HIV-1 INFECTION INDUCED MICRORNA EXPRESSION PROFILE AND ITS DOWNSTREAM EFFECTS ON CELLULAR TRANSCRIPTOME

by

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BS, University of Pittsburgh, 2010

Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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University of Pittsburgh, 2012

HIV-1 infected individuals show a remarkable variation in virus replication and disease progression. Host cellular gene expression as a response to virus infection is directly correlated with disease patterns. Here, we examine the role of microRNAs on mRNA regulation and its effect in HIV-1 disease progression by performing a comparative microRNA and mRNA profiling.

Total RNA was extracted from subjects with high viral load (HVL)(>45,000 copies/ml), low viral load (LVL) (~<40 copies/ml), and uninfected controls. RNA was quality tested and then reverse transcribed and tested using a Megaplex Real-time PCR for the quantification of expression of 754 miRNAs, in addition to controls, using a high throughput array. Transcriptome profiling was carried out using Illumina HT-12 array, which targets more than 28,688 annotated genes with more than 47,231 probes. Computational analysis to find the mRNA targets of significantly differentially regulated miRNAs was performed with the expression values of both miRNA and mRNA, supplemented with a GenMir++.

HIV-1 infection with high viral load significantly dysregulated the miRNA profile in the infected individuals compared to uninfected or LVL group, whereas infection with low viral load produced a less distinctive profile compared to the controls. These differences are independent of age within these populations. Independent validations confirmed the high throughput results in 65-85% in independent donors. GO enrichment analysis further showed 34 significant terms

of biological processes in the high viral load compared to controls. A less distinctive profile was seen in the low viral load samples compared to controls on both the miRNA and mRNA levels. Biological validation studies also confirmed the expression of HIV induced host cellular factors in *in vitro* infected samples. Together, these results indicate that HIV-1 infection differentially regulates host cellular transcriptome through miRNA expression.

Public health relevance: Viral load significantly upregulates miRNA expression, which translates to an altered expression on the mRNA level. The discovery of microRNAs that play a role in HIV infection could lead to the development of new biomarkers that could be used to identify disease progression within the infected individuals as well as the infected individuals' ability to respond to antiviral treatment.

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PREFACE

I would like to thank my advisor and mentor, Dr. Ayyavoo, for her support and guidance. Her mentorship and encouragement helped cultivate my positive attitude toward science and research. She was always available to answer questions and to discuss any problems or troubleshooting that came up during my rotations. She helped me grow as a student and as a scientist, and I hope to use the skills that I have acquired under her wing in my professional career. I would also like to thank Dr. Martinson and Dr. Barchowsky for their insights into the project and for their encouragement and guidance along the way.

I would also like to thank Hai-Son Le and his mentor Dr. Ziv Bar Joseph, Carnegie Mellon University for their collaboration and analysis. I greatly appreciate the amount of work that they performed in an effort to help predict the miRNA-mRNA interactions from my set of data. Without their skills and expertise, much of my project would not be able to be performed.

Additionally, I would like to thank Bill Buchanan and the entire Pitt MACS clinic for working patiently with me to schedule appointments for my selected donors. A big thanks also goes out to all the participants in my study. I hope that the results of my research are a fair repayment for their participation and devoted time.

None of my work could also be accomplished without the support of my fellow lab members. I would especially like to thank Pruthvi for his assistance in learning various

computer software programs, for his availability to discuss the different methods of analyzing my data, and overall for his involvement and help with the project.

The academic life can become overwhelming sometime; therefore, it is important to have a good support system outside of the work and school environment. As a result, I would like to thank my family for their encouragement and support.

Finally, I would like to thank Alex for everything that he has done for me. Thank you for listening to me complain about failed experiments, encouraging me to keep studying for exams when I felt tired, and telling me to relax when I felt stressed. Thank you for being my best friend and for always being there for me. Without you, I would be a complete honey badger!

1.0 INTRODUCTION

An estimated 33.3 million people are living with HIV infection around the world[1]. However, the disease susceptibility and progression within the infected individuals show remarkable variation in virus replication and immune control [2-5]. Even with advancements in diagnostics and therapy, there is a variation in the level of progression of disease. Research has identified several host factors including the *CCR5*-Δ32 as a factor contributing to the resistance against HIV; however, the mutation does not account for the majority of cases that are persistently resistant[6]. Other genetic factors, such as a particular class 1 HLA allele (HLA-B27 and HLA-B57), appear to also play a role[7, 8]. In addition to these factors, it is likely that host differences in gene expression also contribute to the different disease patterns.

Previous studies have shown that the replication and immune evasion of the virus are affected by the host transcriptome and gene regulation in immune cells, particularly those targeted by HIV[9, 10]. Several other genome-wide association studies have examined the resistance of uninfected exposed sex workers; however, they could not fully confirm what makes the group resistant[11]. Other systems biology studies of elite controllers have identified the FOXO3a, STAT5, and Wnt/beta-catenin pathways as signatures associated with the survival of T cells during the infection[12]. This suggests that other host factors, perhaps those involved in regulating host gene expression, may be involved.

Gene expression is regulated on three levels: transcriptional, post-transcriptional, and translational. The promoter and coding regions of host genetics affect the transcriptional and translational controls; whereas, external factors including cellular factors regulate the post-transcriptional controls. One of the ways that gene expression is regulated on the post-transcriptional level is through microRNAs. MicroRNAs (miRNAs) are a class of newly identified non-coding 18-22nt RNA that act as post-transcriptional regulators controlling gene expression. Over 1,000 miRNAs have thus far been identified in animal genomes[13]. MicroRNAs have been implicated to play a role in development, differentiation, apoptosis, metabolism, cancer, and viral infection[14-16]. By binding to the 3' UTR of their target mRNAs, microRNAs cause translational repression or mRNA degradation[17]. It has been reported that miRNAs regulate up to 92% of human genes[18].

Studies comparing miRNA expression in resting and in activated CD4+ T lymphocytes showed that several miRNAs, which target the Nef-3'-UTR contribute to HIV-1 latency[19]. We hypothesize that host cellular gene expression is controlled by a combination of host genetics, post-transcriptional regulators mediated by HIV-1 infection resulting in a loss of immune control, and disease progression. To test this hypothesis, we evaluated the role of microRNAs on the transcriptome in HIV-1 disease progression by performing a comparative microRNA and mRNA profiling.

2.0 BACKGROUND

2.1 THE AIDS EPIDEMIC AND HIV DISEASE PROGRESSION

There are currently 33.3 million HIV infected individuals worldwide, with more new cases reported each year[20]. The virus infects two types of cells in the immune system: CD4+ T cells and macrophages. It is the count of CD4+ T lymphocytes that is currently used as one of the markers of disease progression in HIV infection[21]. The infection is marked by several stages. During the acute infection stage, which occurs 2-4 weeks after infection, an infected individual may experience severe flu-like symptoms. As the virus replicates and the viral load increases, the CD4 count decreases until a viral set point is reached. At this point, the immune response stabilizes the level of the virus and somewhat increases the CD4 count. The second stage of HIV infection is the clinical latency period, which typically lasts about eight to twenty years, depending on the patient's disease progression. It is a stage where HIV replicates at low levels and the individual may exhibit undetectable viral load levels and a healthy CD4 count. Toward the middle/end of this period, the virus resumes its rapid replication causing a decrease in bystander CD4 cells[5]. When the count drops below 200 cells/mm^3, the individual is diagnosed with AIDS (Acquired immune deficiency syndrome), and eventually succumbs to opportunistic infections that he/she would normally be able to fight off[22]. The incubation period between the time of infection to the development of AIDS is 10 years on average[23].

2.1.1 Long-Term Non-Progressors/Elite Controllers

The introduction of HAART has been able to significantly reduce the rate of individuals progressing to the AIDS stage and has been able to slow the progression of disease. However, there are certain groups of individuals, known as long-term non-progresors (LTNPs), who even without therapy maintain stable CD4 counts[2]. A subset of these individuals, known as elite controllers, actually has undetectable levels of viral replication, and therefore exhibits completely atypical patterns of disease progression[3]. Studies on CD4+ T cells from these individuals have found that they are susceptible to HIV[2]. APOBEC3 family members are RNA editing enzymes, which become incorporated into HIV through their interaction with Gag and result in restriction of HIV-1 infection [24, 25]. The level of hypermutation of G to A caused by APOBEC3G and APOBEC3F in elite controllers is not significantly different from people on HAART, indicating that the hyperactivity of these proteins is not a cause of the viral control [2].

2.1.2 Rapid Progressors

On the other hand, there are other infected individuals who progress to AIDS faster than expected. It is estimated that about 10% of HIV-infected individuals fall into this category and progress to AIDS within 3-5 years after infection[4]. These rapid progressors have higher viral load levels and weaker cellular and humoral immune responses than conventional progressors[26]. Physiologically, these individuals exhibit lower levels of cytokines, including RANTES and MIP-1 alpha and beta, which may contribute to the impaired cellular immune response to infection[4].

2.1.3 Highly Exposed Seronegatives

Additionally, a third group of individuals, known as highly exposed seronegatives, indicates the existence of a natural or acquired immunity to HIV [27]. These highly exposed seronegatives have been reported, among other groups, in children born to seropositive mothers [28], in intravenous drug users [29], and in sex workers [30, 31]. However, the exact mechanisms contributing to the various immune defenses in all of these groups of individuals remain unclear.

2.2 POSSIBLE EXPLANATIONS FOR VARYING DISEASE PROGRESSION

The differential progression of disease can be partially explained by genetic factors. The discovery that HIV uses chemokine receptors as co-receptors has led to the identification of CCR5- Δ 32 mutation. The mutation is more common in Caucasians and for people that are homozygous for the mutation represents a lower possibility of susceptibility to infection [32]. Homozygous CCR5-Δ 32 mutation has been found to be highly protective against M-tropic HIV strains; it is, however, ineffective against T-tropic HIV strains [33]. Heterozygous individuals have been found to exhibit slower disease progression [34-36].

Another possible explanation for an increased HIV/AIDS protection is high level of CCR5-binding chemokines that effectively compete with the virus for the CCR5 receptor [37]. Similarly, an increased presence of stromal cell-derived factor-1 (SDF-1), which occurs following an SDF-1 3'α mutation, slows disease progression by competitively inhibiting T-tropic HIV strains from binding to CXCR4 receptors[32].

Different human leukocyte antigen (HLA) alleles, which are associated with the Major histocompatibility complex (MHC) molecule, have also been associated with different disease progressions and susceptibility to infection[32]. Delayed HIV disease progression has been reported in association with HLA-B*57 and B*27[7, 38].

Recent research suggests that host cellular gene expression (transcriptome) profile as a response to virus infection is directly correlated with disease patterns[39-43]. A study of highly exposed seronegatives from a cohort of Nairobi sex workers found a number of differentially expressed genes compared to controls[44]. Additionally, Borjabad et al. have demonstrated the presence of a differential transcriptome profile between HIV-1 HAND (HIV subjects with associated neurocognitive disorders) in the presence or absence of therapy[45]. A study of gene expression between HIV+ therapy naive and HIV- Botswana females found HIV-1 infection to be associated with genes linked to TLR activation, interferon, and antiviral RNA response pathways[46].

Since gene expression is regulated at multiple levels including transcription, post-transcription, and translation, it is possible that host factors, such as microRNAs, are involved in producing the differential gene expression profile.

2.2.1 MicroRNA (miRNA) biology

MicroRNAs (miRNAs) are a new class of 18-22 nt long, non-coding RNAs that act as post-transcriptional regulators and alter the stability of their target mRNAs[47]. It is predicted that more than 60% of all human protein coding genes contain miRNA binding sites in their 3'untranslated region (UTR) for miRNA to bind to[17]. The miRNA-mRNA relationship is

complex in that one miRNA has the potential to target multiple mRNAs and one mRNA can also be targeted by multiple miRNAs[48].

2.2.2 MiRNA processing and function

Genes encoding miRNAs are transcribed by RNA polymerase II as hairpin-containing pri-microRNAs. The hairpin is then processed into the nucleus by RNAse III Drosha enzyme to form pre-microRNAs. These are then transported out of the nucleus via Exportin 5. In the cytoplasm, RNAse III Dicer generates a dsRNA complex composed of a miRNA and miRNA*. One of the strands is selected as a guide strand, while the complementary one is typically degraded. The guide strand is recruited to an RNA-induced silencing complex (RISC). The miRNA-RISC complex then binds the 3'-UTR of mRNAs and inhibits translation. Perfect or near perfect complementarity between the miRNA and the 3'-UTR of the mRNA results in the degradation of mRNA, while mismatched complementarity results in the inhibition of translation due to the inhibition of the circularization of mRNA that is needed for ribosomal attachment [49].

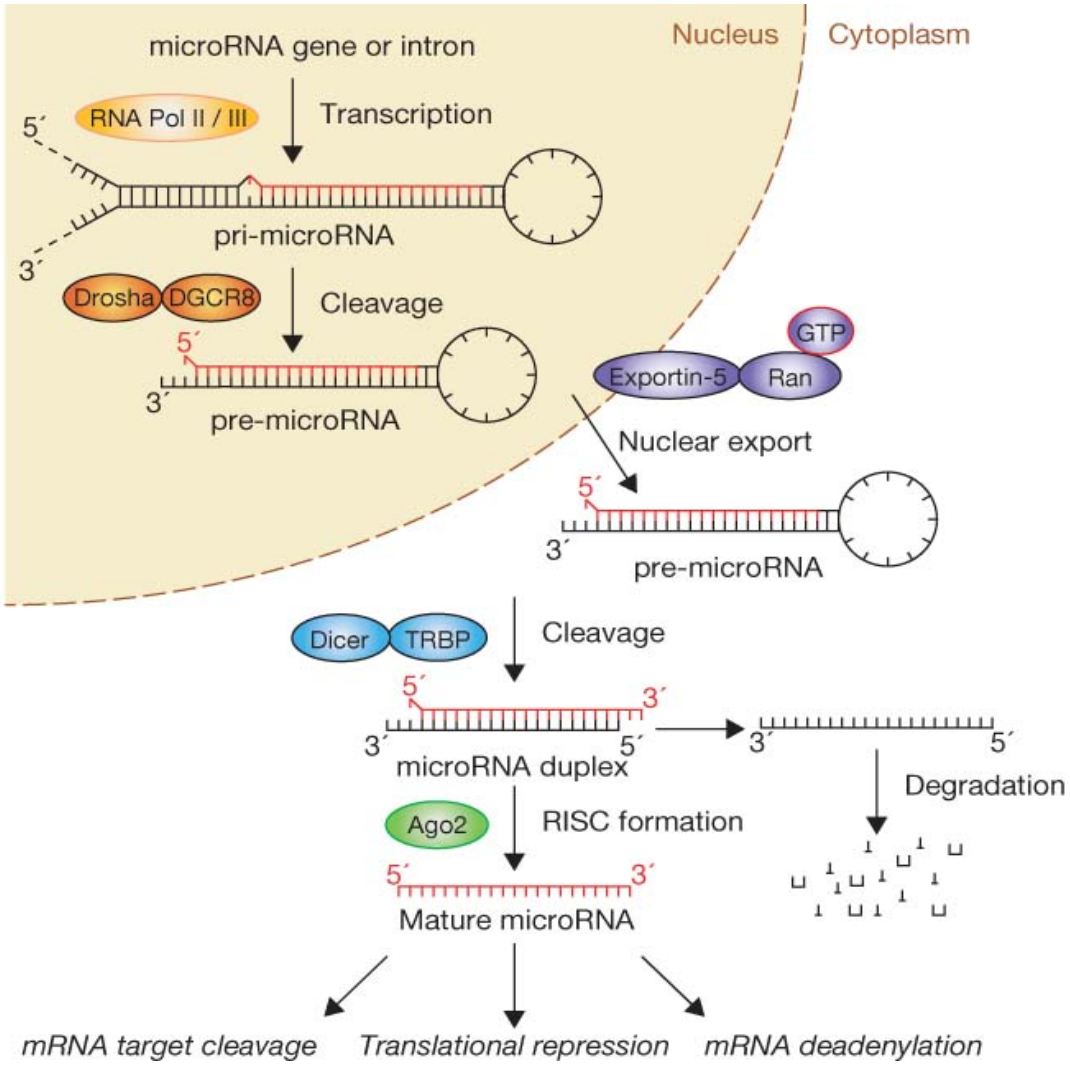

Figure 1. The 'linear' canonical pathway of microRNA processing

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2.2.3 Viral miRNA and HIV

miRNAs are encoded by many viruses, including HIV. HIV is associated with a transactivation response element (TAR), which binds the transcription activator (Tat) in order to initiate transcription from the LTR[50]. A miRNA encoded by HIV-1 TAR has been shown to produce a diminished viral gene expression and, therefore, has been suggested to play a role in viral latency[51] Additionally, Oullet at al have demonstrated the TAR region to be a source of two additional miRNAs: miR-TAR-3p and miR-TAR-5p; the biological role of these miRNAs has yet to be established[52]. Furthermore, using a computer-directed analysis, Bennasser et al have found five HIV encoded pre-miRNAs and a number of 3'UTR binding sites in the human genome, indicating the potential role of these viral miRNAs in regulating cellular transcripts[53]. The HIV-1 accessory protein Nef has also been shown to encode viral miRNAs. One of these Nef-derived miRNAs is miR-N367, which functions not on the post-transcriptional level like ordinary miRNAs but through the suppression of HIV-1 promoter activity[51]. More research is required to learn more about the exact mechanism of the suppression.

