genome cluster as miR-122-5p [8], downregulated in HCV and HEV infections.

Table 8. Top 10 significantly upregulated miRNAs exclusively in EBOV infected cells

miRNA	LogFC			Q-Value
	EBOV	HCV	HEV	
miR-145-3p	6.74833	0.553259	1.725039	7.51E-09
miR-4449	5.996027	0.77897	-0.63744	4.79E-08
miR-4443	5.338923	0.032022	0.298638	6.74E-08
miR-4286	2.611622	-0.0756	0.286311	9.53E-06
miR-4488	1.8111	1.79553	-2.17608	1.49E-04
miR-23a-5p	1.683092	1.021938	-0.14602	1.62E-04
miR-10401-5p	3.303428	-0.2393	-1.41965	2.10E-04
miR-3651	1.534387	-0.74813	-0.65694	2.68E-04
miR-4485-3p	3.835294	3.676645	-2.90637	2.68E-04
miR-132-5p	1.847802	1.214349	0.056475	2.68E-04

Table 9. Significantly downregulated miRNAs exclusively in EBOV infected cells

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
miR-1303	-1.50019	-0.56292	-0.34293	7.54E-06
miR-345-5p	-0.76233	-0.5929	-0.31996	5.94E-04
miR-122b-5p	-1.05028	-0.86796	-0.10034	2.38E-03
miR-106a-5p	-0.75708	-0.34608	-0.79001	3.37E-03

In HCV infections, the most significantly upregulated miRNAs (Table 10) included miR-212-3p, which is implicated in tumour suppression [54]. Significantly downregulated miRNAs included miR-210-5p, which has been shown to be downregulated in previous studies (55].

Table 10. Top 10 significantly upregulated miRNAs exclusively in HCV infected cells

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
miR-1246	0.140996	3.926918	-2.06458	2.41E-06
miR-133b	2.003933	5.389605	0.554305	2.56E-05
miR-133a-3p	2.03125	6.300261	-0.51581	2.66E-05
miR-206	0.837183	4.61865	-0.41357	0.000145
miR-212-3p	0.02185	3.052504	0.05349	0.000269
miR-4463	1.210287	3.461109	-2.0872	0.000269
miR-136-3p	1.210287	3.455094	-0.63108	0.000646
miR-4326	1.210901	3.075783	0.895885	0.000918
miR-4508	1.876666	3.535224	-1.42508	0.001042
miR-127-3p	0.001671	1.679035	0.162971	0.001492

Table 11. Top 10 significantly downregulated miRNAs exclusively in HCV infected cells

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
miR-192-5p	-0.2092	-0.93796	1.503776	3.83E-05
miR-210-5p	-0.49004	-2.40966	1.717507	4.39E-05
miR-210-3p	-0.43849	-2.34181	1.069348	4.97E-05
miR-769-5p	-0.47053	-1.27355	-0.16911	7.63E-05
miR-885-3p	-0.60713	-1.58285	-0.64373	1.95E-04
miR-148a-5p	-0.31407	-0.94895	-0.71345	4.06E-04
miR-181a-2-3p	-0.5957	-0.81054	1.056253	5.51E-04
miR-769-3p	-0.27436	-1.11759	-0.92006	5.51E-04
miR-19a-3p	-0.55935	-1.06822	-0.57303	6.21E-04
miR-301b-3p	-0.02649	-1.08736	0.587685	6.59E-04

The top 10 significantly upregulated miRNAs in only HEV infected cells included miR-21-5p, the elevated expression of which has previously been demonstrated in said viral infections [20], as well as EBOV and HCV [18, 26].

Table 12. Top 10 significantly upregulated miRNAs exclusively in HEV infected cells

miRNA	LogFC			Q-value
	EBOV	HCV	HEV	
miR-27a-3p	0.547757	0.688123	1.795614	1.72E-06
miR-489-3p	0.47244	-0.1277	2.86366	1.72E-06
miR-574-5p	-0.31633	-0.94188	2.781543	3.22E-06
miR-215-5p	0.233697	0.812956	2.416447	3.81E-06
miR-28-5p	0.148858	-0.33933	1.808775	3.81E-06
miR-196a-5p	-0.61702	-0.66092	2.344264	3.81E-06
miR-21-5p	0.045331	0.356125	1.964813	4.04E-06
miR-26b-5p	-0.1352	-0.55671	2.436166	6.24E-06
miR-582-5p	-0.21638	-0.09063	2.140092	9.70E-06
miR-194-5p	-0.15808	-0.66007	1.786591	2.92E-05

Table 13. Top 10 significantly downregulated miRNAs exclusively in HEV infected cells

miRNA	LogFC			Q-value
	EBOV	HCV	HEV	
miR-92a-1-5p	0.011363	-0.26094	-3.3245	6.23E-06
miR-1246	0.140996	3.926918	-2.06458	1.02E-05
miR-3909	-0.13282	-0.3807	-1.80618	3.94E-05
miR-1257	-0.52847	2.138625	-4.07675	1.19E-04
miR-33b-3p	-0.82772	-0.81637	-2.63659	1.67E-04
miR-187-3p	-0.85645	-1.44492	-1.67954	4.06E-04
miR-18a-3p	-0.39807	-0.27044	-1.8978	4.55E-04
miR-1185-2-3p	1.60595	-1.03221	-4.00844	7.12E-04
miR-17-5p	-0.50203	-1.04843	-1.17406	7.73E-04
miR-1185-1-3p	1.208067	-1.03221	-4.00844	8.37E-04

3.4 Predicted targets and functions for differentially expressed miRNAs shared between viral infections

TargetScan was used for significantly up- or downregulated miRNAs to predict their gene targets, the top 50 most significant of which were put into g:Profiler for GO term enrichment. In miRNAs that were upregulated in all viral infections, gene targets for miR-146a-5p (Appendix) included *IRAK1, TRAF6* and *CARD10* implicated in the activation of NF-kB inducing kinase (NIK) (Table 14). No significant biological processes were found for genes targeted by miR-21-3p.

Table 14. GO term enrichment of target genes for significantly upregulated miRNAs in all viral infections

miRNA	Adjusted P-value	GO: Biological Process	Genes
miR-146a-5p	1.86E-02	activation of NF-kappaB-inducing kinase activity	*IRAK1, TRAF6, CARD10*

In EBOV and HCV infections, the target genes for upregulated miRNAs including miR-663a showed significant GO terms for polyadenylate-binding protein (*PABPC*) related to poly (U) RNA binding and miR-4516, where the target genes encoding rhodopsin (*RHO*), osteopontin (*OPN*) and ATP-binding cassette (*ABC*) transporter A4 were implicated in light detection (Table 15). No significant terms were found for miR-12136, miR-27a-5p or miR-4284.

Table 15. GO term enrichment of target genes for significantly upregulated miRNAs shared between EBOV and HCV infections

miRNA	Adjusted P-value	GO: Biological processes	Genes
miR-4516	8.99E-03	protein-chromophore linkage	*RHO, OPN1MW, OPN1MW2*
miR-4516	1.80E-02	phototransduction	*RHO, ABCA4, OPN1MW, OPN1MW2*
miR-663a	2.06E-02	poly(U) RNA binding	*PABPC1L2B, PABPC1L2A, DIS3L2*
miR-4516	4.21E-02	detection of light stimulus	*RHO, ABCA4, OPN1MW, OPN1MW2*

In EBOV and HEV infections, target genes for upregulated miRNAs found miR-145p showed significant GO terms related to functions involving actin filaments, while miR-146a-3p targets genes predominantly encoded zink finger (*ZNF*) proteins involved in the transcriptional activity of RNA polymerase II (Table 16). No terms were found for genes targeted by miRNAs that were downregulated.