2.2.4 Host miRNA and HIV

Although much of the research regarding miRNAs has focused on their association with cancer, miRNAs have also been implicated to play a role in infectious diseases. For instance, miR-32 has been shown to exhibit antiviral activity when a knockout of the expression of the miRNA or the deletion of its target sequence produced an increase in the replication of primate foamy virus 1 [54].

Several groups have begun to examine a possible relationship between host miRNAs and HIV-1 infection. Regarding the possible connection to CD4+T cells, one of the main targets of HIV, miR-28, miR-125b, miR-150, miR-223, and miR-382 of miRNAs were found to be upregulated in resting CD4+ T cells compared to activated CD4+ T cells, suggesting their possible role in HIV latency[19]. The suppression of these miRNAs in monocytes was found to facilitate HIV infection, while an increase in these anti-HIV-1 miRNAs led to an inhibition of viral replication[55]. Triboulet et al discovered that HIV-1 suppressed the miR-17/92 miRNA cluster, thus allowing efficient viral replication to occur[56]. Additionally, miR-29a has been shown to be highly expressed in CD4+ T cells. This miRNA targets both the 3' UTR of HIV -1 transcripts and HIV-1 Nef; thus, it may be a key player in controlling viral replication[57].

However, up till now, there has not been a study that we are aware of that has examined both the miRNA and mRNA profiles in subjects with varying viral loads. For this reason, our examination of both of these profiles and our analysis of how the miRNA and mRNA interplay with each other is a novel study.

3.0 THESIS AIMS

We hypothesize that host cellular gene expression is controlled by a combination of host genetics, and post-transcriptional regulators, that are altered by HIV-1 infection resulting in loss of immune control, cellular dysfunction, and disease progression.

AIM #1: To examine a whole genome miRNA and mRNA profiles of HIV positive subjects along with appropriate uninfected controls in a cross-sectional study

A. To perform the profiling in PBMCs of subjects infected with low viral load, high viral load, and in uninfected controls.
B. To perform miRNA and mRNA validations on independent subjects with similar disease status.

AIM #2: To examine the interplay of miRNA and their target mRNA using computational analyses

A. To perform a computational analysis to predict possible mRNA targets of the significantly dysregulated miRNAs.
B. To perform correlation studies for selected transcripts and CD4, Nadir CD4 counts, and viral load.
C. To perform in-vitro biological functioning studies.

AIM #3: To identify miRNA profiling in CD4 and CD14 cells

A. In subjects with low viral load and uninfected controls.

B. In *in vitro* infected samples representing high viral load and in mock infected samples representing uninfected controls.

4.0 MATERIALS AND METHODS

4.1 STUDY POPULATION AND SELECTION CRITERIA

All donor samples were obtained from the Pittsburgh site of the Multicenter AIDS Cohort Study (MACS). The study population included uninfected seronegative controls (N=42), HIV-1 positive subjects with low viral load (~<40 copies/ml) (N=37), and HIV-1 positive subjects with a high viral load (>45,000 copies/ml) (N=35) at the time of sample collection. Infected subjects were selected and grouped into the appropriate categories (low viral load/high viral load) if they maintained the appropriate viral load for a period of at least a year leading up to the date of collection. Details of the subjects including age, CD4 counts at the time of sample collection, Nadir CD4 counts, viral load at the time of sample collection, viral load for the past 5 years and number of years on ART/HAART are included in Table 1-3.

Table 1. Clinical characteristics of uninfected control samples

ID	AGE	miRNA	mRNA	CD4	CD4 range over last 5 yrs	Nadir CD4
CT1	74	yes	no	1326	1116-1892	1074
CT2	54	yes	yes	644	664-964	240
CT3	55	yes	yes	584	460-584	400
CT4	56	yes	yes	385	351-759	351
CT5	55	yes	yes	889	535-1015	535
CT6	53	yes	yes	702	530-921	474
CT7	57	yes	yes	537	497-732	426
CT8	49	yes	yes	685	438-1082	428
CT9	42	yes	yes	841	556-947	553
CT10	42	yes	yes	1634	1365-1852	757
CT11	28	yes	yes	1178	709-1225	636
CT12	34	yes	no	894	819-1147	819
CT13	36	yes	yes	852	664-1014	664
CT14	36	yes	yes	789	725-1061	725
CT15	52	yes	no	491	491-654	363
CT16	46	yes	yes	918	799-1315	799
CT17	51	yes	yes	2194	715-2194	715
CT18	72	yes	yes	530	470-859	470
CT19	52	yes	yes	733	625-888	625
CT20	64	yes	yes	879	736-1107	737
CT21	60	yes	yes	1019	894-1281	894
CT22	49	yes	yes	589	481-765	481
CT23	38	yes	yes	545	545-1187	545
CT24	28	yes	yes	857	605-947	605
CT25	43	yes	yes	1106	732-1106	732
CT26	50	yes	yes	790	703-1304	571
CT27	56	yes	yes	488	479-703	479

Continued Table 1.

	38	yes	yes	1074	587-1175	587
CT29	52	yes	yes	993	977-1453	840
CT30	33	yes	yes	886	737-1170	737
CT31	77	yes	yes	489	444-569	328
CT32	30	yes	yes	732	588-854	520
CT33	58	yes	yes	568	390-732	390
CT34	51	yes	yes	885	659-1083	659
CT35	50	yes	yes	944	637-1204	523
CT36	50	yes	no	606	550-872	550
CT37	46	no	yes	1054	870-1360	444
CT39	30	no	yes	589	419-979	419
CT40	43	no	yes	1636	1297-1659	1045
CT38	54	no	yes	676	598-771	441
CT41	38	no	yes	1057	784-1071	783
CT42	41	no	yes	1429	683-1551	683

Table 2. Clinical characteristics of low viral load subjects

CODE ID	miRNA	mRNA	AGE	CD4	CD4 range over last 5 yrs	Nadir CD4	ART	yrs on HAART	V. Load
LVL1	yes	no	53	356	252-696	252	yes	5	59
LVL2	yes	yes	57	256	248-435	248	yes	-	40
LVL3	yes	yes	50	334	321-433	184	yes	6	40
LVL4	yes	yes	61	662	330-662	109	yes	7	40
LVL5	yes	yes	59	552	496-690	93	yes	13	51
LVL6	yes	yes	52	304	249-607	249	yes	13	40
LVL7	yes	yes	39	366	91-816	13	yes	12	122
LVL8	yes	yes	43	1066	672-1148	436	yes	8	40
LVL9	yes	yes	39	753	414-753	325	yes	12	40
LVL10	yes	yes	52	766	693-1187	492	yes	14	40
LVL11	yes	yes	34	597	65-597	65	yes	2	40
LVL12	yes	no	44	952	558-952	70	yes	11	40
LVL13	yes	yes	59	502	502-973	502	yes	13	4615
LVL14	yes	no	57	193	75-275	75	yes	10	2580
LVL15	yes	yes	47	746	627-868	543	yes	8	40
LVL16	yes	yes	61	927	871-1195	639	yes	11	40
LVL17	yes	yes	53	471	304-757	304	yes	13	40
LVL18	yes	yes	49	379	272-482	272	yes	13	40
LVL19	yes	yes	49	1421	582-1459	582	yes	4	40
LVL20	yes	yes	61	509	431-671	350	yes	14	40
LVL21	yes	yes	57	674	315-674	315	yes	2	40
LVL22	yes	yes	55	845	508-932	99	yes	8	40
LVL23	yes	yes	ND	459	406-598	238	yes	14	40
LVL24	yes	yes	41	595	463-756	463	yes	9	40
LVL25	yes	yes	49	654	672-971	267	yes	12	40
LVL26	yes	yes	55	862	570-1084	231	yes	13	40

Continued Table 2.

LVL27	yes	yes	41	826	680-1103	393	yes	13	40
LVL28	yes	yes	62	680	557-726	272	yes	14	40
LVL29	yes	yes	46	488	496-797	207	yes	13	40
LVL30	yes	yes	51	769	615-864	281	yes	14	40
LVL31	yes	yes	51	960	543-1000	231	yes	14	40
LVL32	yes	yes	50	782	636-785	191	yes	14	40
LVL33	no	yes	52	552	287-642	279	yes	11	40
LVL34	no	yes	50	915	473-860	461	yes	11	40
LVL35	no	yes	53	771	689-1146	333	yes	5	40
LVL36	no	yes	57	879	721-960	100	yes	9	40
LVL37	no	yes	38	377	331-807	331	yes	11	497

Table 3. Clinical characteristics of high viral load subjects

CODE ID	miRNA	mRNA	AGE	CD4	CD4 range over last 5 yrs	Nadir CD4	Yrs on ART/HAART	V. load
HVL1	yes	yes	46	219	77-219	77	7	336300
HV2	yes	no	37	222	222-520	222	7	96534
HV3	yes	no	37	246	246-524	246	7	47083
HV4	yes	no	43	173	173-456	173	4	79454
HV5	yes	no	64	142	142-495	142	1	250617
HV6	yes	yes	62	279	275-1175	275	2	151396
HV7	yes	yes	37	337	320-576	320	1	76521
HV8	yes	no	31	162	29-323	29	1	198196
HV9	yes	no	36	312	231-894	231	5	71580
HV10	yes	no	65	233	142-495	142	2	49430
HV11	yes	yes	51	136	136-449	135	2	561627
HV12	yes	yes	50	120	120-804	120	1	297672
HV13	yes	yes	37	378	378-1025	378	6	289528
HV14	yes	no	35	38	38-472	38	0	167261
HV15	yes	yes	39	96	79-365	79	1	47310
HV16	yes	yes	63	268	251-495	251	0	123700
HV17	yes	no	47	151	151-453	151	0	146580
HV18	yes	yes	42	232	232-634	232	0.75	252692
HV19	yes	yes	45	218	218-582	218	0	48352
HV20	yes	yes	37	120	120-196	119	0	370999
HV21	yes	yes	49	336	515-787	336	0	161325
HV22	yes	no	62	147	147-483	147	3	320455
HV23	yes	yes	49	215	215-998	215	0	184411
HV24	yes	no	39	188	188-483	188	0	201158
HV25	yes	yes	50	120	120-804	120	1	297672
HV26	yes	yes	39	188	188-483	188	0	112327

Continued Table 3.

HV27	yes	yes	28	234	234-779	234	0	46053
HV28	yes	yes	53	100	100-460	100	0	111314
HV29	yes	yes	33	310	149-1245	149	0	127376
HV30	yes	yes	42	272	265-480	235	0	50530
HV31	yes	yes	40	351	308-697	308	0	116293
HV37	no	yes	38	312	312-1001	312	0	108499
HV38	no	yes	40	172	172-603	172	0	48494
HV39	no	yes	39	188	188-765	188	0	112327
HV40	no	yes	38	116	24-281	24	11	1157700

4.2 PBMC ISOLATION

Blood samples were obtained from the Pittsburgh site of the Multicenter AIDS Cohort Study and processed within 12 hours of delivery. The blood was first spun with a lymphocyte separation solution (Cellgro) to obtain a buffy coat layer, which was subsequently diluted with PBS and washed twice with PBS. Isolated PBMCs were then counted and either stored in RNA later (Ambion) in -80°C or immediately used for RNA isolation and/or other cell type isolation.

4.3 CD4+ AND CD14+ CELL ISOLATION

CD14+ cells were isolated from PBMCs by positive selection using CD14 MicroBeads according to the manufacturer's instruction (Miltenyi Biotec). CD4+ cells were then isolated from the non-CD14 elute by positive selection using CD4 MicroBeads (Miltenyi Biotec). Isolated cells were tested by flow cytometry for the purity. Briefly, the cells were first washed twice with FACS buffer and stained with anti-human CD4-PE (Immunotech) and CD14-PE (Immunotech), or IgG-PE (Pharmingen) for 60 minutes in ice, and then again washed twice with PBS. The purified CD4+, CD14+, and remaining non-target cells were immediately processed for RNA isolation.

4.4 RNA ISOLATION

PBMCs (fresh or frozen) or isolated CD4+, CD14+, and non-target cells were used for RNA isolation using the MirVana kit (Applied Biosystems), according to the manufacturer's protocol. Briefly, 5-10 million cells were lysed with 600µl of lysis buffer. For fewer cells, only 300µl of lysis buffer was added. Next, RNA homogenate at a volume equaling 1/10 of lysis buffer was added to the cells, and the cells were incubated on ice for 10 minutes. This was followed by an addition of acid phenol chloroform at a volume equal to that of the lysis buffer. The samples were then thoroughly vortexed for 30 seconds and centrifuged for five minutes at room temperature at 12,000 rpm. The aqueous phase was then removed. Next, 100% ethanol was added. Using a filter cartridge, the samples were spun and washed three times with wash solutions. Finally, the samples were eluted with 90µl of pre-heated elution solution. RNA concentration was determined with NanoDrop2000 spectrophotometer, and the samples were stored at -80°C.

4.5 RNA QUALITY CONTROL

RNA (100ng) was used to test the expression of endogenous controls RNU48, miR-26b, or U6snRNA. Briefly, the RNA was first converted into cDNA using the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems) according to Table 4 and run at 16°C for 30 mins, 37°C for 30 mins, 85°C for 5 mins, and indefinitely held at 4°C. The cDNA was either stored at -20°C or immediately used for a qRT PCR. The qRT PCR reaction was run in 20µl triplicates (according to Table 5), using the ABI Prism 7000 Sequence detection system, set at

50°C for 2 mins, 95° for 10 mins, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The expression of the endogenous controls was analyzed using CT values obtained with an auto threshold of 0.2. If a particular RNA sample showed endogenous control expression, at least three times, with a value of 25 or greater, the particular sample was eliminated from further analysis. The RNA quality was also determined using Agilent Bioanalyzer 2100 and the protocol developed by the Core. RNA samples with RNA Integrity Number (RIN) 5-10 were determined to be of good quality and were used for further processing.

Table 4. Reverse transcription reactants per reaction for miRNA detection

Reagents	Volume (µl)
100mM dNTPs with dTTP	0.15
Multiscribe Reverse Transcriptase 50 U/ul	1
10X Reverse Transcription Buffer	1.5
RNase Inhibitor, 20U/ul	0.19
Nuclease-free water	4.16
RNA	5
Taqman MicroRNA assay (5x)	3
Total	15

Table 5. Real Time PCR reactants per reaction for miRNA detection

Reagents	Volume (µl)
Taqman MicroRNA assay (20X)	3.6
cDNA	4.8
Taqman 2X Univesal PCR Master Mix II, No UNG	36
Nuclease-free water	27.61
Total	70

4.6 MICRORNA PROFILING AND DATA ANALYSIS

4.6.1 MicroRNA profiling

Microfluidic cards - Human MicroRNA card set v3.0 (purchased from Applied Biosystems) were used for miRNA profiling of samples. This set, composed of 2 cards (Pool A and Pool B), enables quantitation of 768 miRNA in total, which include 754 targets, 4 endogenous controls (MammuU6/U6 snRNA run in quadruplicates, and RNU44, RNU48, and ath-miR-159a run in duplicates). One µg of RNA was reverse transcribed using Taqman microRNA reverse transcription kit (Applied Biosystems, CA) along with 1.5 Reverse transcription master mix (Table 6) at a volume equal to 1.5 times the volume of RNA. The PCR was run for 40 cycles at 16°C for 2 mins, 42°C for 1 min, 50°C for 1 sec, followed by 85°C for 5 minutes and indefinite holding at 4°C. The resulting PCR product was either stored at -20°C or directly loaded on to a 4.5array cards with 450µl Taqman Universal Master Mix II-No UNG, and nuclease-free water to make the final volume equal 900µl. The cards were run on ViiA7 Real-Time PCR system according to manufacturer's protocol, using comparative CT and standard settings.

Table 6. Reverse transcription reagents for microRNA high throughput miRNA profiling

Reagents	Volume (µl)
Megaplex RT primers (10X)	0.8
dNTPs with dTTP (100mM)	0.2
Multiscribe Reverse Transcriptase 50U/ul)	1.5
10X RT buffer	0.8
MgCl2 (25mM)	0.9
RNase Inhibitor, 20U/ul	0.1
Nuclease-free water	0.2
Total	4.5

4.6.2 Data Analysis

Initial miRNA expression data were analyzed using integrated ViiA7 software (Applied Biosystems). Each run was exported separately using an auto detection threshold. The data were then uploaded to RealTime StatMiner software (Integromics, PA) for further analysis. Detectability threshold for miRNA assays was set to Ct value less than or equal to 35 and at least 25% of all samples in each group. We used Grubbs method to eliminate outliers within technical replicates and Genorm method to select endogenous control that is U6-snRNA/mammU6 for normalization based on stability scoring across the samples in each group. Differentially expressed miRNA between different groups were identified using parametric t-test or LIMMA (one factor analysis) and were then sorted using Benjamini-Hochberg false discovery rate (FDR) method with adjusted p-value <0.05. The fold changes were obtained using linear RQ values. Hierarchical clustering for differentially expressed miRNA was performed with their corresponding dCt values across the samples in different groups with 'Complete linkage' clustering method and 'Euclidean' distance measure for dendrograms.

4.7 GENE EXPRESSION PROFILING AND DATA ANALYSIS

4.7.1 Gene Expression Profiling

For whole genome transcriptome analysis, we used Illumina HT-12 V4 array bead chips (Illumina, Inc., San Diego, CA, USA) for mRNA profiling of different groups (Control, LVL and HVL) samples in the study. Each array targets about 47,231 probes that include 28,688 well-

characterized or annotated coding transcripts along with 11,121 coding transcripts with provisional annotation and remaining being non-coding transcripts and splice variants. RNA (1 μg) samples were labeled using 'TotalPrep RNA' labeling kit (Ambion), reverse transcribed to cDNA, hybridized onto array bead chips overnight on rocker and scanned on 'iScan system' according to the manufacturer's protocols as well as standardized protocols developed by Genomics and Proteomics Core Laboratories.

4.7.2 Data Analysis

Data analysis was performed using the Illumina software to delineate the false discovery rate (FDR) and differences with statistical significance ($p<0.05$). Initial raw data analysis and cubic spline normalization was done using BeadStudio Gene expression module (Illumina, Inc.). The normalized sample probe profile and control probe profile were then uploaded to Integrated Biomarker Discovery (IBD) (Integromics) for further data analysis. A total of 21,852 probe sets were detected in all samples of different groups (Control, LVL and HVL) with present call filter set to probes detected in at least 75% of samples in each group. Linear models for microarray data (LIMMA) (one factor analysis) was used to identify differentially expressed genes/probes between different groups. These genes were further sorted based on Benjamini-Hochberg false discovery rate (FDR) method with adjusted p-value <0.05 and fold change cut-off of at least 2-fold up/down regulation. Hierarchical clustering for differentially expressed genes/probes was performed using their corresponding detection signal values across the samples in different groups with 'Complete linkage' clustering method and 'Euclidean' similarity measure for dendrograms.

4.8 VALIDATION OF SELECTED DIFFERENTIALLY REGULATED MIRNAS

Based on the data analyses, selected miRNA were verified by qRT-PCR using specific Taqman based primers and probes (Applied Biosystems). We used independent RNA samples (n=5) from the miRNA microarray profiling to validate the high throughput microarray results. Selected primers were used to generate cDNA from 33ng of RNA using the miRNA Reverse Transcription Kit (Applied Biosystems). The procedure was identical to that described in Quality control RNA section. Following normalization to U6, the data were exported and analyzed in RealTime Statminer software, as described previously.

4.9 VALIDATION OF SELECTED DIFFERENTIALLY REGULATED MRNAS

cDNA was generated from 100ng of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to Table 7. The PCR was run at 25°C for 10 mins, 37°C for 120 min, 85°C for 5mins, and held indefinitely at 4°C. The cDNA was then either kept at -20°C or immediately used for qRT PCR. The qRT PCR was run in 20µl triplicates (according to Table 8), using the ABI Prism 7000 Sequence detection system, set at 50°C for 2 mins, 95° for 10 mins, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative expression (ddCT) was calculated after normalization to RPLPO. The data were analyzed using Realtime StatMiner following an individual export of each sample, set an auto threshold.

Table 7. Reverse transcription reagents for mRNA validations

Reagents	Volume (µl)
RNA	10
10X RT buffer	2
25x dNTP	0.8
Random Primer	2
Multiscribe Reverse Transcriptase	1
Nuclease-free water	4.2
Total	20

Table 8. Real Time PCR reagents for mRNA validations

Reagents	Volume (µl)
cDNA	20
Taqman Gene Expression Master Mix	35
mRNA assay	3.5
Nuclease-free water	11.5

4.10 PATHWAY ANALYSES

To determine gene interactions and correlation networks, we used STRING. The cutoff values for inclusion in these analyses were differential mRNA expression, with p-value <0.05 and ± 2.0 in fold change. Since no mRNAs were differentially regulated between the LVL and Control groups at this fold change cutoff, all mRNAs with a $p<0.05$ (regardless of fold change) were considered for the pathway analysis between these groups. Gene Ontology Enrichment (GO) analysis tool within IBD was used to detect biological annotations that are statistically over-represented in the list of differentially regulated genes/probes between different groups (e.g. Control and LVL). With minimum number of overlapping genes equal to 3 and FDR threshold of <0.05, both singular and concurrent enrichment analyses were carried out. Significant terms for biological processes (BP), molecular functions (MF) and cellular components (CC) were retrieved, arranged in an order based on p-value for the corresponding term.

4.11 COMPUTATIONAL ANALYSIS FOR MIRNA-MRNA INTERACTION PREDICTIONS

Computational analysis was kindly performed by our collaborators Haison Le and Dr. Ziv Bar-Joseph from Carnegie Mellon University. Using the mRNA data from Illumina, they used the R package samr to detect differentially expressed mRNAs, which were then combined with the differentially expressed miRNAs from our analysis and used as input for GenMIR++, using MicroCosm Targets database to make the miRNA-mRNA predictions. Predictions were filtered to the union of the significantly differentially regulated miRNA and mRNA (mRNA and miRNA present in at least 75% of the samples, without FDR correction) or to the specific list of significantly differentially regulated miRNA and mRNA from each group analysis.

4.12 INFECTION OF PBMCS

Isolated PBMCs were stimulated with PHA (1μl/1ml R10 media) for three days and subsequently infected with 500pg equivalent p24 of EGFP reporter NL43 (CXCR4 tropic) or YU2 (CCR5 tropic) virus per 10^6 cells. Seven days post-infection, EGFP fluorescence was observed under a microscope and examined with HIV-1 p24 ELISA, and CD4 and CD14 cells were isolated, as described previously.

4.13 BIOLOGICAL VALIDATION OF MRNA BY QRT-PCR USING PBMCS INFECTED IN VITRO

mRNA expression was tested in an *in vitro* model of uninfected PBMCs and PBMCs infected with CXCR4 (NL43) or CCR5 (YU2)-coreceptor utilizing viruses (n=3). It was also assessed in normal donor PBMCs or in a set of independent donors (n=5). cDNA was generated from 100ng of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). QPCR was then performed using the Taqman Gene Expression Master mix (Applied Biosystems) and the appropriate gene assay (Applied Biosystems), as suggested by the manufacturer using the ABI Prism 7000 Sequence Detection System. Relative expression (ddCT) was calculated after normalization to RPLPO.

4.14 BIOLOGICAL VALIDATION OF HIV-1 REGULATED FACTORS BY ELISA

HIV-1 induced cytokines and chemokines were tested in an *in vitro* model by infecting normal donor blood derived PBMCs infected with CXCR4 (NL43)- or CCR5 (YU2) coreceptor utilizing viruses as described above. Infection was confirmed via a HIV-1 p24 ELISA. Seven days postinfection, supernatant was assessed for the production of CCL2, CCL8, IL-6 and IL-8 by ELISA (BD Biosciences, CA),

4.15 WESTERN BLOT ASSESSMENT FOR NEUROGRANIN (NRGN)

Whole cell lysates were prepared using RIPA buffer (containing 50mM Tris (pH 7.5), 150mM NaCl, 1% Triton X-100, 1mM sodium orthovanadate, 10mM sodium fluoride, 1mM phenylmethyl-sulfonylfluoride, 0.05% deoxycholate, 10% SDS, 0.07 trypsin and protease inhibitors 1ug/ml) and 20 μg equivalents of protein were separated by 12% SDS–PAGE, transferred to nitrocellulose membrane (Bio-Rad), and probed with anti-NRGN (Millipore) for 4°C over night, and then washed with PBS and 0.1% Tween20 and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Caltag) for 1hr at room temperature. The membrane was developed using an ECL enhanced chemiluminescence kit (Pierce). Densitometry analysis was performed using Image J software.

5.0 RESULTS

5.1 AIM #1: TO EXAMINE WHOLE GENOME MIRNA AND MRNA PROFILES OF SUBJECTS IN A CROSS-SECTIONAL STUDY

5.1.1 To perform miRNA profiling in PBMCs of subjects infected with low viral load, high viral load, and in uninfected controls

To examine the expression of miRNAs in the uninfected controls, low viral load, and high viral load donors, we isolated RNA from PBMCs and tested its quality with Nanodrop 2000 spectrophotometer and Taqman assays for endogenous controls U6, RNU48, or miR-26b. If the expression of the endogenous controls in a sample exceeded Ct of 25, that sample was eliminated from further processing.

The miRNA profiling consisted of running two microfluidic cards per sample for the combined effect of the ability to detect 754 miRNA and 4 controls. Once again, for RNA quality purposes, samples where the endogenous controls exceeded Ct of 25 were eliminated. Our endogenous control analyses indicate that the Ct values for all our samples range from 15-22 and none of them are more than 25 (Fig. 2).

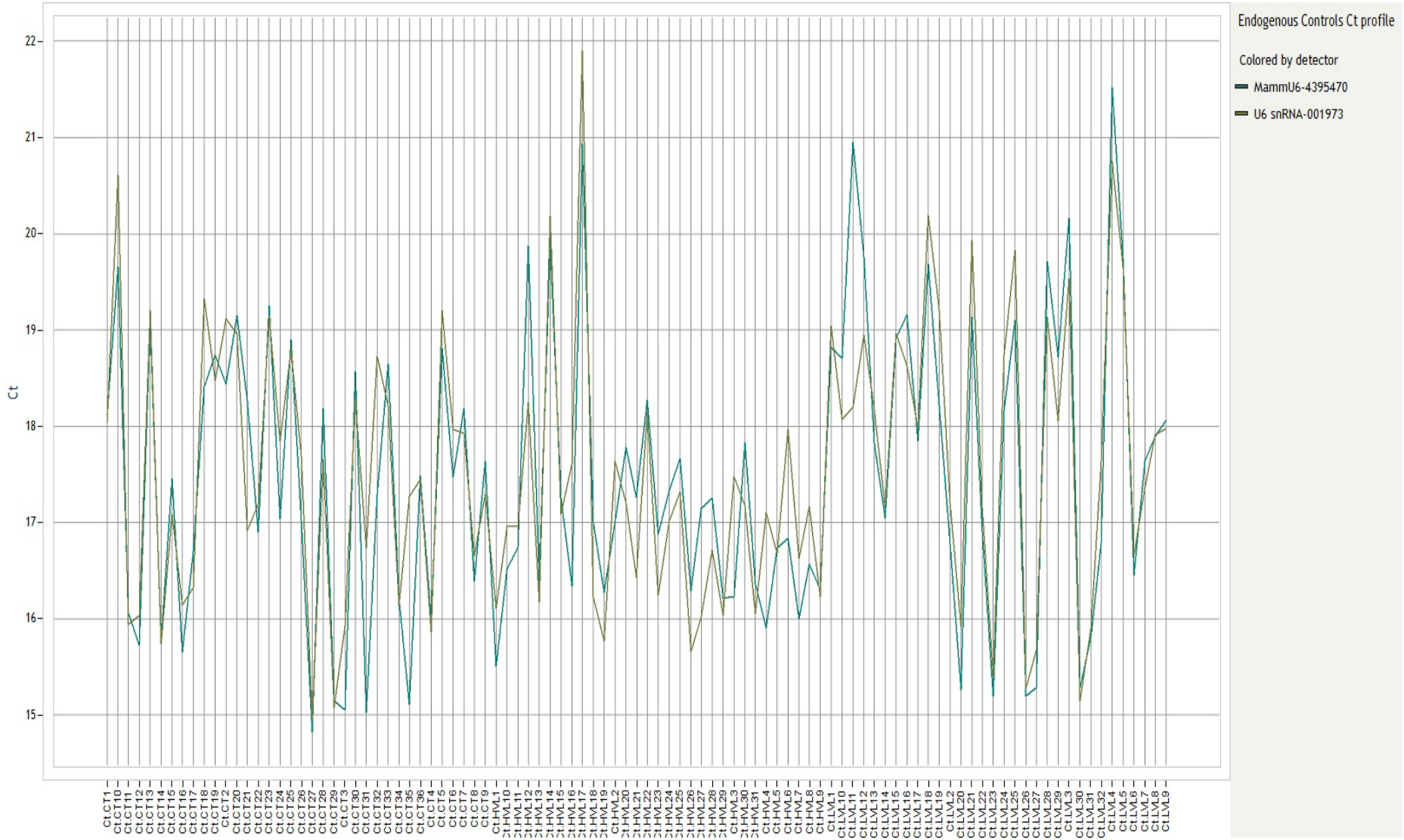

Figure 2. Endogenous control profile of samples used for microRNA profiling

Endogenous CT values for each sample were obtained using RealTime StatMiner to determine the quality of the sample. Green depicts MammU6 (Pool A), while brown depicts U6 snRNA (Pool B).

Results from high and low viral groups were compared with the uninfected control group as well as within the HIV-1 positive groups. Differentially regulated miRNAs between these groups were analyzed using two different software packages using appropriate settings required for each software to maximize the confidence (Table 9, 10, and 11). The first program used was DataAssist (Applied Biosystems), and the settings were default, as suggested by the manufacturer. Briefly, maximum allowable Ct value was set at 40, meaning that any miRNAs with a Ct>40 were assigned Ct of 40, so that fold change could be calculated. Benjamini-Hochberg False Discovery Rate adjustment was also applied.

The second program used was Realtime StatMiner (Integromics) in which we considered Ct values that are >35 as non-specific. In our analyses, we excluded miRNAs that show a raw Ct value of >35 in more than 75% of the subjects in each group as suggested[58]. This resulted in elimination of 177, 173 and 160 miRNAs from each comparison (control vs. LVL; control vs. HVL; LVL vs. HVL) respectively. The remaining miRNA were assessed using a statistical package within Realtime StatMiner to identify the significantly regulated miRNA within and between these groups. Results indicate that HIV-1 infection differentially regulated expression of several miRNAs (+/- 1.72-fold with $p<0.05$ using Benjamini-Hochberg method within each group analysis). The level of expression in infected subjects was compared with that in uninfected controls, and fold differences were calculated based on normalization with endogenous control, U6 snRNA/MammU6, as suggested by the manufacturer.