Table 16. GO term enrichment of target genes for significantly upregulated miRNAs shared between EBOV and HEV infections

miRNA	Adjusted P-value	GO: Biological Process	Genes
miR-146a-3p	4.64E-05	regulation of transcription by RNA polymerase II	*ZNF23, ZNF233, ZNF300, ZNF655, ZNF544, ZNF487, ZNF225, ZNF667, ZNF24, ZNF616, ZNF286A, ZNF286B, GRIK1-AS2, KLF12, MDM4, ZKSCAN1, ZNF268, ZNF140, ZNF30, ZNF26, ZFP69B, ZNF83, ZNF471*
miR-145-5p	8.34E-03	actin filament-based process	*FSCN1, MYO5A, ABRACL, SWAP70, GMFB, ACTG1, AKAP9, RTKN, ADD3, SRGAP2, ACTB*

In HCV and HEV infections, shared upregulated miRNAs such as miR-10-5p had multiple gene targets with GO terms related to signalling transduction in the cellular response to endogenous stimuli, including mitogen-activated protein kinase-3 7 (*MAP3K7*), while miR-584-5p targeted genes were involved in promoting prostaglandin biosynthesis, including annexin A1 (*ANXA1*) and vasopressin receptor A1 (*AVPR1A*) (Table 17).

Table 17. GO term enrichment of target genes for significantly upregulated miRNAs shared between HCV and HEV infections

miRNA	Adjusted P-value	GO: Biological process	Genes
miR-10-5p	2.73E-03	cellular response to endogenous stimulus	*BDNF, VWC2L, GATA6, HCN1, NR6A1, NCOR2, FLRT2, RORB, RORA, KLF11, MAP3K7, UBE2I, XRN1, GABRB2,*
miR-584-5p	4.16E-02	positive regulation of prostaglandin biosynthetic process	*ANXA1, AVPR1A*

The only downregulated miRNA shared in HCV and HEV infections where gene targets gave significant go terms included miR-122-5p (Table 18), which was implicated in the activity of protein tyrosine/threonine phosphatase through dual-specificity phosphatase (*DUSP*) genes and drug transmembrane transporter activity in the solute carrier (*SLC*) family of genes.

Table 18. GO term enrichment of target genes for significantly downregulated miRNAs shared between HCV and HEV infections

miRNA	Adjusted P-value	GO: Molecular function	Genes
miR-122-5p	**1.43E-02**	protein tyrosine/threonine phosphatase activity	*DUSP4, DUSP2*
miR-122-5p	**4.54E-02**	drug transmembrane transporter activity	*SLC52A2, CD320, SLC1A5, SLC7A1*

3.5 Predicted targets and functions for differentially expressed miRNAs exclusive to one viral infection

Upregulated miRNAs exclusive to EBOV infection (Table 19) included miR-443, which targeted the genes *CD209* and *CLEC4M* implicated in peptide antigen transport, suggesting the activity of antigen-presenting cells (APCs) involved in T cell stimulation may be supressed in EBOV infection [56].

Table 19. GO term enrichment of target genes for significantly upregulated miRNAs exclusive to EBOV infections

miRNA	Adjusted P-value	GO: Biological process	Genes
miR-443	1.46E-02	peptide antigen transport	*CD209, CLEC4M*

In HCV infections, the exclusively upregulated miR-133a-3p was found to have gene targets including protein phosphatases (*PPPs),* implicated in peptidyl-serine dephopshporylation (Table 20). In downregulated miRNAs, terms found for gene targets were vague or not obviously related to viral infection, including peptide cross-linkage in miR-769-3p, gene suppression in miR-301b-3p and nitrogen compound biosynthesis in miR-19-3p (Table 21).

Table 20. GO term enrichment of target genes for significantly upregulated miRNAs exclusive to HCV infections

miRNA	Adjusted P-value	GO: Biological process	Genes
miR-133a-3p	0.02873	peptidyl-serine dephosphorylation	*PPP2CA, PPP2CB, PPP2R2D*

Table 21. GO term enrichment of target genes for significantly downregulated miRNAs exclusive to HCV infections

miRNA	Adjusted P-value	GO: Biological process	Genes
miR-769-3p	2.28E-02	peptide cross-linking	*LCE3C, LCE1A, TGM2, EVPL*
miR-301b-3p	2.21E-02	negative regulation of gene expression	*MDM4, PAN3, CPEB1, IRF1, RPS6KA5, MAF, TSHZ1, EREG, NFIA, ZBTB18, IGF1, GJA1, PPARG, DDX6, ESR1,T SC1*
miR-19-3p	4.71E-02	cellular nitrogen compound biosynthetic process	*CTDSPL2, KBTBD8, ZMYND11, QKI, ZBTB18, HBP1, NEUROD1, SPTSSB, PLA2G10, CDS1, TIA1, SGK1, RAP2C, KLF7, IMPDH1, GJA1, NFIB, RBBP8, TRAK2, MDM4, TMBIM6, ID2, BNC2, PTEN, CCNL1, MRPL17, BAMBI*

In HEV infected cells, upregulated miRNA targeted genes (Table 22) also had GO terms that were vague, including the regulation of signal transduction in miR-21-5p, which is supported by previous studies showing it supresses IFN-α signalling [26]. GO terms with no obvious connection to viral infections were found for targets of the upregulated miR-296-5p, which included multiple homeobox (*HOX)* genes implicated in skeletal system development and anterior/posterior patterning in embryogenesis. Targets for miR-26-5p included high-mobility group proteins (*HMGAs),* involved in chromatin remodelling and tumorigenesis. The downregulated miR-33b-3p was shown to target genes directly related to the immune response, including natural killer cell activation, B cell proliferation and responses to exogenous dsRNA.

Table 22. GO term enrichment of target genes for significantly upregulated miRNAs exclusive to HEV infections

miRNA	Adjusted P-value	GO: Biological process	Genes
miR-296-5p	1.83E-03	embryonic skeletal system development	*HOXB8, HOXA7, HOXA9, HOXB7, HOXA5, HOXB6*
miR-21-5p	1.90E-03	regulation of signal transduction	*IL12A, FASLG, FGF18, CCL1, PLEKHA1, RSAD2, PELI1, SKP2, NTF3, TIMP3, SMAD7, PHF14, FAM13A, RTN4, ARHGAP24, DUSP8, PDCD4, SPRY1, RALGPS2, GLIS2, SPRY2, ELF2, RECK*
miR-296-5p	2.32E-03	anterior/posterior pattern specification	*HOXC8, HOXB8, HOXA7, HOXA9, HOXB7, HOXA5, HOXB6*
miR-26-5p	1.64E-02	oncogene-induced cell senescence	*HMGA2, HMGA1*
miR-26-5p	2.91E-02	heterochromatin organization	*HMGA2, HMGA1, LOXL2*

Table 23. GO term enrichment of target genes for significantly downregulated miRNAs exclusive to HEV infections

miRNA	Adjusted P-value	GO: Biological process	Genes
miR-33b-3p	8.87E-04	natural killer cell activation involved in immune response	*IFNA2, IFNA17, IFNA4, IFNA16*
miR-33b-3p	2.31E-03	B cell proliferation	*GAPT, IFNA2, IFNA17, IFNA4, IFNA16*
miR-33b-3p	4.91E-03	response to exogenous dsRNA	*IFNA2, IFNA17, IFNA4, IFNA16*

4. Discussion

4.1 Experimental methods have a greater impact on overall miRNA expression than viral infections

As all cells were derived from the same cell line, we expected miRNA expression profiles in control cells from different experimental groups to be similar with large variations between virally infected cells, however our study showed the variation between cells from different experiment was far greater than the variation between the infected and non-infected cells, suggesting experimental conditions play a greater role in overall miRNA expression than viral infection. A recent study in HeLa cells, another human cancer cell line, showed that transcriptomic and proteomic analyses across different laboratories lead to distinct profiles of mRNA expression, protein quantification and protein turnover [57], with variations possibly occurring to varying protocols in handling the cells including cryopreservation, transfer between different environments, the allowance of contamination and their correct identification [58]. As the datasets for each viral infection were derived from different experimental groups, it is possible that varying protocols for handling the cells across different labs may play a role the large variation in miRNA expression between the groups.

As each experimental group in our investigation also used different miRNA-seq protocols to produce the miRNA libraries, it is possible that this may have had an impact on the quantity of miRNAs in our differential expression analysis. Studies have shown that different miRNA-seq protocols are biased towards different sequences leading to varying quantifications of miRNAs, which has been attributed mainly to the use of alternate adapters as well as other factors such as the temperature of ligation reactions [59]. Furthermore, different miRNA-seq protocols used within the same lab led to a greater variation in miRNA quantification than when using the same protocol in different labs and this variation appears greater in miRNA-seq than in mRNA-seq [60].

Our study highlights the importance of using the same cell lines, laboratories and miRNA-seq protocols while also adhering to specific guidelines for managing cells [58] when investigating the differential expression of miRNAs in different viral infections for an accurate comparison. Furthermore, if large variations in miRNA expression between cells of the same line can arise due to different laboratory conditions, it is likely that large variations can arise in patients with the same viral infections due to multiple factors such as age, body mass, lifestyle, etc. [3]. For this reason, using miRNA levels as biomarkers for disease or as targets in therapeutic strategies may not be effective for diagnoses or treatments in all patients.

4.2 Upregulated miRNAs were shared between all viral infections

The universal upregulation of miR-146a-5p in our analysis is supported by studies in many viral infections, including, HCV and HBV [36, 61], where viral core proteins appear activate the NF-kB transcription factor to promote miR-146a-5p expression, which in turn positively regulates autophagy-induced replication of the viruses in hepatocytes. Despite miR-146a-5p being positively regulated by the activation of NF-kB, our study predicted genes involved in the activity of NIK were targeted by the miRNA, 2 of which, including *IRAK1* and *TRAF6* have been shown to be important co-regulators in the NIK-mediated expression of NF-kB [62], which could imply miR-146a-5p self-regulates its expression.

Studies involving adenoviral vectors encoding kinase-inactive NIK used in HeLa cells showed an inhibition of NF-kB activation in response to the normally positively regulatory cytokines TNF-α and IL-1 [63], suggesting that since HCV and HBV can promote NF-kB expression independently from NIK,

the NF-kB pathway in infected cells may be more tightly regulated by the virus and not inflammatory cytokines of the immune response. This is supported by the fact suppression of NF-kB activity by miR-146a-5p in non-infected cells has been shown to promote tumour suppression and reduce inflammation [62], while HCV and HBV infections are highly tumorigenic and inflammatory.

In EBOV infections, miR-146a-5p is also upregulated, but its function in the pathogenesis of the disease is unknown [18]. It is possible that it is not directly regulated by the virus, but instead by inflammatory cytokines promoting NF-kB activity, thus the expression of the miRNA. While there is little evidence for its role in HEV infection, the upregulation of miR-146a-5p in HEV infected cells in our study suggests it may have a similar mechanism as in HCV infections, as the viruses are believed to share similarities in pathogenesis.

We expected to find all infected cells would display a universal upregulation of miR-21-5p, responsible for evading the host immune response [18, 20, 26], however this was only the case for HEV in our study. Interestingly the alternate species, miR-21-3p, was found to be upregulated in all infected cells, which has previously been implicated in promoting replication in influenza A virus by altering the regulation of chromatin accessibility [64]. It is possible that this deviation from the literature is a result of the different experimental conditions between cells.

We also expected many upregulated miRNAs to inhibit tumour suppressors and promote the development of HCC, however in HCV and HEV infected cells, miR-486-5p was the only shared upregulated miRNA that is believed to directly promote inflammation and tumorigenesis by inhibiting the suppression of NF-kB signalling [65]. Conversely, some differentially expressed miRNAs may have a tumour supressing function, as our analysis of HEV infected cells showed the upregulated miR-26-5p is predicted to target members of the *HMGA* family, which is well characterised as an oncogene that inhibits the tumour suppressing activity of *TET1* and *HOXA9*, leading to an increased risk of tumorigenesis [66]. This suggests that the promotion of tumorigenesis may result from a select few miRNAs, core viral proteins or both.

4.3 Downregulated miRNAs were shared between only EBOV + HCV and HCV + HEV infections

Our analysis showed markedly fewer miRNAs were significantly downregulated compared to those that were upregulated in all viral infected cells, which is consistent with the large number of studies reporting overexpressed miRNAs in EBOV, HCV and HEV infections and the small number reporting repressed miRNAs. Studies in rat hepatocytes would initially appear to contradict this, as stimulation by cytokines implicated in the immune response, such as IL-1α, IL-6, INF-β and TGF-β1, which would be speculated to be abundant in infected cells, are typically downregulated by the expression of miRNAs [67], but it is possible that the effect of these cytokines is not significant to the overall miRNA expression levels compared to those directly regulated by viral proteins, many of which are upregulated [36].

We showed miR-122-5p was under-expressed in both HCV and HEV infections, which supports the current view that it is downregulated in said infections [29] as opposed to previous contentions suggesting the expression was not significantly altered [28]. Its decreased expression is believed to

be directly regulated by HCV proteins [29], despite the fact it appears to positively promote HCV replication, as it has been suggested miR-122-5p binds to sites at the 5' of the viral genome, where it stimulates the folding of an internal ribosome entry site (IRES) required for the translation of HCV proteins [31].

The downregulation of miR-122-5p in hepatocytes has been associated with the development of HCC, with liver-specific knockouts of the *Mir122* locus in mice leading to an increased risk of hepatic tumorigenesis, which was attributed both to increased activation of pro-inflammatory cytokines and the increased expression of genes involved in cell proliferation, including *IGF2*, *RAS*, *EPCAM, C-MYC* and those involved in the β-catenin pathway (35). This suggests miR-122-5p is a tumour suppressor, and its downregulation in HCV or HEV infection may be a leading factor in HCC development.

Hepatic steatosis, or non-alcoholic fatty liver disease (NAFL), is characterised by an accumulation of lipids in hepatocytes, resulting in necrosis and fibrosis of the liver, of which HCV has been known to be a contributing factor to the inflammatory processes leading to the disease [68]. The study in transgenic mice by *Hsu et al.* also showed that miR-122-5p may have multiple target genes related to triglyceride biosynthesis, including *AGPAT1* and *MOGAT1* as well as decreased levels of cholesterol, suggesting miR-122-5p may contribute to NAFL. Our analysis supports the use of low serum levels of miR-122-5p as a biomarker for chronic hepatitis infections, including HCC in HCV [37] and acute-phase HEV infected cells [69], but only in conjunction with other diagnostic methods.

The dependence of HCV replication on miR-122-5p has made it a potential target for treating the viral infection, with the use of a modified DNA phosphorothioate antisense oligonucleotide having been shown to sequester miR-122-5p, leading to the reduction of HCV RNA and elimination of the virus in infected patients [38], however this may come at the expense of increasing the risk of the development of HCC. In fact, delivery of miR-122-5p genes to tumorigenic murine hepatocytes through adeno-associated viral vectors (AAVs) [70] or cationic lipid nanoparticles [71] resulted in a major reduction in HCC, which would not be suitable for HCV or HEV infections, as it may promote the replication and pathogenesis of the viruses. This suggests altering the activity of miRNAs to treat diseases should be used with care, as it could result in adverse effects.

4.4 Mechanisms of up- or downregulation in RNA virus infections

While our study is intended to analyse the expression of miRNAs in response to viral infections, different quantities of miRNAs extracted from cells does not necessarily imply there is a difference in their transcription and biosynthesis. It is believed the decreased abundance and activity of miR-122-5p in HCV results from it having a high affinity for the 5' end of the viral genome, leading a miR-122-5p "sponge" effect [72] and there is only a small amount of evidence showing the HCV core proteins may alter miR-122-5p transcription through histone modifications [73]. It is possible the downregulation of other miRNAs in our study may also result from post-transcriptional events.

Epigenetic reprogramming in viral infections through histone modifications or DNA methylation can affect the expression of many host genes, some of which may contribute to the pathogenesis of the disease [74]. Up- or downregulation of gene expression in HBV and HCV infection have been shown

to result from increasing or decreasing the accessibility to chromatin by histone modifications and transcriptional regulation through DNA demethylation or hypermethylation of the promoter regions [2, 75-77]. This is believed to play a role in the development of HCC in viral hepatitis, as the epigenetic modifications affect a large number of oncogenes and tumour suppressors.