Table 9. Differentially regulated miRNA in low and high viral load compared to uninfected control group and within the infected groups using DataAssist

	Differentially regulated miRNA	Differentially regulated miRNA $p<0.05$	Differentially regulated miRNA $p<0.01$	Differentially regulated miRNA +/- 2-fold $p<0.05$	Differentially regulated miRNA +/- 2-fold $p<0.01$
Control vs.	N/A	39	8	38	8
Control vs.	N/A	245	164	244	163
LVL vs. HVL	N/A	194	93	183	90

Table 10. Differentially regulated miRNA in low and high viral load compared to uninfected control group and within the infected groups using RealTime StatMiner

	Differentially regulated miRNA	Differentially regulated miRNA $p<0.05$	Differentially regulated miRNA $p<0.01$	Differentially regulated miRNA +/-2-fold $p<0.05$	Differentially regulated miRNA +/-2-fold $p<0.01$
Control vs.	577	41	14	41	14
Control vs.	580	221	138	218	137
LVL vs. HVL	593	190	100	182	99

Table 11. Commonly differentially regulated miRNAs between DataAssist and RealTime StatMiner

	Differentially regulated miRNA	Differentially regulated miRNA $p<0.01$
Control Vs.	21	8
Control vs.	191	118
LVL vs. HVL	158	80

Among the 754 miRNAs tested, 21 miRNAs were significantly ($p<0.05$) regulated in low viral load individuals compared to uninfected controls by both DataAssist and StatMiner. Of these, 2 miRNAs were downregulated (with a StatMiner derived fold change ranging from -27.6

to –1117.81) and 19 miRNAs were upregulated, with a StatMiner derived fold change ranging from 3.09 to 20.48 (Appendix A1).

However, in case of HVL group, the number of differentially regulated miRNAs was higher than that in the LVL group. A total of 191 miRNAs were differentially regulated in high viral load subjects compared to uninfected controls by both StatMiner and DataAssist. Of these, 13 were downregulated (with a StatMiner derived fold change ranging from -2.05 to -89.82). In the 178 miRNAs that were upregulated, the StatMiner derived fold change ranged from 1.77 to 4078.26 (Appendix A2).

Similarly, we also assessed the differentially regulated miRNA in low viral and high viral load groups that resulted in 158 miRNAs. Of these, 27 were downregulated (with a StatMiner derived fold change ranging from -69.08 to -1.86). In the 131 upregulated miRNAs, the StatMiner derived fold change ranged from 1.73 to 6460.41 (Appendix A3). These results indicate that the number of miRNAs that are dysregulated in high viral load group is much higher that in low viral load.

Intersection between these groups indicates that there are several miRNAs that are common in the context of HIV-1 infection independent of viral load (Fig. 3). Figure 3 exhibits the miRNAs that are specific to each group as well as the overlap between the groups. When uninfected group versus HIV-1 infected samples were compared, 17 miRNAs are commonly regulated in HIV-1 infected subjects (LVL and HVL combined) compared to uninfected controls. Among the 21 miRNAs that are differentially regulated in CT vs. LVL, 3 were unique to the LVL group, whereas 62 were specific to HVL group among the 191 observed in CT versus HVL group. Similarly, we also compared all three groups and found that 4 miRNA are commonly regulated in all three groups. Further examination indicates that miR-1275, miR-483-

5p, and miR-650 show similar upregulation compared to control or LVL group, whereas miR-1262 shows downregulation in control versus HIV-1 positive group, and were upregulated in HVL group compared to LVL group. Together, these results suggest that HIV-1 infection (irrespective of viral burden) and high viral load have specific regulatory effects on miRNA expression profile.

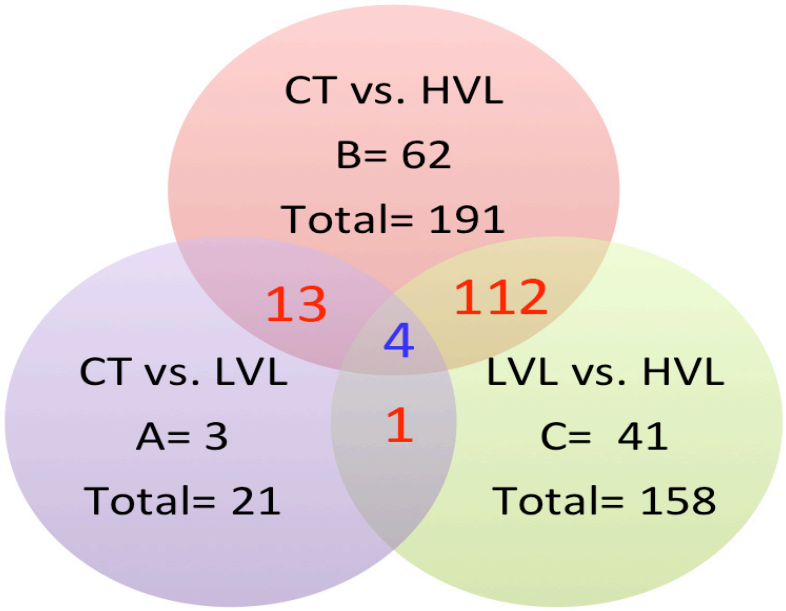

Figure 3. Venn diagram illustrating the number of significantly differentially regulated miRNAs between low viral load, high viral load, and uninfected controls

The Venn diagram displays the number and overlap of significantly differentially expressed miRNA (Benjamini-Hochberg adjusted, $p<0.05$) among the LVL and HVL groups relative to the CT and within the infected groups. miRNAs represent those miRNAs that were found to be significantly differentially regulated by both RealTime StatMiner and DataAssist.

To further assess the clustering of subjects within these groups and across the groups, hierarchical clustering was performed between control versus low viral load (Fig. 4) and control versus high viral load (Fig. 5). Results indicate that when control versus LVL group is compared, subjects from each group formed several clusters and these clusters dispersed intermittently. The

control groups formed 3 major clusters; whereas, LVL showed 2 majors clusters with a few outliers in each group. The clustering appears to be independent of any patterns in the clinical characteristics (Table 1 and 2). However, when we compared uninfected versus HVL group, these subjects clustered distinctly from each other based on their miRNA profile. Few exceptions were present in the HVL cluster. One group of control samples, including CT20, CT21, CT7, CT9, CT25, CT13, and CT23, clustered with the HVL samples. A second major group, which also clustered with the HVL samples, was composed of CT19, CT1, and CT2. A closer look at the clinical characteristics (CD4 and Nadir CD4 counts and age) of these subjects does not reveal a pattern that makes them different from the rest of the controls (Table 1). Majority of the subjects within each group, however, exhibit a distinct profile and a single cluster with mixed population (Fig. 5). Similar distinction was observed when LVL group was compared to HVL group (Fig. 6). Importantly, these results suggest that miRNA expression profile within the infected subjects with high viral load is specific. This pattern is different in HIV-1 subjects with undetectable viral load and high CD4 counts which maintains the expression profile similar to uninfected controls. More importantly, this distinct profile is observed in most of the subjects (based on the clustering) and further confirms the specificity of miRNA expression profile.

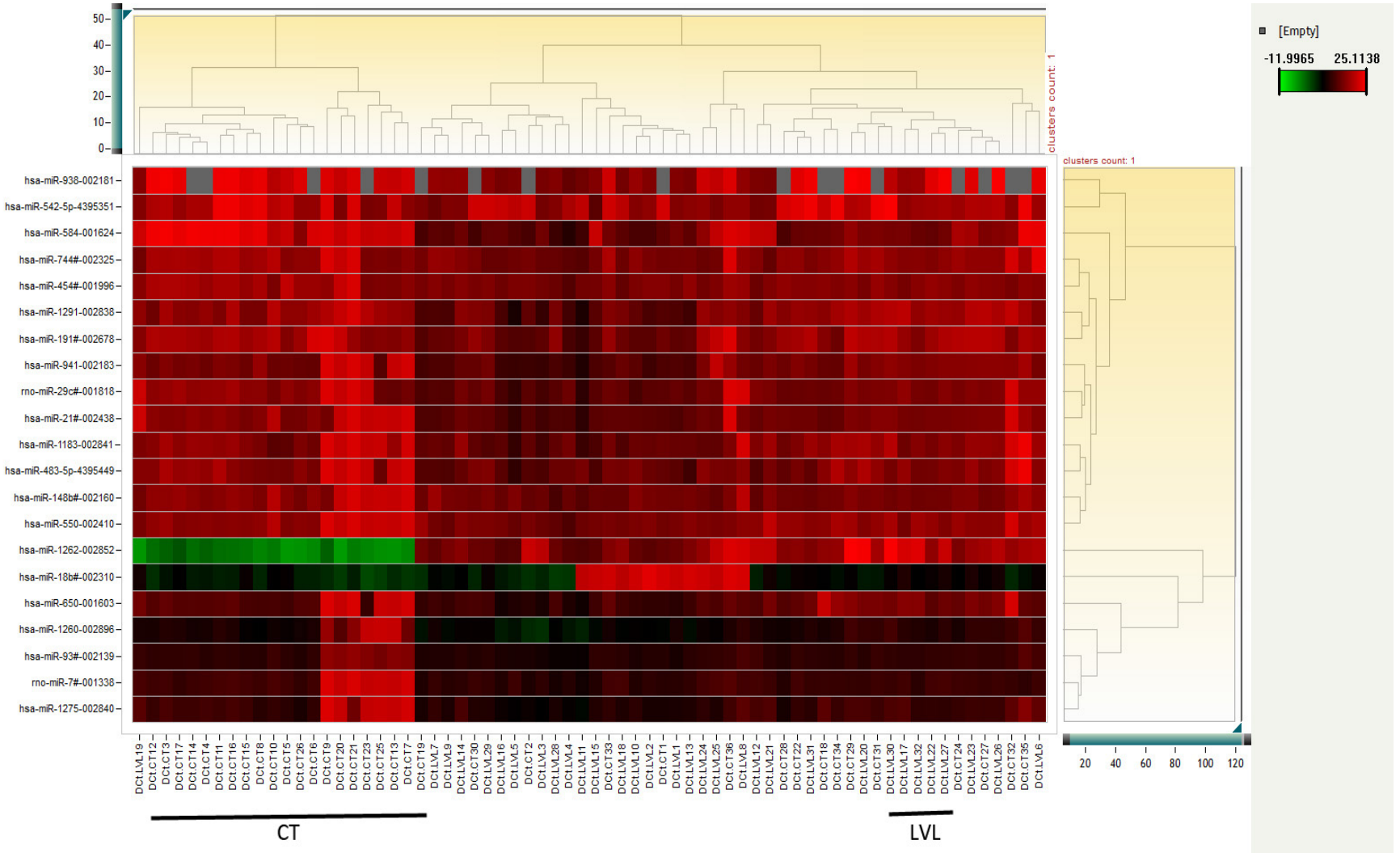

Figure 4. miRNA hierarchical clustering between low viral load samples and uninfected controls

Hierarchical clustering of miRNA between CT and LVL. miRNA in the clustergram are dysregulated at a significance cutoff of $p<0.05$. The dendrogram depicting the clustering of samples is calculated using Complete linkage with Euclidian distance measure values. Color ranging from green to red indicates minimum to maximum dCT. Gray indicates empty wells. Numbers on X axis represent subject group. CT, uninfected controls; LVL, low viral load subjects.

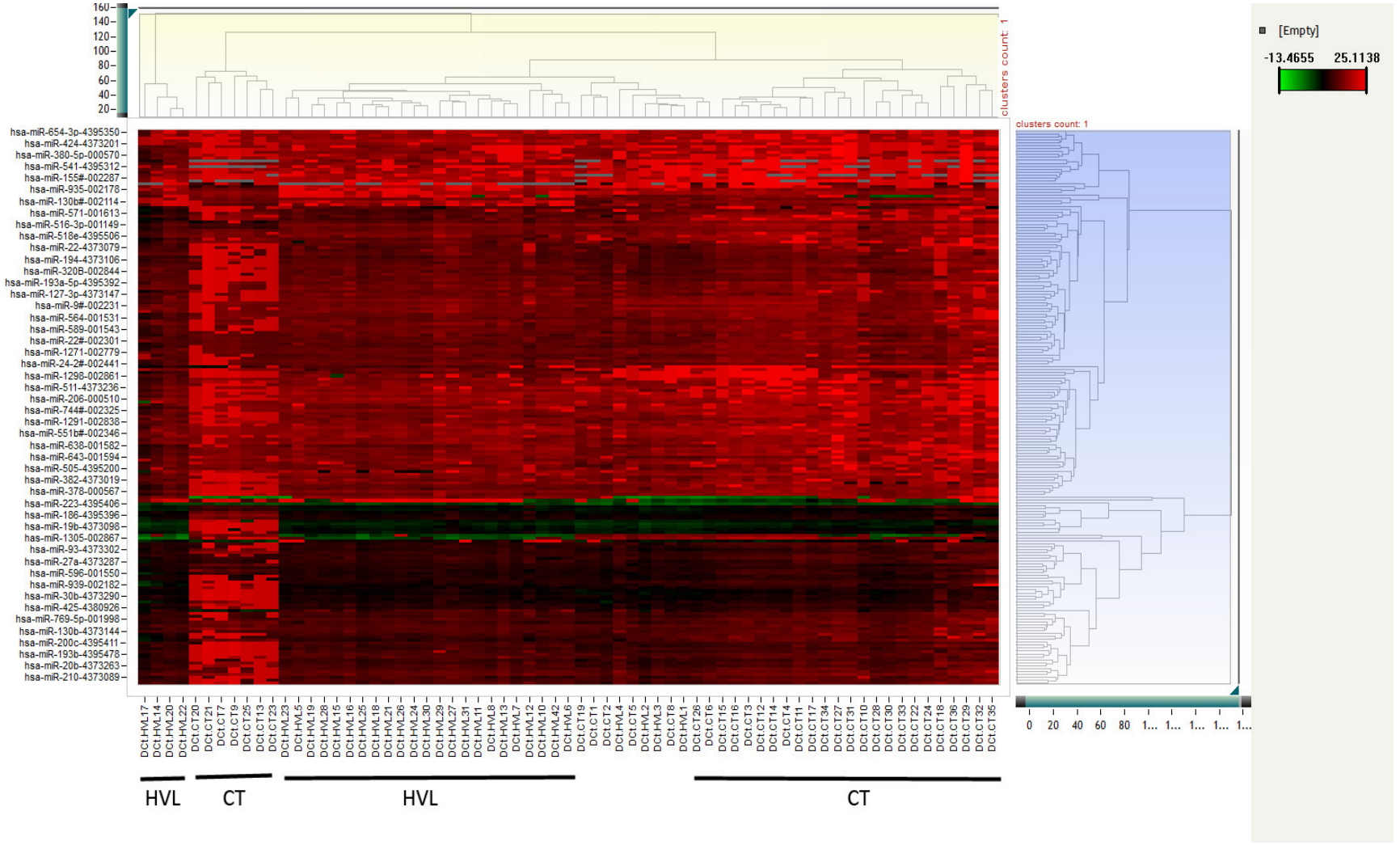

Figure 5. miRNA hierarchical clustering between high viral load samples and uninfected controls

Hierarchical clustering of miRNA between CT and HVL. miRNA in the clustergram are dysregulated at a significance cutoff of $p<0.05$. The dendogram depicting the clustering of samples is calculated using Complete linkage with Euclidian distance measure values. Color ranging from green to red indicates minimum to maximum dCT. Gray indicates empty wells. Numbers on X axis represent subject group. CT, uninfected controls; HVL, high viral load subjects.