The expression of miRNAs is highly regulated by epigenetic modifications, with downregulation of miR-122-5p in HBV infections having been shown to result from the HBV derived X protein (HBX) inhibiting the peroxisome-proliferator-activated-receptor-γ (PPARγ) associated to nuclear proteins at the miR-122-5p promoter region, leading to a histone methyl transferase (HMT) mediated repression of the miRNA [75]. A similar process may occur to regulate miR-122-5p transcription in HCV infections [73] and core HCV derived proteins similar to HBX bind to PPARγ complexes to regulate transcriptional activity in a similar manner [2], with miR-152-5p also having been shown to have reduced expression in hepatocyte tumorigenesis driven by HCV core proteins [76]. DNA methylation has also been shown to be involved in regulating miRNA expression, as HCV infected cells showed a downregulation of miR-124 which was reversed with a DNA methyltransferase 1 (DNMT1) knockdown [77].

While there are few studies in focusing on the relationship between epigenetic mechanisms and miRNA expression in EBOV or hepatitis virus infected cells, it is possible that viral mediated epigenetic modifications may be responsible for significant up- or downregulations of miRNAs found in our analysis. We found at least one upregulated miRNA in HEV, miR-26-5p that may target and supress *HMGA* genes involved in chromatin remodelling [66], which could potentially alter the expression of other miRNAs.

As we only analysed miRNA-seq datasets, we cannot determine if changes to chromatin accessibility, DNA methylation status or the activity of HMT, DNMT or other epigenetic enzymes resulted in the differential expression of miRNAs in our study. Potential methods for doing so include chromatin immunoprecipitation sequencing (ChiP-seq), where antibodies for differentially modified histones can be mapped to specific genomic regions and has been used previously to identify the histone mediated regulation of chromatin accessibility in miRNA expressing regions [78], or bisulfite sequencing (BS-seq), where bisulfite treatment of DNA allows the location of methylated sites across the genome, which has been combined with miRNA-seq data to determine the effect differentially methylated regions have on miRNA expression [79].

4.5 GO term enrichment of predicted miRNA target genes found few process relevant to viral infection

We found only a small number of significant GO terms for predicted genes targeted by differentially expressed miRNAs of which only a few were obviously relevant to viral infection. The upregulation of miR-443 in EBOV infection was predicted to inhibit genes *CD209* and *CLEC4M*, which were suggested to drive peptide antigen transport, an essential process in T cell activation by APCs [56], thus potentially positively regulating viral replication, while the HEV mediated downregulation of miR-33b-3p was predicted to de-repress multiple *IFN* genes with immune functions that may play a negative role in viral replication, including T killer cell activation, B cell proliferation and a response to exogenous dsRNA, which may include that of the virus.

Many terms for target genes could potentially be implicated in viral pathogenesis, such as the regulation of RNA polyemerase II activity targeted by miR-146a-3p, cellular response to endogenous stimuli by miR-10-5p, regulation of gene expression by miR-301-3p and regulation of signal transduction by miR-21-5p, however they are too vague to determine this. Some of the targets had

terms that were unlikely to be implicated in infection, such as embryogenesis functions by miR-296-5p, which supports the view that miRNA dysregulation by viral infections can impact physiological processes beyond inflammation or tumorigenesis, as shown by development of microcephaly in ZIKV infections [34].

While *in silico* miRNA target prediction methods are fast and convenient, *in vivo* investigations have identified gene transcripts targeted by miRNAs that were not found in computational target prediction [80], suggesting it is not always accurate. Luciferase assays are commonly used to validate the miRNA mediated suppression of their predicted mRNA targets, where predicted target genes are engineered to include a luciferase reporter, which can be used to detect whether or not the translation of the mRNA is suppressed [81].

5. Conclusion

We have shown that cells derived from the same line can vary greatly in their miRNA expression profiles due to laboratory protocols, even more so than between those that are non-infected and infected with EBOV, HCV or HEV with the same protocols. Despite this, we have shown some miRNAs shared between viral infections, such as the upregulated miR-146a-5p in all viruses or the downregulated miR-122-5p in HCV and HEV, have expression profiles consistent with previous studies. Others however, such as miR-21-5p, predicted to be upregulated in all infections, were only highly expressed in HEV infections, suggesting the future use of miRNAs as therapeutic targets or biomarkers are not reliable as the sole methods of treatment or diagnosis for diseases and that they should be used in conjunction with other methods.

We further suggest future studies comparing the miRNA expression profiles of RNA virus infected cells should use datasets derived from the same laboratory using the same protocols. To understand more about the molecular mechanisms leading to differential expression of miRNAs in infected cells, researchers could use miRNA-seq analyses alongside BS- or ChiP-seq to determine how epigenetic modifications may influence miRNA expression. Predicted targets for differentially expressed miRNAs should also be validated through the use of luciferase assays.

6. References

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7. Appendices

Appendix 1. Processing of miRNA-seq datasets

1A. Conversion of FASTQ files into FASTA format

A simple BASH command was used to convert our datasets, which were downloaded as FASTQ files, into a FASTA format.

```
sed '/^@/!d;s//>/;N' #FASTQ FILE# > #FASTA FILE#
```

1B. Quantification of sequences in FASTA files

Python was used to write a function that quantifies all the reads of FASTA file.

```
def count_dic(self):

    #COUNTER PACKAGE USED TO COUNT READS#

    from collections import Counter

    #USE ADDITIONAL SCRIPT FOR PATHS OF FASTA FILES#

    for file in #PATHS OF FASTA FILES#:

        readit = open(file, "r")
        filename = (file.split("/")[-1])
        output = (self.dirout + "/" + filename)
        writeit = open(output, "w")


        res = []

        for line in readit:

            #REMOVE ">" AND "@"#
            #FILTER OUT READS <16 NTS or > 31 NTS#

            if not line.startswith(">"):
                if not line.startswith("@"):
                    if len(line) >=16:
                        if len(line) <= 31:
                            if "N" not in line:

                                strippedline = line.strip("\n")
                                res.append(strippedline)

        counts = Counter(res)

        #WRITE A FASTA FILE OF THE SEQUENCES AND THEIR COUNTS#

        for i, e in counts.items():
            writeit.write(">"+i+"("+str(e)+")"+"\n"+i+"\n")
```

Appendix 2. Alignment of datasets in FASTA format to mature miRNA sequences derived from miRBase

2A. Use of PatMaN alignment tool to map reads from the datasets to mature miRNAs in miRBase

We used Python to run the PatMaN alignment tool, which mapped reads from each dataset to all known mature miRNAs in miRBase. PatMaN was ran through a subprocess, set to allow no mismatches or gaps.

```
    def run_patman(self):
        import subprocess

       #MAKES AN OUTPUT FOLDER#
        self.make_outputdir_pat("#PATMAN OUTPUT FOLDER")

       #SELECTS DATASETS IN FASTA FORMAT#
        fastafile = self.pathfinder_fasta()

       #RUNS PATMAN FOR EACH DATASET#
        for item in fastafile:
            split = (item.split(".")[-2]).split("/")[-1]

       #PATMAN IS RUN WITH NO MISMATCHES AND NO GAPS#
            subprocess.call(["patman", "-e", "0", "-g", "0", "-D",
           "%s" % #FILE CONTAINING MICRORNAS#, "-P", item, "-o",
           self.output + "#PATMAN OUTPUT FOLDER" + "%s" % split +
           ".txt"])
```

2B. Filtering out non-human miRNAs from PatMaN output files

A Python script was used to filter out miRNAs that did not begin with “hsa”, as this indicated they were not human miRNAs.

```
    def select_humans(self):

       #MAKES AN OUTPUT FOLDER#
        self.make_outputdir_pat("#HUMAN OUTPUT FOLDER")

       #MAKES A NEW FILE WITH ONLY HUMAN MICRORNAS#
        for file in #PATMAN OUTPUT FOLDER#:
            filename = (file.split(".")[-2]).split("/")[-1]

           writehsa = open(self.output + "hsaPATout/hsa" + filename
           + ".txt", "w")

            for line in open(file, "r"):
                if line.startswith("hsa"):
                    writehsa.write(line + "\n")
```