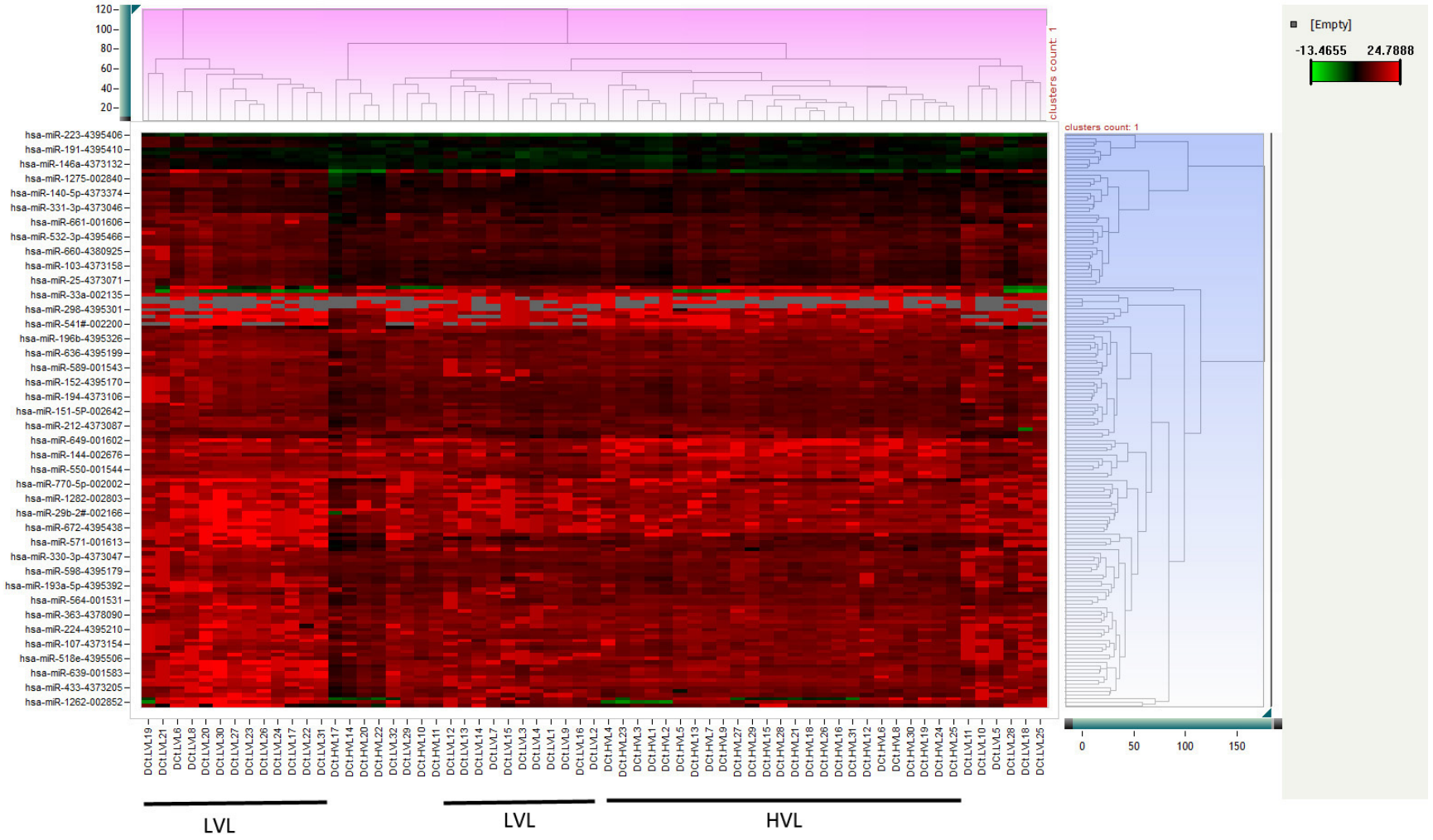

Figure 6. miRNA hierarchical clustering between high viral load and low viral load samples

Hierarchical clustering of miRNA between LVL and HVL. miRNA in the clustergram are dysregulated at a significance cutoff of $p<0.05$. The dendrogram depicting the clustering of samples is calculated using Complete linkage with Euclidian distance measure values. Color ranging from green to red indicates minimum to maximum dCT. Gray indicates empty wells. Numbers on X axis represent subject group. LVL, low viral load subjects; HVL, high viral load subjects.

5.1.2 To perform mRNA profiling in PBMCs of subjects infected with low viral load, high viral load, and in uninfected controls

MicroRNAs produce translational inhibition by binding to the 3'-UTR of their target mRNAs [59]. Therefore, we next performed mRNA profiling in PBMCs of the three groups of subjects to assess whether a direct correlation exists between the expression patterns of miRNA and mRNA. Microarray profiling of mRNA samples was normalized with the internal endogenous control and cross-compared between the two groups. Among the 47,000 transcripts tested, 21,852 were detected in all samples. Among the total detected probes, 47, 11,510 and 10,007 probes were significantly regulated (with a fold change ranging from +/- 0.2 to 6) with the p-value of <0.01 in control versus LVL, control versus HLV and LVL versus HVL, respectively. When we narrowed the probes with a fold change of +/- 2-fold ($p<0.01$), it resulted in 0, 309 and 182 probes in the 3 groups, respectively (Table 12).

Table 12. Differentially regulated mRNA probes in low and high viral load groups compared to uninfected control group and within the infected groups with statistical significance

	Total Detected probes	$p \leq 0.05$ (All probes)	$p \leq 0.05$ (Known probes)	+/- 2 fold and $p \leq 0.05$ (All probes)	+/- 2 fold and $p \leq 0.05$ (Known probes)
Control Vs. LVL	21,852	47	41	0	0
Control Vs. HVL		15,736	13,866	309	280
LVL Vs. HVL		14,432	11,014	182	166

	Total Detected probes	$p \leq 0.01$ (All probes)	$p \leq 0.01$ (Known probes)	+/- 2 fold and $p \leq 0.01$ (All probes)	+/- 2 fold and $p \leq 0.01$ (Known probes)
Control Vs. LVL	21,852	47	41	0	0
Control Vs. HVL		11,510	9,137	309	280
LVL Vs. HVL		10,007	7,820	182	166

Among the detected 47 probes (41 annotated) in the low viral load samples compared to uninfected controls (FDR corrected with p-value of <0.01), 36 were upregulated and 11 were downregulated (Appendix B1). None of the transcripts show more than 1 fold difference compared control suggesting that there is not a greater difference between uninfected versus subjects with no detectable viral load.

In contrast, 309 probes (280 annotated) were differentially regulated in the high viral load samples compared to the uninfected controls (FDR corrected with a p-value of <0.01 with +/- 2-fold regulation). Of these, 125 were upregulated, and 184 were downregulated (Appendix B2).

Similarly, we also compared the differentially regulated probes between LVL and HVL groups and the results indicate that 182 probes (166 are annotated) are regulated. Within the differentially regulated transcripts, a majority of them (113) are downregulated and 69 were upregulated (Appendix B3).

Further distribution of these probes within the groups indicate that 178 probes were commonly shared between control versus HVL and LVL versus HVL, whereas none of the mRNAs are shared by all the groups or between control versus LVL group (Fig. 7). More importantly, the remaining 131 probes are very specific to HVL group. Together these results suggest that transcriptome profile in subjects with high viral load or AIDS exhibits a unique transcriptome profile compared to the low viral load group.

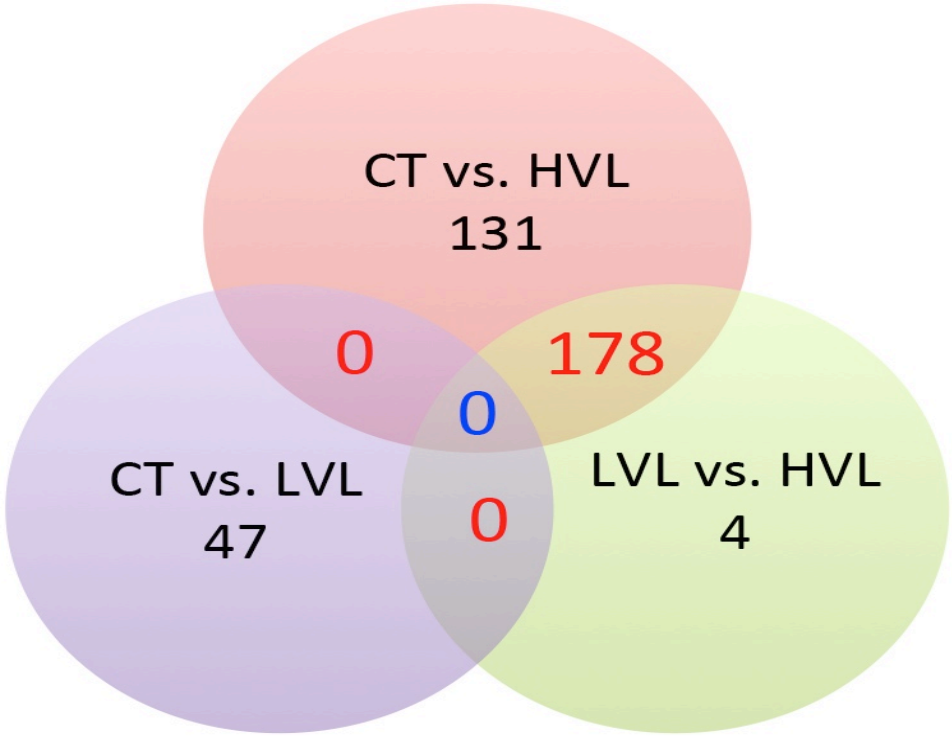

Figure 7. Venn diagram illustrating the number of significantly differentially regulated mRNA probes between low viral load, high viral load, and uninfected control groups.

The Venn diagram displays the number and overlap of significantly differentially expressed mRNA (Benjamini-Hochberg adjusted, $p<0.05$, +/- 2-fold for CT vs. HVL and LVL vs. HVL) among the LVL and HVL groups relative to the CT and among the infected groups.

Clustergram analysis of differentially regulated mRNA profile between control and LVL did not show distinct clustering (Fig. 8), whereas control versus HVL and LVL versus HVL group segregated distinctly indicating a different transcriptome profile mediated by virus replication within this group (Fig. 9 and 10). Samples LVL2, LVL4, LVL6, LVL15, and LVL11 did cluster with the HVL group; however, these clusters did not exhibit a clinical characteristic pattern that would explain why these samples resembled the HVL donors.

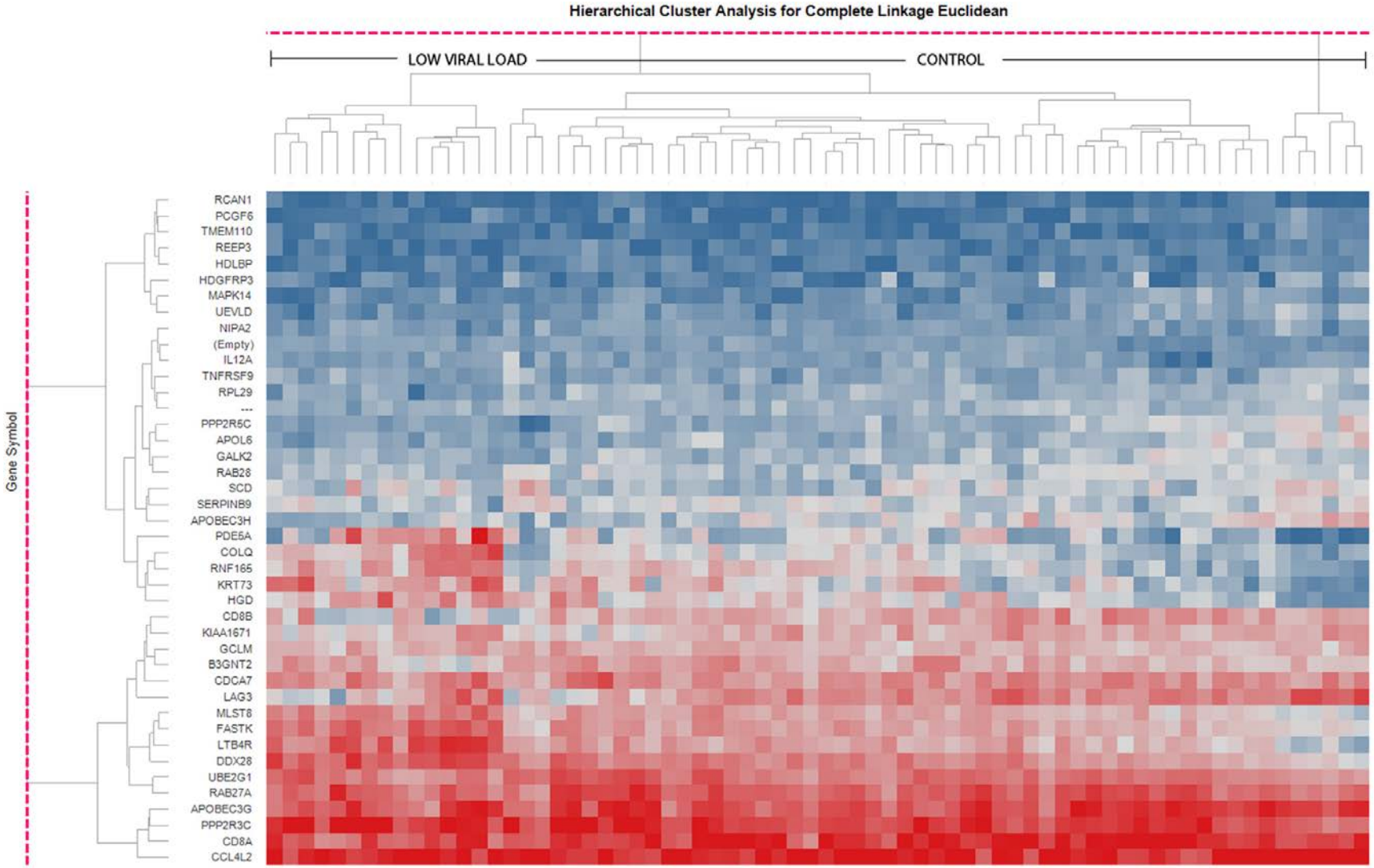

Figure 8. mRNA hierarchical clustering between control and low viral load groups

Probes in this clustergram are differentially regulated ($p<0.05$). Red indicates high, blue indicates low, and gray stands for average level of expression. Sample category is indicated on top.

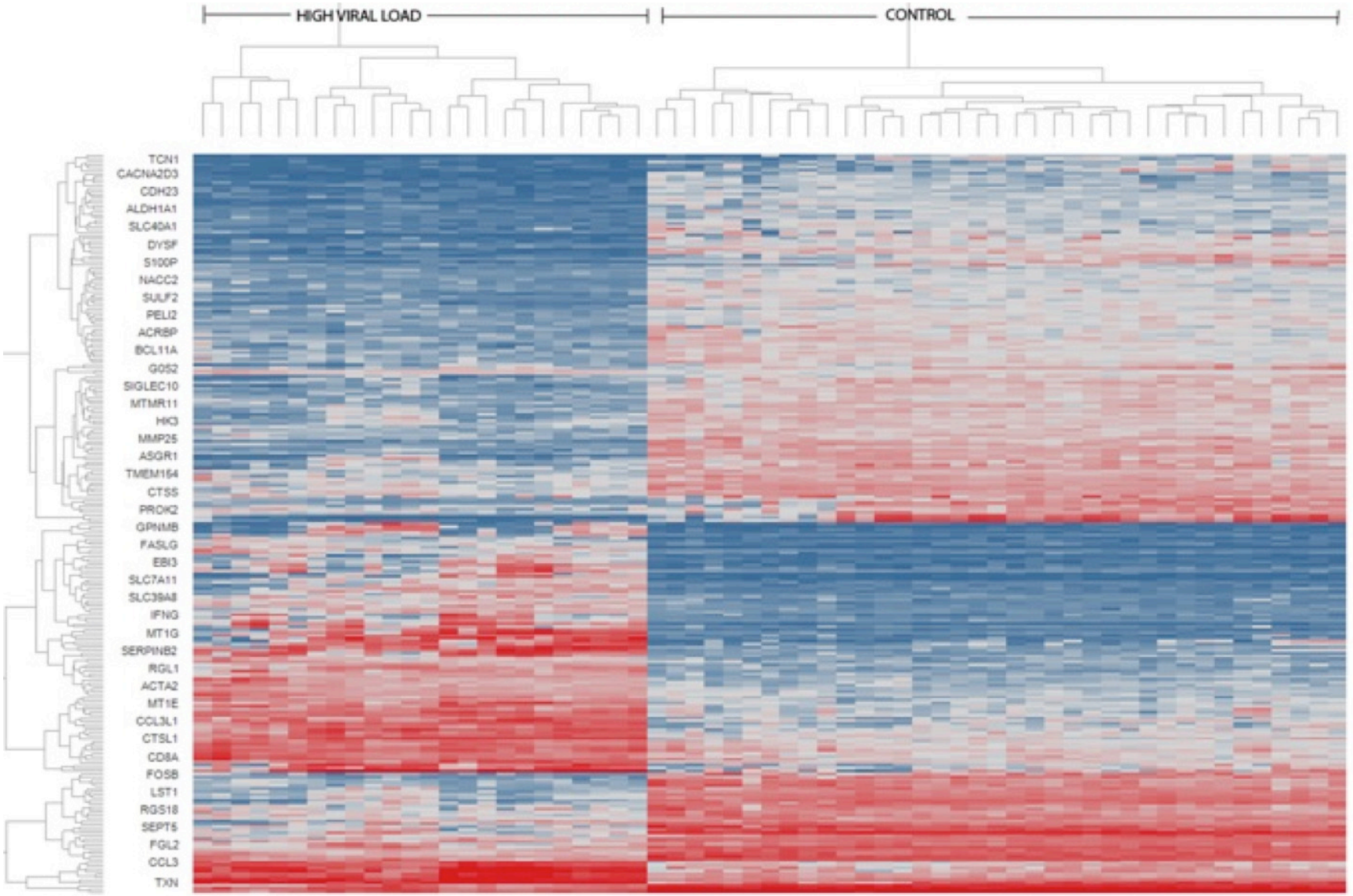

Figure 9. mRNA hierarchical clustering between control and high viral load groups

Probes in this clustergram are differentially regulated with a fold change>2 ($p<0.05$). Red indicates high, blue indicates low, and gray stands for average level of expression. Sample category is indicated on top.

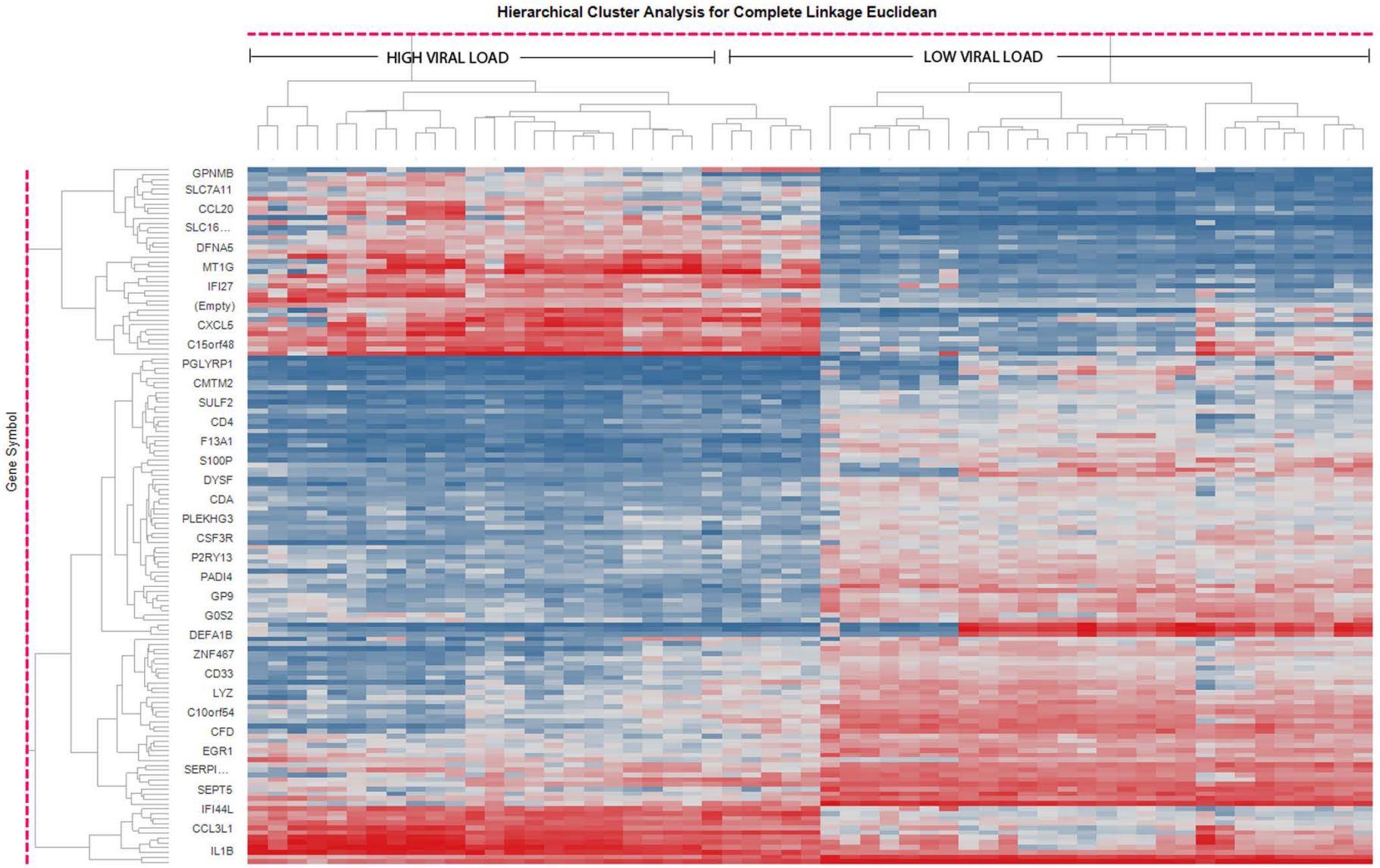

Figure 10. mRNA hierarchical clustering between low and high viral load groups

Probes in this clustergram are differentially regulated with a fold change>2 ($p<0.05$). Red indicates high, blue indicates low, and gray stands for average level of expression. Sample category is indicated on top.

5.1.3 To analyze pathways and biological processes altered by HIV-1 infection

In order to examine the pathways and biological processes involved in HIV-1 infection, we analyzed the significantly differentially regulated mRNAs from each analysis with STRING and Gene Enrichment Ontology. All significantly upregulated and downregulated mRNAs were grouped together and analyzed. Results of STRING analysis indicate that LVL vs. uninfected controls did not show significant changes in cellular pathways (Fig 11).

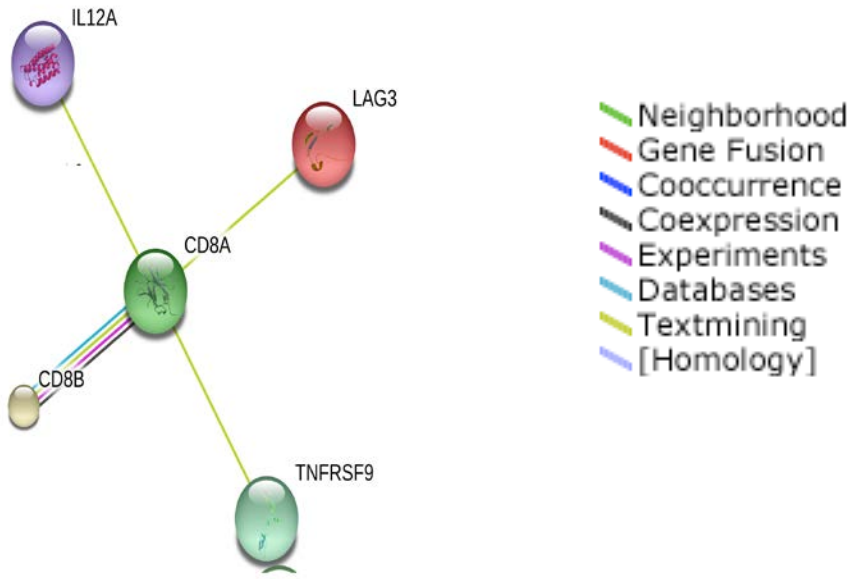

Figure 11. STRING analysis of Predicted interaction networks of genes significantly dysregulated in LVL relative to CT.

The interactions between genes were identified using STRING software. Node color has no meaning and is simply used as a visualization tool. Colored lines indicate different sources of evidence for each interaction.

This contrasts the HVL, which in comparison to the uninfected controls, exhibited distinct pathway regulation (Fig. 12). STRING analysis shows four major clusters: chemokines and its receptors, pro-inflammatory cytokines, interferon induced genes, and metallothionein genes. Among these clusters, the most significantly regulated molecules include inflammatory factors, cytokines/chemokine, cell cycle and apoptosis related proteins, cell signaling molecules, factors expressed in response to virus/bacterial infection, innate factors and cell-to-cell interaction. Most importantly, many of the inflammatory factors and cytokines (CXCL5, CCL2, CCL8, CXCL10, CCL7, IL-1α, IL-1β, IL-6 and IL-8) are upregulated in HVL group, whereas antiviral factors and innate immune molecules (members of Defensin family, IFN) were significantly downregulated.

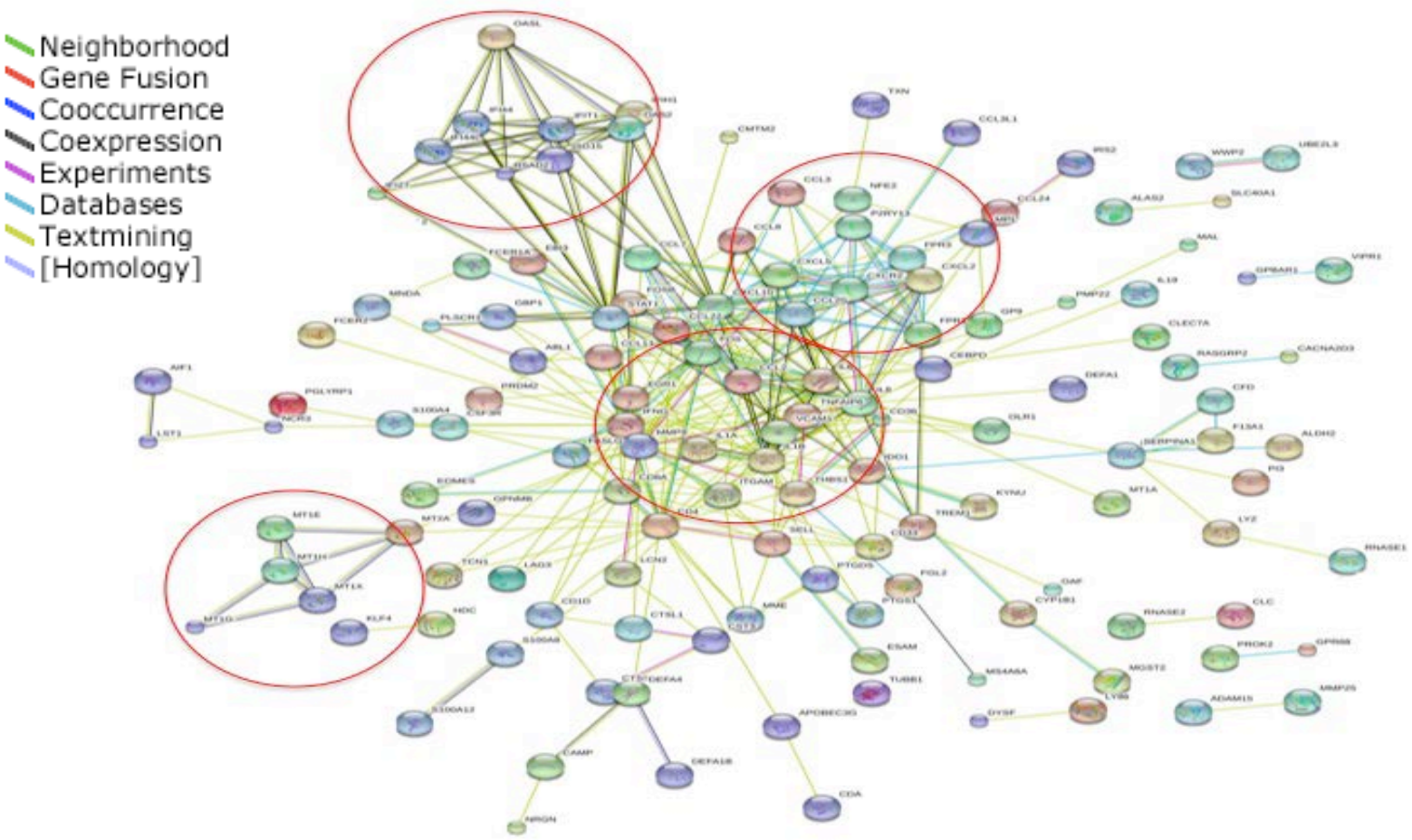

Figure 12. STRING analysis of predicted interaction networks of genes significantly dysregulated in HVL relative to CT.

The interactions between genes were identified using STRING software. Node color has no meaning and is simply used as a visualization tool. Colored lines indicate different sources of evidence for each interaction.

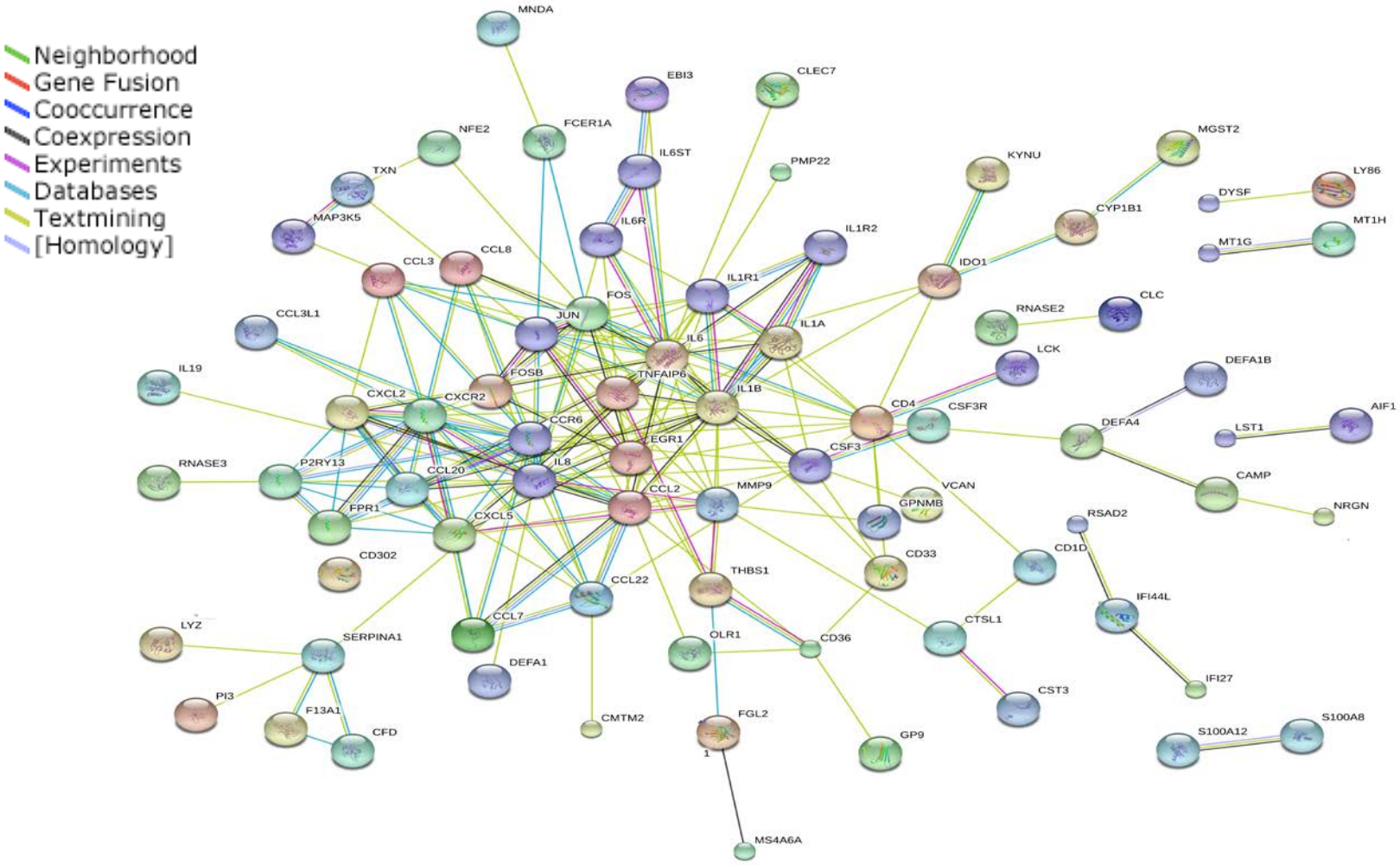

Figure 13. STRING analysis of predicted interaction networks significantly dysregulated in HVL relative to LVL

The interactions between genes were identified using STRING software. Node color has no meaning and is simply used as a visualization tool. Colored lines indicate different sources of evidence for each interaction.

Finally, the comparison between the HVL and LVL shows a similar pathway with an evident presence of pro-inflammatory cytokines and chemokines (Fig. 13).

We next chose to study the functionality of the significantly dysregulated genes with a focus on those genes that were statistically over represented. An analysis of biological functions, using Gene Ontology Enrichment, shows only four significant functions including response to virus, IFNγ mediated signaling pathway, immune response, and cellular component movement for the comparison between LVL and controls (Fig. 14A). This contrasts with 34 significant functions, including inflammatory response, chemotaxis, and immune response, produced from the comparison between HVL and the control group (Fig. 14B). Interestingly, only four significant functions were obtained from the comparison between HVL and LVL (Fig. 14C). These functions relate mostly to viral replication and include translational elongation, viral transcription, and translational termination. Overall, these results suggest that high viral load alters several genes and cellular processes related to HIV-1 induced disease progression.