Appendix 3. Compilation of miRNA counts from all datasets into CSV format

3A. Quantification of human miRNAs in the PatMaN output

Python was used to produce a dictionary with the dataset name as the key and a list including the miRNA name and its quantity.

```
    def get_mircounts(self):
        import re

        self.make_outputdir_pat("#OUT PUT FOLDER#")

       #FINDS THE PATH OF PATMAN OUTPUT FILES#
        read_hsa = self.pathfinder_hsa()

       #MAKES A DICTIONARY WITH ALL HUMAN MIRNAS AS A KEY AND 0 AS A
       VALUE#
        mirdic = self.make_mirdic()
        dictionary = dict()


       #WHERE file IS EACH DATASET#
        for file in read_hsa:
            tempdic = dict()
            filename = (file.split(".")[-2]).split("/")[-1]
            empty = ""

           #MAKES A NEW DICTIONARY USING ALL HUMAN MIRNAS, SETTING
           THE VALUE AS AN INTEGER#
            for key, value in mirdic.items():
                tempdic[key] = int(value)

            for line in open(file, "r"):
                mir_name = line.split(" ")[0]
                allseqcount = line.split()
                if len(allseqcount) > 0:
                    seqcount = allseqcount[-5]
                    count = re.findall("\d+", seqcount)[0]


                      #IF A MIRNA IS IN OUR DATASET, IT'S COUNT WILL
                      BE ADDED TO THE DICTIONARY VALUE#
                       if mir_name in tempdic:
                            tempdic[mir_name] =
                       int(tempdic[mir_name]) + int(count)


           #MAKES A DICTIONARY OF THE DATASET NAMES WITH THE MIRNAS
           AND THEIR COUNTS#
            for key, value in tempdic.items():
                if filename not in dictionary:
                    dictionary[filename] = [[key, value]]
                else:
                    dictionary[filename].append([key, value])


        return dictionary
```

3B. Compilation of miRNA counts into a CSV file

Python was used again to read the dictionary from Appendix 3A and create a CSV file of the counts for each miRNA in each replicate. Replicates were set as the column names and all human miRNAs were set as the row names.

```
    def compile_dics(self):

        #MAKES A LIST FOR THE COLUMN NAMES#
         rescell = ["mirna"]

        #MAKES A LIST FOR THE ROWS#
         resdata = []

        #MAKES A NEW DICTIONARY WITH THE MIRNA NAMES AS THE KEY AND
        COUNTS FROM ALL DATASETS AS THE VALUE#
         compiledic = dict()

         getdic = self.get_mircounts()


         for key, value in getdic.items():

             rescell.append(key)

             for mirname, count in value:
                 if mirname in compiledic:
                     compiledic[mirname].append(count)

                 else:
                     compiledic[mirname] = [mirname, count]

                #ADDS THE MIRNA NAME AND ALL COUNTS TO THE ROW LIST#
                 for key, value in compiledic.items():
             resdata.append(value)

        #WRITES A CSV FILE, WITH THE MIRNAS AS THE ROWS AND THE
        DATASETS AS THE COLUMNS#
         import csv
         file = open(self.output + "/hsamirs.csv", "w")
         writer = csv.writer(file)
         print(rescell)
         for item in resdata:
             writer.writerow(item)
```

Appendix 4. Filtering of lowly expressed miRNAs and calculation of normalisation factors

RStudio was used with the EdgeR package to convert miRNA counts to counts per million (CPM), where only miRNAs with a CPM greater than 1 in at least 2 replicates were used in our analysis, and the rest were filtered out. The count data was processed using the DGEList function, where the normalisation factors were calculated to remove composition bias between libraries.

```
#OPENS THE edgeR PACKAGE#
Library(edger)

#OBTAIN COUNTS PER MILLION FROM CSV FILE DATA#
myCPM <- cpm(hsamirs)

#DETERMINES WHICH VALUES IN myCPM ARE GREATER THAN 1#
thresh <- myCPM > 1

#DETERMINES WHICH MIRNAS HAVE AT LEAST 2 REPLICATES WITH A
CPM > 1#
keep <- rowSums(thresh) >= 2

#FILTERS OUT MIRNAS WITHOUT COUNTS PER MILLION GREATER THAN
IN AT LEAST 2 REPLICATES#
counts.keep <- hsamirs[keep,]

#STORE COUNT DATA AS A DGEList OBJECT#
dmire <- DGEList(counts.keep)

#CALCULATE NORMALISATION FACTORS TO ELMINATE COMPOSITION BIAS
BETWEEN LIBRARIES#
dmire <- calcNormFactors(dmire)
```

Appendix 5. Voom transformation

The limma package was used in RStudio to perform a voom transformation on the counts, where they were normalised as log-2 CPM and the mean-variance relationship was calculated.

```
#OPENS THE limma PACKAGE#
library(limma)


#SPECIFIES A DESIGN MATRIX#
design <- model.matrix(~ 0 + groups)

#NAMES COLUMNS AFTER GROUPS#
colnames(design) <- levels(groups)

#TRANSFORMS READ COUNTS INTO NORMALISED log-2 COUNTS PER
MILLION AND DETERMINES THE MEAN-VARIANCE RELATIONSHIP#
vdmire <- voom(y,design,plot = FALSE)
```

Appendix 6. MDS plotting and hierarchical clustering

Using EdgeR, an MDS plot was produced using the count data, showing the variation between replicates. Using the gplots package, a hierarchical clustering was performed, generating a heatmap of the top 500 most variable miRNAs.

```
#OPENS THE gplots PACKAGE#
Library(gplots)

#SETS DIFFERENT COLOURS TO CONTROL AND INFECTED GROUPS#
col.inforcon <- c("red", "darkgreen")[infcongroup]

#PRODUCES AN MDS PLOT USING THE COUNT DATA#
plotMDS(dmire, col=col.status, pch=c(1, 2, 14)[group]),
legend("bottomleft", legend=c("EBOL", "HCV", "HEV"), pch=c(1,
2, 14),
legend("bottomright", legend=c("Control", "Infected),
col=c("darkgreen", "red"), pch=c(15)

#GET log-2 COUNTS PER MILLION
logcounts <- cpm(dmire, log=TRUE)

#ESTIMATE THE VARIANCE IN ROWS FOR logcounts#
var_mirs <- apply(logcounts, 1, var)

#FIND THE TOP 500 VARIABLE MIRNAS AND SUBSET logcounts#
select_var <- names(sort(var_mirs, decreasing=TRUE))[1:500]
highly variable_mirlcpm <- logcounts[select_var,]

#PRODUCES A HEATMAP USING log-2 COUNTS PER MILLION#
heatmap.2(highly_variable_mirlcpm, col=rev(morecol(50)),
trace="none", ColSideColors=col.inforcon, scale="row)
```

Appendix 7. Identifying differentially expressed miRNAs

Using *limma*, we made a comparison between the expression of replicates in control cells to those of infected cells within their respective experimental group. We further performed an empirical Bayes shrinkage of the variances and estimated moderated t-statistics, then made a summary of the significantly differentially expressed miRNAs in the comparison.

```
#FITS DATA TO LINEAR MODEL#
fit <- lmFit(vdmire)

#PRODUCES A MATRIX FOR OUR COMPARISONS BETWEEN CONTROL AND
INFECTED REPLICATES#
contrast.matrix <- makeContrasts(EBOV=ebol-ebol.control,
HCV=hcv-hcv.control, HEV=hev-hev.control, levels=design)

#FITS THE LINEAR MODEL TO THE CONTRAST MATRIX#
fit.contrasts <- contrasts.fit(fit, contrast.matrix)

#EMPIRICAL BAYES SHRINKAGE AND ESTIMATION OF T-STATISTICS#
fit.contrasts <- eBayes(fit.contrasts)

#SUMMARY OF THE DIFFERENTIALLY EXPRESSED MIRNAS IN THE
CONTRASTS#
summary.fit <- decideTests(fit.cont)

#PLOTS A VENN DIAGRAM OF THE SIGNIFICANTLY DIFFERENTIAL
MIRNAS#
vennDIagram(summary.fit, include=c("up", "down"),
counts.col=c("darkgreen", "red")
```

Appendix 8. Identifying differential miRNAs that are shared between infections or exclusive to specific infections

We used a logical matrix in RStudio to find miRNAs that were expressed in all, two or one infection(s).