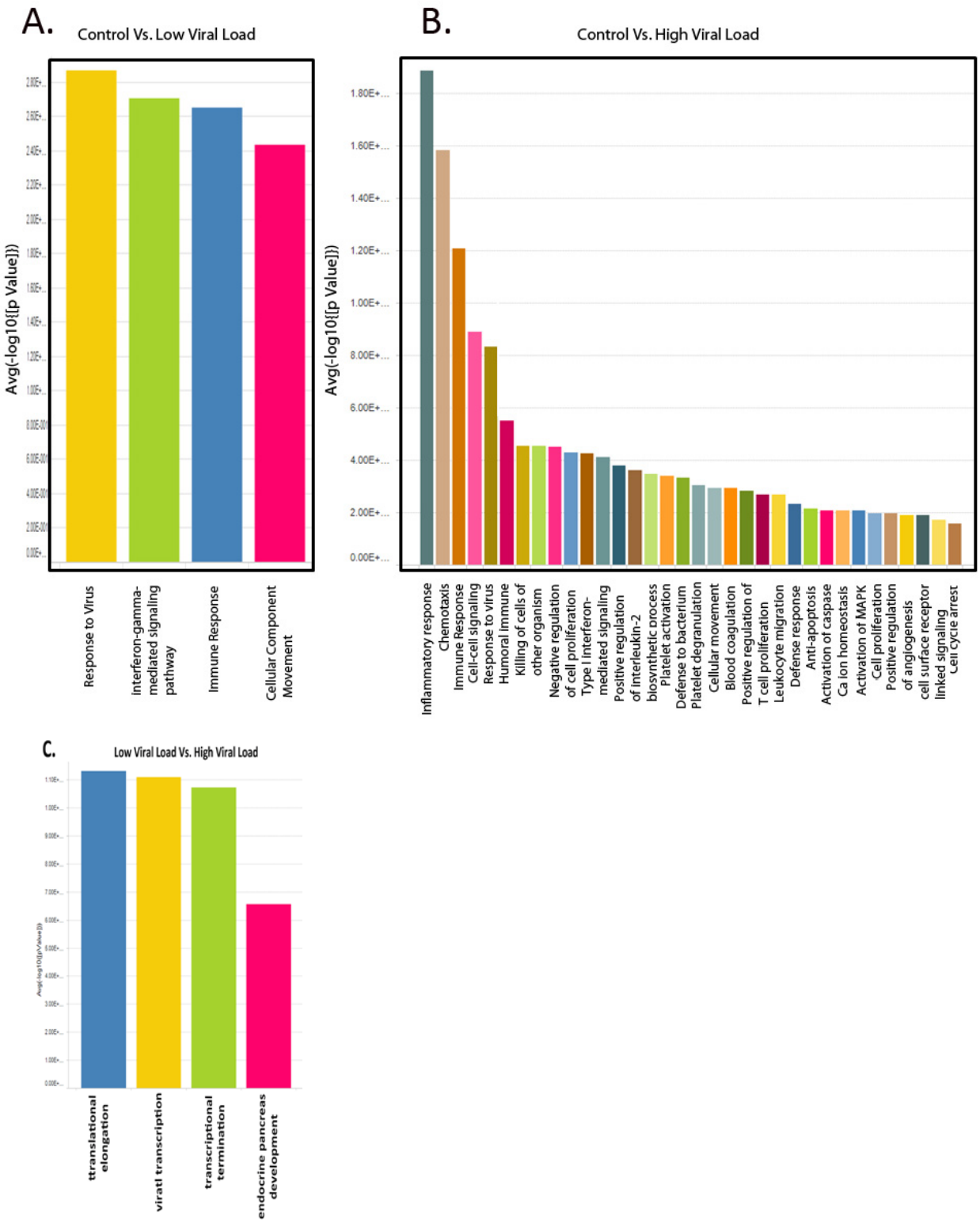

Figure 14. Gene Ontology Enrichment Analysis for biological processes using significantly differentially regulated genes

Bar graphs were generated using average –lop of p value for each term on y-axis and term name on x-axis using Gene Ontology Enrichment tool in IBD.

5.1.4 To perform miRNA and mRNA validations on independent subjects.

Because the high throughput platforms simultaneously test for the expression of many miRNAs or many mRNA, we chose to validate selected miRNAs and mRNAs, ones that we determined to be significant (>2-fold change, $p<0.05$), by qRT PCR in a set of independent donors. This was performed to increase the confidence in our results and to reduce the possibility of platform related errors.

5.1.4.1 miRNA validations

To validate the differentially regulated miRNAs from the microarray results, we randomly selected miRNAs (miRNAs with >2-fold change and $p<0.05$) and tested them in independent subjects (using similar selection criteria) using miRNA specific Taqman primers and probes by qRT-PCR (Fig. 15A). Results from the comparison of the low viral load and uninfected controls indicate that among the six miRNAs tested, four miRNAs (miR-483-5p, miR-18b*, miR-938, and miR-1260) exhibit similar fold change pattern as the high throughput results with miR-483-5p showing significance ($p<0.001$). For instance, three of these miRNAs are upregulated in high throughput and also upregulated in qRT PCR assay, while fourth miRNA is downregulated in both platforms. Validation of two miRNAs (miR-1262 and miR-21*) showed an opposite fold change pattern to that of the high throughput results. Specifically, miR-1262 was downregulated and miR-21* upregulated in high throughput, while in the qRT PCR, they were upregulated and downregulated, respectively.

We also tested selected miRNAs in a set of independent high viral load donors relative to uninfected controls (Fig. 15B). This comparison indicated that among the seven miRNAs tested,

all but two miRNA (miR-21* and miR-18b*) positively validated the high throughput results. All but one of these (miR-935) also produced significance ($p<0.05$).

Overall, validation of miRNA in both sample groups confirms >65-85% of the mRNAs, suggesting that the array analyses in our samples are reproducible.

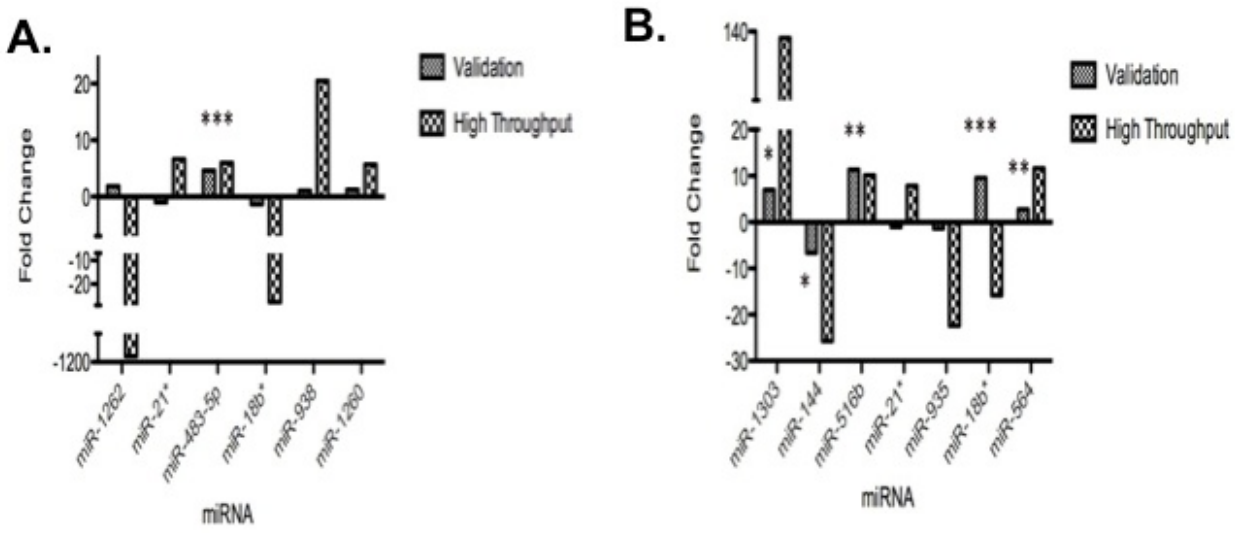

Figure 15. Independent validations of randomly selected miRNAs

qRT-PCR was used to validate the expression of selected miRNAs between CT and LVL **(A)** and CT and HVL **(B)** from the high throughput results (derived by StatMiner) using a specific primer and probe for each miRNA. Fold increase/decrease was calculated based on normalization to U6. Average fold change for each miRNA represents fold change obtained from independent donors (CT: N=5 or 4, LVL: N=5, HVL: N=5). *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

5.1.4.2 mRNA validations

Since all of the significant mRNAs in the comparison between low viral load and uninfected controls had a very low fold change (-0.768 to 1.03), none of them met our selection criteria for validation. We still chose to validate certain transcripts, which were previously shown to be associated with HIV infection (Fig. 16A). We chose to examine CCL2, or CC Chemokine Ligand 2, which is a chemoattractant produced by macrophages following inflammation and is a

factor that has been associated with disease progression [60]. We also examined the expression of CCL8, a CCR5 binding ligand and, therefore, an inhibitor of HIV-1 viral entry[61]. Furthermore, we examined the expression of IFNγ, a cytokine produced by T lymphocytes, which has been shown to be upregulated following HIV infection[62]. We also examined the expression of IL1β, an inflammatory cytokine previously shown to be stimulated in monocytes and macrophages by extracellular HIV Tat[63]. Finally, we chose to examine the expression of Neurogranin (NRGN). Neurogranin is a cellular factor that plays a role in the calcium-calmodulin signaling pathway and has been suggested to play a role in learning and memory[64]. We chose to explore its expression because its neurocognitive effects could be linked to HAND (HIV associated neurocognitive disorders). Additionally, NRGN acts as a substrate for protein kinase C, which has been shown to be involved in HIV-1 gp120-induced apoptosis[65]. Our qPCR results indicate that CCL2, CCL8, and IL1β were downregulated, while IFNG and NRGN were upregulated in donors with low viral load compared to uninfected controls (Fig 16A).

Since our microarray data for the comparison between HVL and the uninfected controls yielded significantly differentially regulated mRNAs at a > +/-2 fold change, we were able to validate several of these mRNAs and compare our validation results to those of the microarray (Fig. 16B). In addition to CCL2 and NRGN, which we also tested in the low viral load subjects, we examined the expression of AIF1 and IL-6. AIF1 or Allograft inflammatory factor 1 is a protein that is induced by cytokines and interferon and is involved in the anti-inflammatory response in blood vessels [66]. We also examined the expression of IL-6, whose expression has for a very long time been known to be elevated in HIV infected individuals [67]. Three out of the four mRNAs selected exhibited the same fold change pattern as in the microarray data. NRGN was the only mRNA that exhibited the opposite fold change pattern in that in the

validation results, it produced a fold change of 2.3, compared to -4.029. However, it is important to note that the fold change is not very high and may not be significant.

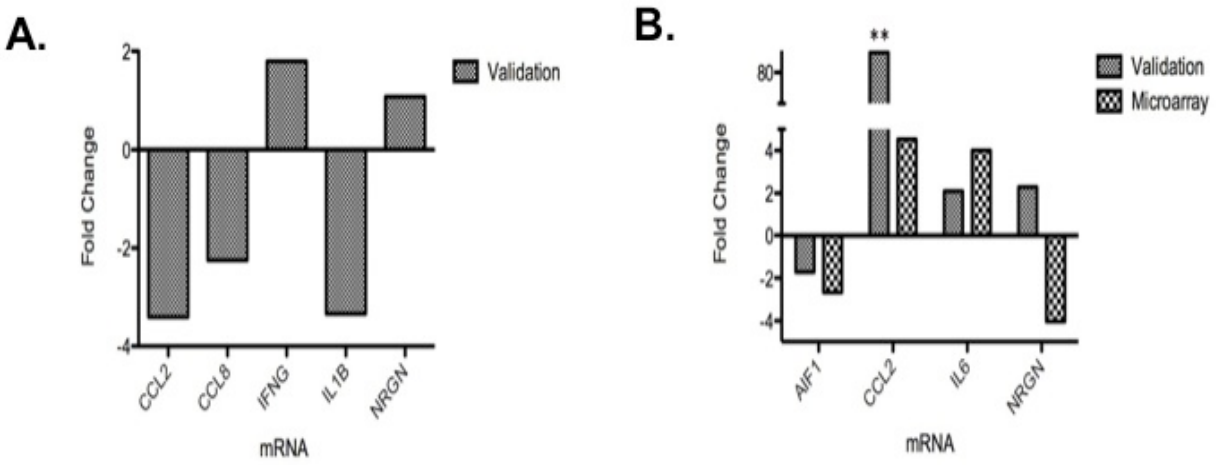

Figure 16. Independent validations of randomly selected mRNAs

qRT-PCR was used to validate the expression of selected mRNAs between CT vs. LVL **(A)** and CT vs. HVL **(B)** from the high throughput results (derived by StatMiner) using a specific primer and probe for each mRNA. Fold increase/decrease was calculated based on normalization to RPLPO. Average fold change for each miRNA represents fold change obtained from independent donors (CT vs. LVL: N=5 vs. N=5; CT vs. HVL: N=3 vs. N=3). **=$p<0.01$.

5.1.5 Summary of AIM #1

Viral load significantly alters the miRNA and mRNA profiles in infected individuals. It is high viral load, in particular, that has the greatest effect on producing a differential profile. Low viral load individuals exhibit profiles that in general resemble those of uninfected individuals. This contrasts to high viral load donors, who produced higher numbers of significantly differentially regulated miRNAs and mRNAs in comparison to both the uninfected controls and the low viral load samples. We hypothesize that the altered miRNA profile differentially regulates the mRNA profile. The study of the interplay of the two profiles is the focus of Aim #2.

5.2 AIM #2: TO EXAMINE THE INTERPLAY OF MIRNA AND THE TARGETED MRNA

Since miRNAs' function is to inhibit translation by targeting mRNAs, our goal was to identify which differentially regulated microRNAs targeted which mRNAs. Several online databases are available for predicting the miRNA-mRNA interplay. One of these databases is Targetscan, which predicts miRNA targets by searching for conserved 8mer and 7mer sites in the mRNA that would match the seeding region in the miRNA, mismatches that are compensated by a conserved 3'pair; non conserved sites are also predicted[68]. An additional database is miRDB, which is based on support vector machines to improve generalization on unseen data[69]. Furthermore, there is MicroCosm Targets, which uses a miRanda scoring algorithm and scans a single organism's genome and all of its miRNA sequences against 3' UTR sequences[70]. However, there is not always a uniform consensus between the different databases and the predictions they make. Zhu et al have, through their study of mouse mammary tumors, also demonstrated that the online databases can generate a large number of false positives[71]. As a result, we chose to perform a computational analysis using our two sets of array data. This follows the example of Wang et al who used a similar strategy to identify an intrinsic miRNA-mRNA correlation during monocytic differentiation[72].

5.2.1 To perform a computational analysis to predict possible mRNA targets of the significantly dysregulated miRNAs.

To predict the targets of our significantly differentially regulated miRNAs, we performed a computational analysis that takes into account our expression data and a database of predicted

miRNA-mRNA interactions. Table 13 shows the predicted miRNA-mRNA interactions following an analysis examining the union of all significant miRNAs and mRNAs and an analysis examining the individual group significant miRNAs and mRNAs. Although multiple miRNAs were predicted to have potential binding sites, many miRNAs show a clear inverse correlation between the miRNA-mRNA interaction pairs. For instance, the downregulated NRGN was shown to be targeted by miR-564, which was upregulated in our miRNA analyses.

Table 13. Predicted miRNA-mRNA interacting pairs with high confidence

		Control vs. LVL		Control vs. HVL		LVL vs. HVL	
miRNA	**mRNA**	**Fold change**	**p value**	**Fold change**	**p value**	**Fold change**	**p value**
miR-376a	**IL-6**	-1.264	0.697	-2.639	0.0185	-2.088	0.0797
miR-210	**NRGN**	2.931	0.162	10.536	0.000121	3.595	0.00276
miR-564	**NRGN**	2.467	0.229	11.667	0.00000170	4.730	0.00306
miR-483-5p	**AIF1**	5.969	0.00410	17.089	0.000000205	2.863	0.00259
miR-605	**AIF1**	1.164	0.872	29.059	0.0000000127	24.962	0.0000000554
miR-222	**ALDH1A1**	1.710	0.229	4.887	0.00000170	2.858	0.000129
miR-152	**KLF4**	1.243	0.799	5.263	0.00190	4.233	0.000544
miR-34b	**NCR3**	1.080	0.893	3.234	0.000494	3.000	0.0182
miR-660	**NCR3**	2.276	0.393	6.452	0.00778	2.835	0.0233
miR-518f	**CCL2**	-5.801	0.315	-3.178	0.442	1.826	0.699
miR-518e	**CCL2**	-1.480	0.614	4.285	0.00896	6.342	0.00145
miR-518f	**CCL8**	-5.801	0.315	-3.178	0.442	1.826	0.699

miRNA-mRNA predicted pairs were selected from the GenmiR++ analysis. Two different analyses were performed: first one utilized the union of significant miRNA and mRNA, the second examined the significant miRNA and mRNA by individual group comparisons. Fold change and p-value refer to those of the miRNA in the appropriate analysis. Red indicates significant p-values ($p<0.05$).