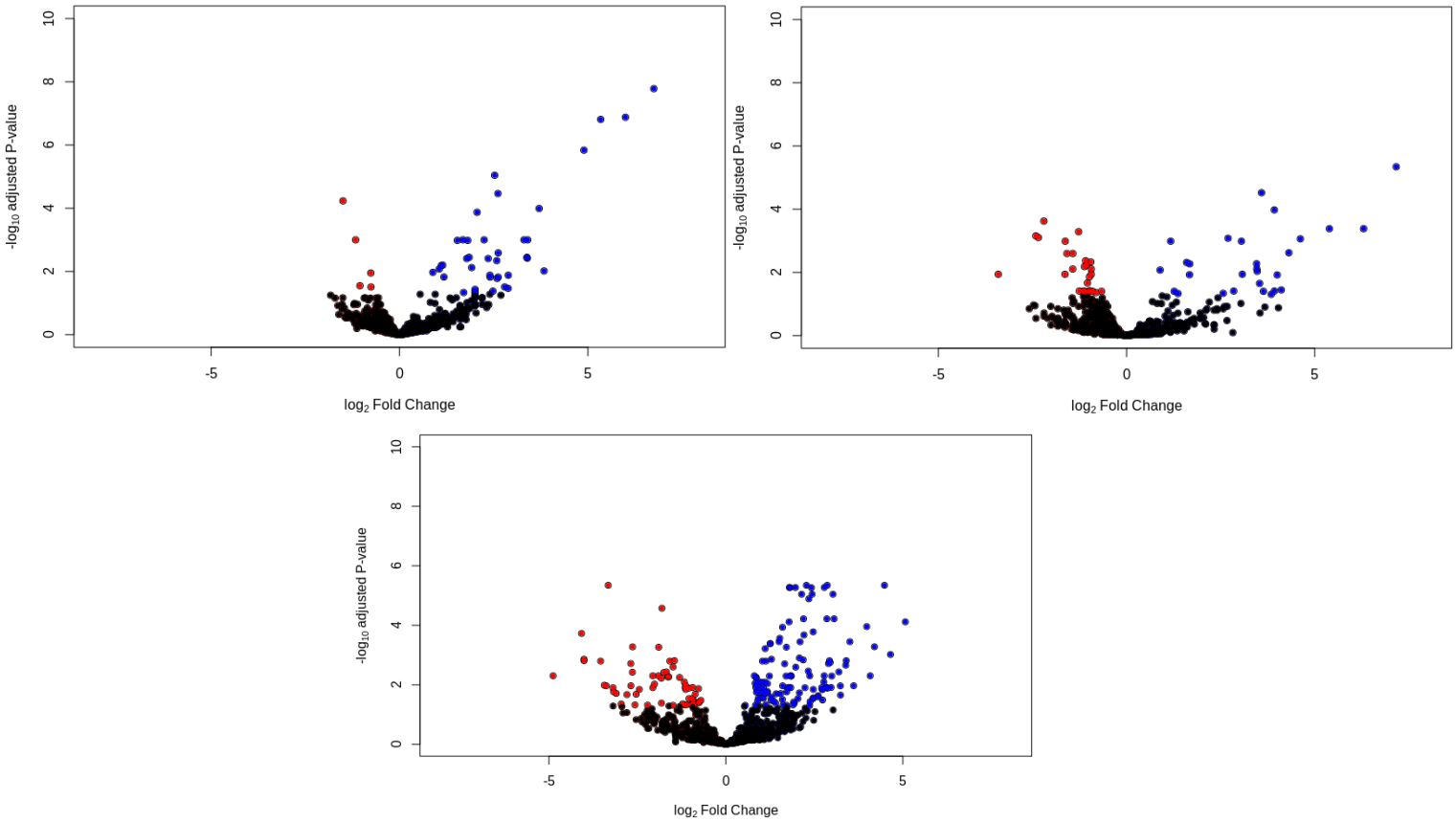
```
#PRODUCES LOGICAL MATRIX WITH TRUE FOR UPREGULATED MIRNAS
USING A THRESHOLD OF > 0#
suma.thresh.positive <- summary.fit > 0

#PRODUCES LOGICAL MATRIX WITH TRUE FOR DOWNREGULATED MIRNAS
USING A THRESHOLD OF < 0#
suma.thresh.negative <- summary.fit < 0

#SELECTS MIRNAS WITH 3,2 or 1 TRUES IN EACH ROW OF THE
THRESHOLD#
keep3 <- rowSums(#THRESH#) == 3
keep2 <- rowSums(#THRESH#) == 2
keep1 <- rowSums(#THRESH#) == 1

#FINDS THE NAMES OF SELECTED MIRNAS#
suma3 <- row.names(summary.fit[keep3,])
suma2 <- row.names(summary.fit[keep2,])
suma1 <- row.names(summary.fit[keep1,])

#WRITES A CSV FILE OF THE log-2 FOLD CHANGE AND ADJUSTED P-
VALUE FOR SELECTED MIRNAS#
write.csv(topTable(fit.contrasts, n=Inf)[suma#NUMBER#,],
"#OUTPUTNAME#.csv")
```



Appendix 9. Volcano plots of differentially expressed miRNAs in each viral infection including EBOV (A), HCV (B) and HEV (C). Only miRNAs with an adjusted P-value (Q < 0.05) were considered significant, including those that were up- (blue) or downregulated (red). Non-significant miRNAs (Q >= 0.05) are shown in black.

Appendix 10. Differentially expressed miRNAs in EBOV infection

10A. Significantly upregulated miRNAs in EBOV infection

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
miR-145-3p	6.74833	0.553259	1.725039	7.51E-09
miR-143-3p	2.525543	1.072625	4.480795	1.43E-08
miR-4449	5.996027	0.77897	-0.63744	4.79E-08
miR-4443	5.338923	0.032022	0.298638	6.74E-08
miR-12136	4.893818	1.266745	-1.63695	7.25E-08
miR-146a-5p	1.111138	0.892156	2.274723	7.25E-08
miR-145-5p	2.24554	0.45765	3.026289	3.77E-07
miR-143-5p	3.400033	0.553259	5.070597	1.86E-06
miR-4516	3.704678	4.312909	-0.62559	1.86E-06
miR-4286	2.611622	-0.0756	0.286311	9.53E-06
miR-21-3p	0.884415	1.171939	0.898915	9.53E-06
miR-27a-5p	2.05858	1.367763	0.339352	9.73E-06
miR-193b-5p	1.053746	0.711397	1.66041	6.29E-05
miR-4488	1.8111	1.79553	-2.17608	1.49E-04
miR-663a	3.389883	4.110826	0.154249	1.62E-04
miR-23a-5p	1.683092	1.021938	-0.14602	1.62E-04
miR-10401-5p	3.303428	-0.2393	-1.41965	2.10E-04
miR-146a-3p	1.701549	1.061487	2.778064	2.47E-04
miR-3651	1.534387	-0.74813	-0.65694	2.68E-04
miR-4485-3p	3.835294	3.676645	-2.90637	2.68E-04
miR-132-5p	1.847802	1.214349	0.056475	2.68E-04
miR-9901	2.618978	2.048636	-0.50937	3.31E-04
miR-30b-3p	1.143878	-0.41012	0.823826	6.60E-04
miR-4284	2.88265	3.928663	-0.7014	7.31E-04
miR-4497	2.353118	0.687838	0.942867	8.41E-04
miR-619-5p	3.380497	0.967642	0.166026	8.85E-04
miR-663b	2.88581	2.568039	-0.63108	1.01E-03
miR-7704	1.783408	0.586346	-0.31498	1.04E-03
miR-3663-3p	2.580438	-0.2393	-0.63108	1.23E-03
miR-5701	1.91474	0.64128	0.557455	1.64E-03
miR-3148	2.40401	0.921028	1.095299	2.86E-03
miR-5100	2.617735	1.164741	-1.32181	3.39E-03
miR-29b-1-5p	1.179038	-0.07972	-0.43783	3.76E-03
miR-765	2.398749	-0.2393	-0.25733	4.31E-03
miR-1291	2.587117	0.497789	-0.63108	4.91E-03
miR-9899	2.789846	0.008845	0.154249	1.07E-02
miR-612	2.003933	-0.2393	-0.63108	1.07E-02
miR-1229-5p	2.002994	-0.2393	-0.63103	1.22E-02
miR-552-5p	2.477773	-0.2393	-0.63108	1.27E-02
miR-4716-3p	2.002994	-0.2393	-0.63108	1.32E-02

10B. Significantly downregulated miRNAs in EBOV infection

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
hsa-miR-483-5p	-1.16498	-1.62891	0.149474	6.99E-06
hsa-miR-1303	-1.50019	-0.56292	-0.34293	7.54E-06
hsa-miR-345-5p	-0.76233	-0.5929	-0.31996	5.94E-04
hsa-miR-122b-5p	-1.05028	-0.86796	-0.10034	2.38E-03
hsa-miR-106a-5p	-0.75708	-0.34608	-0.79001	3.37E-03

Appendix 11. Differentially expressed miRNAs in HCV infection

11A. Significantly upregulated miRNAs in HCV infection

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
hsa-miR-1-3p	2.196519	7.165276	4.197624	1.01E-07
hsa-miR-12136	4.893818	1.266745	-1.63695	1.01E-07
hsa-miR-146a-5p	1.111138	0.892156	2.274723	1.01E-07
hsa-miR-486-5p	-0.09677	3.587168	1.598476	2.11E-06
hsa-miR-4516	3.704678	4.312909	-0.62559	2.34E-06
hsa-miR-1246	0.140996	3.926918	-2.06458	2.41E-06
hsa-miR-9985	1.210287	2.697185	2.077037	5.33E-06
hsa-miR-21-3p	0.884415	1.171939	0.898915	9.08E-06
hsa-miR-27a-5p	2.05858	1.367763	0.339352	9.08E-06
hsa-miR-133b	2.003933	5.389605	0.554305	2.56E-05
hsa-miR-133a-3p	2.03125	6.300261	-0.51581	2.66E-05
hsa-miR-663a	3.389883	4.110826	0.154249	1.43E-04
hsa-miR-10a-5p	-0.803	1.59663	3.239012	1.44E-04
hsa-miR-584-5p	0.501745	1.673188	2.369547	1.44E-04
hsa-miR-206	0.837183	4.61865	-0.41357	1.45E-04
hsa-miR-212-3p	0.02185	3.052504	0.05349	2.69E-04
hsa-miR-4463	1.210287	3.461109	-2.0872	2.69E-04
hsa-miR-136-3p	1.210287	3.455094	-0.63108	6.46E-04
hsa-miR-4284	2.88265	3.928663	-0.7014	6.46E-04
hsa-miR-4326	1.210901	3.075783	0.895885	9.18E-04
hsa-miR-4508	1.876666	3.535224	-1.42508	1.04E-03
hsa-miR-127-3p	0.001671	1.679035	0.162971	1.49E-03
hsa-miR-3195	1.317041	4.003245	-1.42508	1.63E-03
hsa-miR-4492	2.313178	3.63755	-0.17618	2.52E-03
hsa-miR-486-3p	-0.00277	3.471449	0.352823	2.55E-03
hsa-miR-320e	0.814004	2.568039	-1.60864	2.62E-03
hsa-miR-1973	0.763422	3.845933	-2.17514	3.92E-03
hsa-miR-410-3p	1.211175	2.848103	-0.63108	4.81E-03

11B. Significantly downregulated miRNAs in HCV infection

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
hsa-miR-483-5p	-1.16498	-1.62891	0.149474	3.83E-05
hsa-miR-4454	0.275907	-2.19656	-0.97664	3.83E-05
hsa-miR-192-5p	-0.2092	-0.93796	1.503776	3.83E-05
hsa-miR-122-5p	-0.76975	-1.42995	-1.58345	3.83E-05
hsa-miR-210-5p	-0.49004	-2.40966	1.717507	4.39E-05
hsa-miR-210-3p	-0.43849	-2.34181	1.069348	4.97E-05
hsa-miR-769-5p	-0.47053	-1.27355	-0.16911	7.63E-05
hsa-miR-885-3p	-0.60713	-1.58285	-0.64373	1.95E-04
hsa-miR-148a-5p	-0.31407	-0.94895	-0.71345	4.06E-04
hsa-miR-19b-3p	-0.52246	-1.13978	-0.70605	5.51E-04
hsa-miR-181a-2-3p	-0.5957	-0.81054	1.056253	5.51E-04
hsa-miR-769-3p	-0.27436	-1.11759	-0.92006	5.51E-04
hsa-miR-19a-3p	-0.55935	-1.06822	-0.57303	6.21E-04
hsa-miR-20a-5p	-0.60403	-1.122	-0.92456	6.21E-04
hsa-miR-301b-3p	-0.02649	-1.08736	0.587685	6.59E-04
hsa-miR-335-3p	-0.00173	-1.42784	-0.47103	6.59E-04
hsa-miR-885-5p	-0.67554	-1.63528	0.578935	8.40E-04
hsa-miR-301a-3p	-0.10806	-0.90271	1.096543	1.16E-03
hsa-miR-1908-5p	-0.44441	-0.98744	-0.17916	1.46E-03
hsa-miR-26a-5p	-0.31725	-0.66547	0.53316	1.49E-03
hsa-miR-30e-3p	0.171569	-1.0627	0.102833	1.49E-03
hsa-miR-1247-5p	-0.6328	-1.25476	-0.65598	1.83E-03
hsa-miR-7706	-0.03662	-0.9375	0.80717	2.58E-03
hsa-let-7a-5p	-0.31842	-0.98848	0.640178	2.78E-03
hsa-miR-4689	-0.31223	-3.40824	-0.79386	2.78E-03
hsa-miR-148a-3p	-0.28269	-1.03838	0.33878	3.57E-03
hsa-let-7c-5p	-0.35018	-0.96467	0.263613	5.33E-03
hsa-miR-3059-5p	-0.03267	-1.0987	-0.66173	9.07E-03

Appendix 12. Differentially expressed miRNAs in HEV infection

12A. Top 50 significantly upregulated miRNAs in HEV infection

miRNA	logFC			Q-value
	EBOV	HCV	HEV	
hsa-miR-143-3p	2.525543	1.072625	4.480795	8.44E-08
hsa-miR-1-3p	2.196519	7.165276	4.197624	2.21E-07
hsa-miR-146a-5p	1.111138	0.892156	2.274723	4.28E-07
hsa-miR-145-5p	2.24554	0.45765	3.026289	1.72E-06
hsa-miR-27a-3p	0.547757	0.688123	1.795614	1.72E-06
hsa-miR-489-3p	0.47244	-0.1277	2.86366	1.72E-06
hsa-miR-574-5p	-0.31633	-0.94188	2.781543	3.22E-06
hsa-miR-215-5p	0.233697	0.812956	2.416447	3.81E-06
hsa-miR-486-5p	-0.09677	3.587168	1.598476	3.81E-06
hsa-miR-28-5p	0.148858	-0.33933	1.808775	3.81E-06
hsa-miR-196a-5p	-0.61702	-0.66092	2.344264	3.81E-06
hsa-miR-143-5p	3.400033	0.553259	5.070597	3.81E-06
hsa-miR-21-5p	0.045331	0.356125	1.964813	4.04E-06
hsa-miR-26b-5p	-0.1352	-0.55671	2.436166	6.24E-06
hsa-miR-582-5p	-0.21638	-0.09063	2.140092	9.70E-06
hsa-miR-9985	1.210287	2.697185	2.077037	9.82E-06
hsa-miR-21-3p	0.884415	1.171939	0.898915	1.82E-05
hsa-miR-194-5p	-0.15808	-0.66007	1.786591	2.92E-05
hsa-miR-192-5p	-0.2092	-0.93796	1.503776	3.00E-05
hsa-miR-20b-5p	-0.72284	-0.40919	3.057335	3.00E-05
hsa-miR-210-5p	-0.49004	-2.40966	1.717507	4.40E-05
hsa-miR-574-3p	-0.40112	-0.42165	2.852906	4.50E-05
hsa-miR-210-3p	-0.43849	-2.34181	1.069348	5.46E-05
hsa-miR-30d-5p	0.053198	-0.14788	2.192952	6.08E-05
hsa-miR-193b-5p	1.053746	0.711397	1.66041	9.66E-05
hsa-miR-363-3p	-0.16195	-0.06147	3.9793	1.17E-04
hsa-miR-193b-3p	0.003217	-0.15637	1.600534	1.24E-04
hsa-miR-26a-2-3p	0.940608	-1.14702	3.387428	1.30E-04
hsa-miR-374b-5p	-0.39892	-0.44146	1.253479	1.33E-04
hsa-miR-374c-3p	-0.39892	-0.44146	1.253479	1.33E-04
hsa-miR-190a-5p	-0.16555	-0.76746	3.503671	1.47E-04
hsa-miR-186-5p	0.042587	-0.34368	2.206148	1.47E-04
hsa-miR-30a-5p	-0.16415	0.040173	2.466121	1.51E-04
hsa-miR-222-3p	0.004923	0.339367	1.52214	2.32E-04
hsa-miR-10a-5p	-0.803	1.59663	3.239012	2.37E-04
hsa-miR-584-5p	0.501745	1.673188	2.369547	2.37E-04
hsa-miR-29c-3p	-0.30792	-0.3211	2.095661	2.73E-04
hsa-miR-196b-5p	-0.32464	0.288611	1.709396	3.05E-04
hsa-miR-146a-3p	1.701549	1.061487	2.778064	3.37E-04
hsa-miR-125a-5p	-0.29821	-0.02752	1.109892	3.64E-04
hsa-miR-217-5p	-1.04981	-0.69501	2.926322	3.85E-04
hsa-miR-935	1.210287	1.608441	3.400883	4.77E-04
hsa-miR-10b-5p	-0.33095	-0.68157	2.186272	4.81E-04
hsa-miR-365a-5p	1.340958	0.241425	2.328588	5.66E-04
hsa-miR-181a-2-3p	-0.5957	-0.81054	1.056253	6.13E-04
hsa-miR-653-3p	-0.15696	-0.57308	4.650563	8.31E-04

hsa-miR-29a-3p	-0.03809	0.307056	1.288253	1.10E-03
hsa-miR-451a	-0.74622	1.164463	2.947012	1.16E-03
hsa-miR-23a-3p	0.179619	0.306718	1.132181	1.26E-03
hsa-miR-454-3p	-0.15726	-0.80268	1.174767	1.53E-03

12B Top 50 significantly downregulated miRNAs in HEV infection

miRNA		**logFC**		**Q-value**
	EBOV	**HCV**	**HEV**	
hsa-miR-12136	4.893818	1.266745	-1.63695	5.54E-07
hsa-miR-92a-1-5p	0.011363	-0.26094	-3.3245	6.23E-06
hsa-miR-1246	0.140996	3.926918	-2.06458	1.02E-05
hsa-miR-4454	0.275907	-2.19656	-0.97664	3.94E-05
hsa-miR-3909	-0.13282	-0.3807	-1.80618	3.94E-05
hsa-miR-122-5p	-0.76975	-1.42995	-1.58345	5.37E-05
hsa-miR-1257	-0.52847	2.138625	-4.07675	1.19E-04
hsa-miR-33b-3p	-0.82772	-0.81637	-2.63659	1.67E-04
hsa-miR-187-3p	-0.85645	-1.44492	-1.67954	4.06E-04
hsa-miR-18a-3p	-0.39807	-0.27044	-1.8978	4.55E-04
hsa-miR-1185-2-3p	1.60595	-1.03221	-4.00844	7.12E-04
hsa-miR-17-5p	-0.50203	-1.04843	-1.17406	7.73E-04
hsa-miR-1185-1-3p	1.208067	-1.03221	-4.00844	8.37E-04
hsa-miR-19b-3p	-0.52246	-1.13978	-0.70605	8.54E-04
hsa-miR-20a-5p	-0.60403	-1.122	-0.92456	1.22E-03
hsa-miR-1247-3p	0.924445	-1.551	-4.88109	1.73E-03
hsa-miR-586	0.23126	-0.90769	-3.53811	1.75E-03
hsa-miR-25-5p	0.021387	0.746517	-2.68622	1.75E-03
hsa-miR-532-5p	0.134593	-0.01954	-1.45282	1.89E-03
hsa-miR-641	-0.3559	-0.63752	-1.30842	1.96E-03
hsa-miR-18a-5p	-0.48435	-0.94818	-0.86573	2.01E-03
hsa-miR-1295a	-0.65835	-0.34855	-1.90585	2.21E-03
hsa-miR-93-3p	-0.19264	0.014603	-1.49203	2.99E-03
hsa-miR-573	0.045485	-0.36336	-2.6411	4.71E-03
hsa-miR-1180-3p	-0.42309	-0.78838	-0.9284	4.71E-03
hsa-miR-671-3p	-0.12159	0.052222	-1.75498	4.71E-03
hsa-miR-93-5p	-0.28724	-0.72473	-0.75649	5.09E-03
hsa-miR-7974	-0.46757	-0.51502	-2.01626	5.09E-03
hsa-miR-19b-1-5p	-0.10289	-0.63115	-1.61257	5.09E-03
hsa-miR-128-1-5p	0.227598	-0.41762	-1.82395	5.16E-03
hsa-miR-629-3p	-0.46155	-0.12057	-1.14195	5.56E-03
hsa-miR-4710	-0.08151	-1.65482	-3.43353	5.56E-03
hsa-miR-1914-3p	-0.5167	1.345662	-3.38162	6.03E-03
hsa-miR-5688	1.210287	-1.09641	-2.21139	6.20E-03
hsa-miR-27b-5p	0.06416	-0.55986	-1.13377	6.86E-03
hsa-miR-4699-3p	-0.1162	-1.1917	-3.18888	9.14E-03
hsa-miR-551b-5p	1.027607	-0.05941	-2.06384	9.50E-03
hsa-miR-193a-5p	-0.15013	-0.29067	-0.93841	9.56E-03
hsa-miR-362-5p	-0.29116	-0.50292	-0.95478	1.00E-02
hsa-miR-423-3p	-0.15987	-0.17007	-0.77332	1.03E-02
hsa-miR-19a-5p	0.100564	-0.20378	-2.6832	1.10E-02
hsa-miR-616-5p	-0.08085	0.179868	-1.15296	1.37E-02

hsa-miR-9-3p	0.215338	0.166435	-1.04238	1.37E-02
hsa-miR-548w	-0.21761	-1.39439	-1.22004	1.42E-02
hsa-miR-1304-3p	-0.38779	0.341953	-1.11192	1.63E-02
hsa-miR-135a-2-3p	0.211818	-0.05466	-2.44539	1.63E-02
hsa-miR-3940-5p	-0.11615	0.553109	-3.17013	1.84E-02
hsa-miR-101-2-5p	-0.59345	-0.2393	-2.79442	1.87E-02
hsa-miR-191-3p	0.273653	-0.50923	-1.16773	1.87E-02
hsa-miR-1910-3p	0.815545	-1.03014	-1.81982	2.03E-02