We were able to generate a visual representation of the predicted miRNA-mRNA interactions between HVL and the uninfected controls (Fig. 17). However, due to the large number of predictions, the entire interactome is difficult to read. Here, we chose to highlight 4 miRNAs and their mRNA predictions (Fig. 18A-D). MiR-92a, miR-564, and miR-130b are examples of miRNAs that are associated with the presence of high viral load, while miR-483-5p is an example of a miRNA that is associated not only with high viral load but also with infection in general. The interactions were ranked based on confidence. From the 24 interactions shown for miR-92, 21 interactions for miR-564, 14 interactions for miR-130b, and 14 interactions for miR-483-5p, 6, 4, 1, and 3 of the interactions had a confidence greater than 0.5 for each of the miRNAs, respectively.

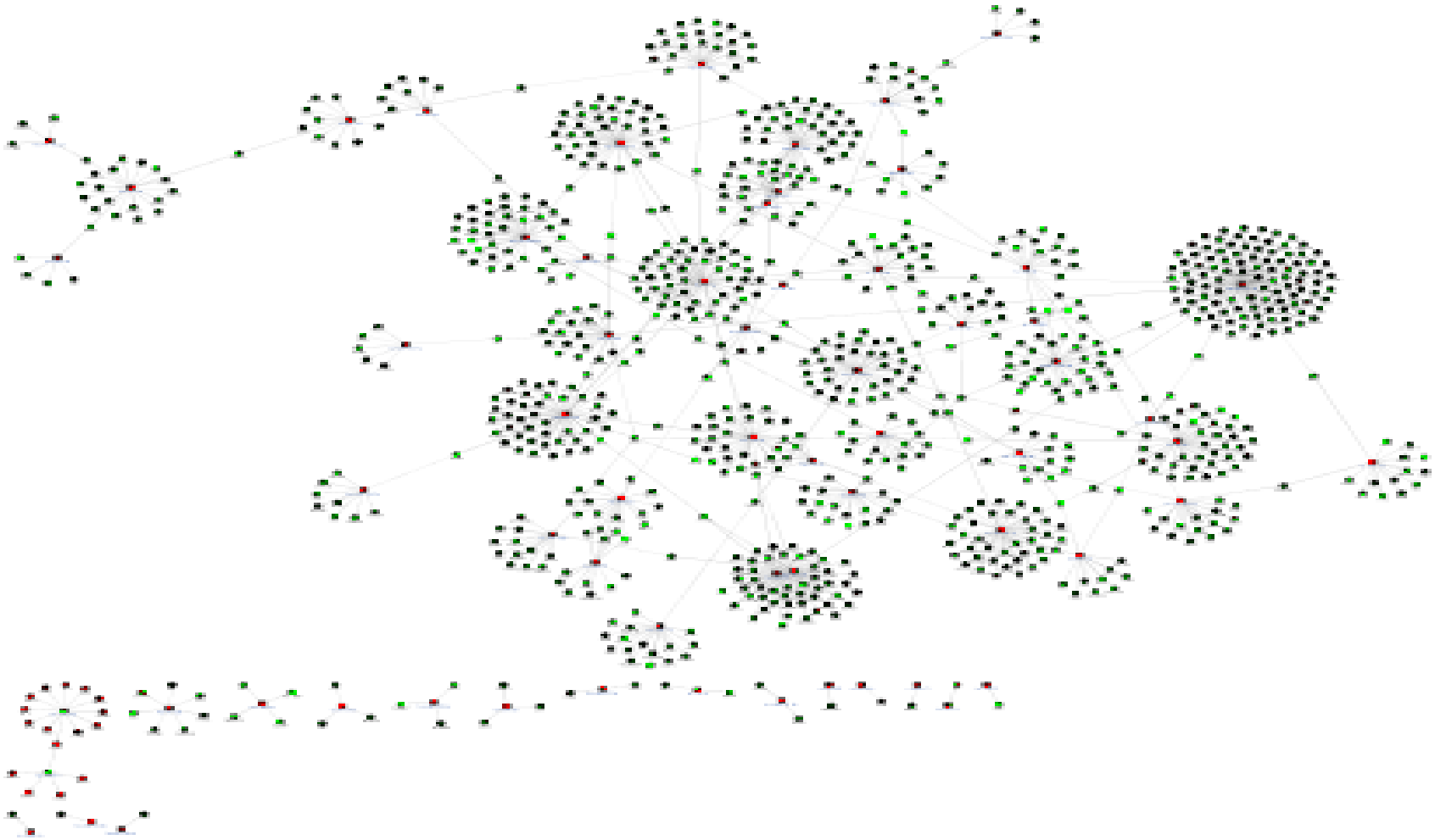

Figure 17. Predicted miRNA-mRNA interactions

Predicted miRNA-mRNA interactions between HVL and CT groups were formulated using array data and GenMIR++ and visualized by Cytoscape. Red indicates upregulation, green stands for downregulation.

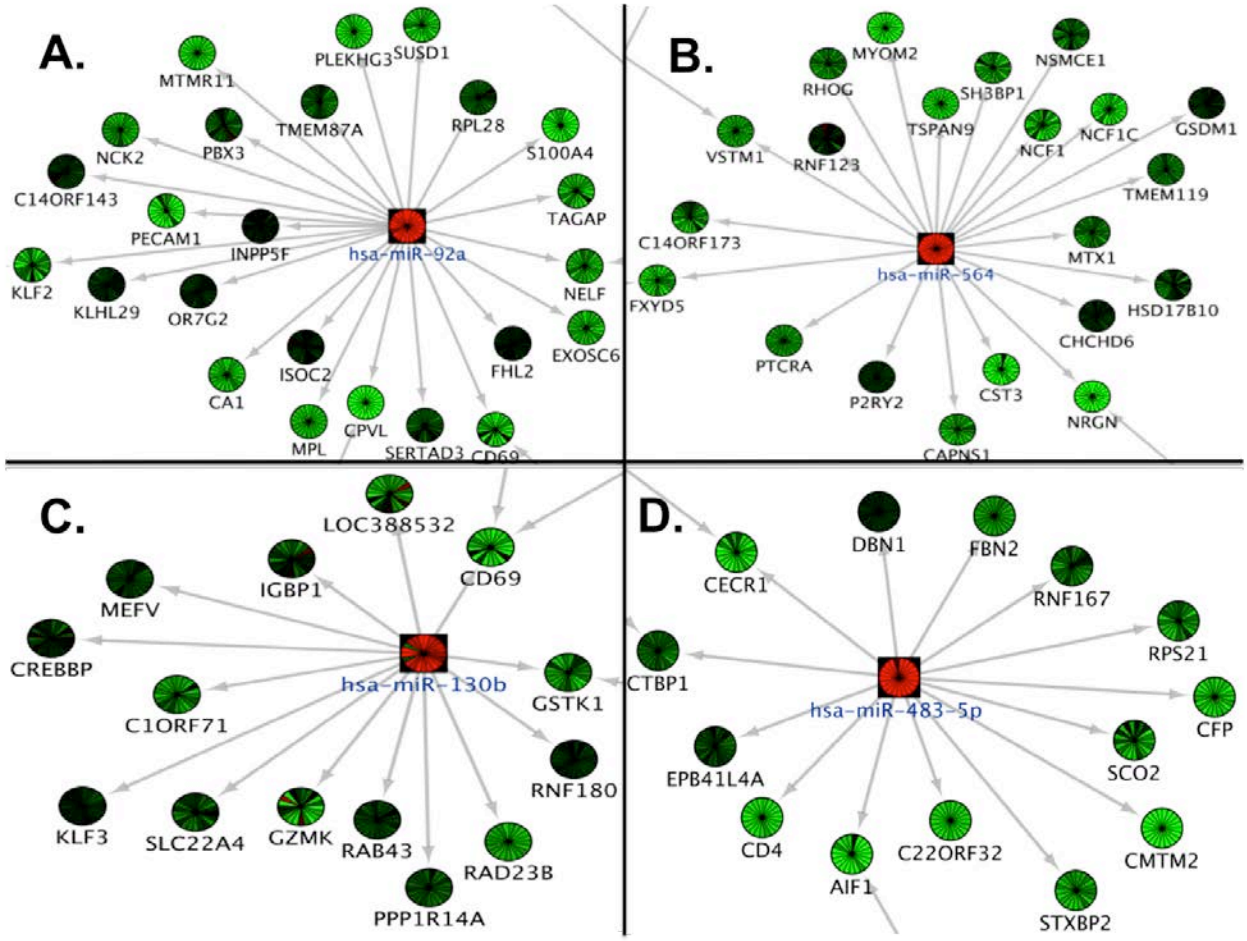

Figure 18. Selected predicted miRNA-mRNA interactions

Selected miRNA-mRNA predicted interactions were chosen from a list of 129 miRNAs and their targets. The analysis was performed using the expressions of significantly differentially regulated miRNAs and mRNAs between HVL and control and the data from MicroCosm Targets dabatase. Red indicates upregulation, green stands for downregulation. Each slice of a circle represents one HVL sample.

5.2.2 To examine the relationship between CD4 count, Nadir CD4 count, and viral load and selected transcripts

To examine whether other factors, besides the presence of high viral load, play a role in producing a differential mRNA profile, we selected a number of cellular molecules from our predictions and assessed their expression between the three groups of donors and also assessed their possible correlation with CD4 count, Nadir CD4 count, and viral load level. In addition to selected cellular factors, which we previously described, we chose to focus on IL-8, whose expression is elevated in the sera of HIV infected individuals [73]. We also focused on CXCL5, which has been inferred to have an influence on the CXCR1 gene, the receptor for IL-8 [74].

Results show that the signals of all of the transcripts are significantly different for the high viral load compared to both the uninfected control and the low viral load group ($p<0.0001$). Furthermore, NRGN and IL-6 signals in the low viral load also significantly differ from the uninfected control group (Fig. 19).

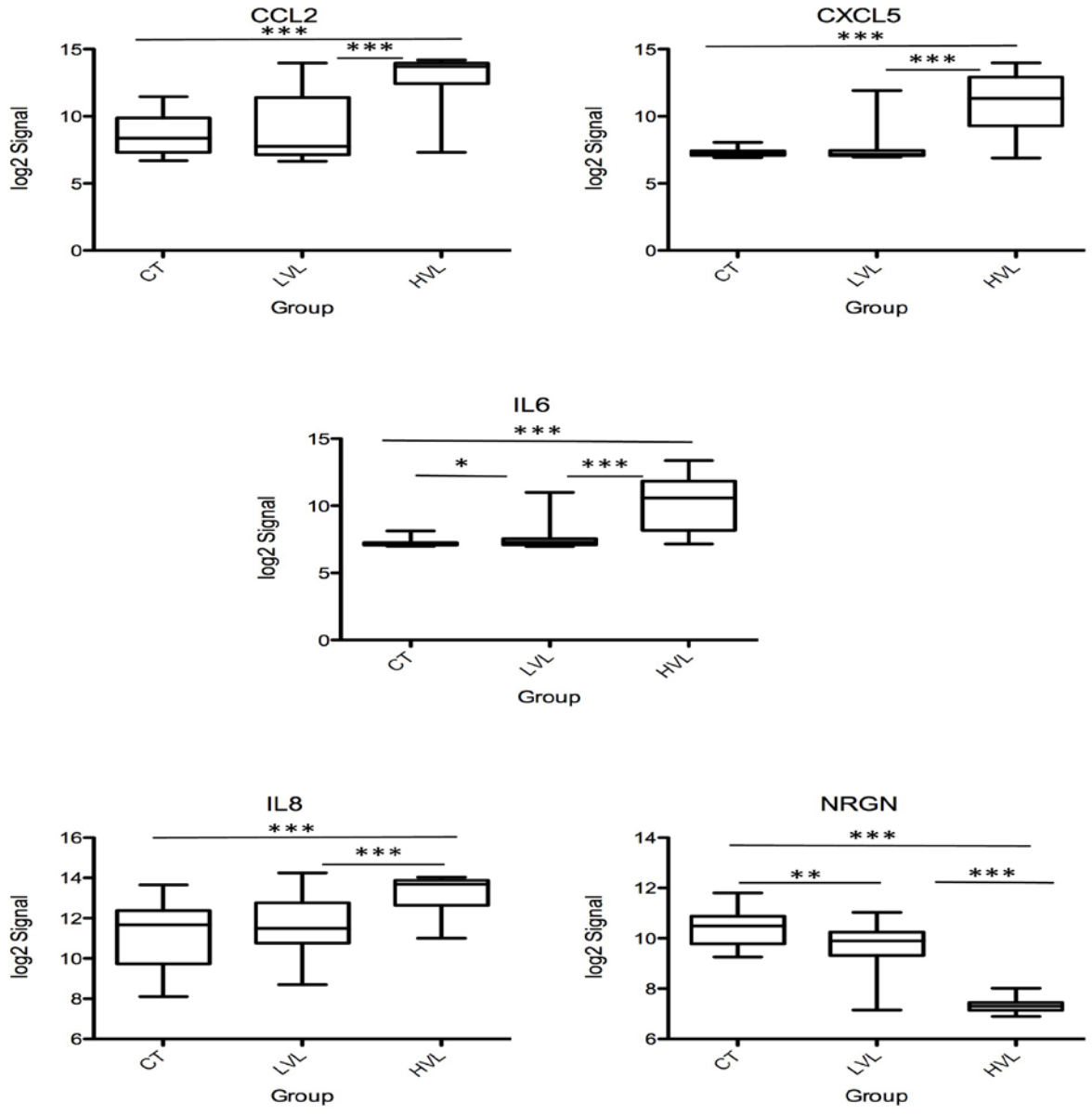

Figure 19. Expression of selected transcripts among the CT, LVL, and HVL groups
Unpaired Student's t-test was used to assess significance between the three groups (CT vs. LVL, CT vs. HVL, and LVL vs. HVL) (*=$p<0.05$, **=$p<0.01$, ***=$p<0.0001$)

To see if CD4 count, the major determinant of HIV infection, plays a role in altering the transcriptome profile, we performed Spearman correlations between the CD4 count and the signal intensity of each selected transcriptome within the three groups of subjects. Similarly, we also assessed the role of Nadir CD4 count, which is the lowest CD4 count of an individual up to the date of collection (Table 14). Results indicate that none of the transcripts significantly correlate with either CD4 count or Nadir CD4 count within the uninfected control group. (Table 14A) Within the LVL group, IL-6 and NRGN significantly correlate with CD4 ($p<0.01$ and $p<0.05$). If we consider a strong relationship to be one with a correlation coefficient between +/-1 and +/-0.7, neither of these relationships is a strong one. None of the transcripts within LVL correlate with the Nadir CD4 count (Table 14B). In the HVL group, IL-8 was the only transcript that had a significant correlation with CD4 count ($p<0.05$), but once again the relationship was not strong. With respect to Nadir CD4 count, IL-6 and IL-8 correlated significantly ($p<0.01$) but without a strong negative relationship (Table 14C).

High viral load group consisted of subjects whose viral load ranged from 46,053 to 561,627 copies/ml, with the exception of one donor with 1,157,700 copies/mL (this outlier sample was eliminated from this analysis). To assess, whether viral load amount within the HVL group is directly or inversely correlated with host cellular factors expression, we also performed Spearman correlation with viral load. Results indicate, however, that there is no significant correlation between viral load and the signal of any of our selected cellular transcripts (Table 14C).

Table 14. Spearman correlations between selected transcript signals and CD4 count, Nadir CD4 count, and viral load.

A.

	CD4 Count		Nadir CD4	
Transcript	Spearman r	p-value	Spearman r	p-value
CCL2	-0.00296	0.986	0.156	0.358
CXCL5	0.0385	0.821	0.101	0.553
IL-6	-0.0236	0.877	0.0424	0.799
IL-8	0.0234	0.891	0.227	0.176
NRGN	-0.186	0.278	-0.172	0.309

B.

	CD4 Count		Nadir CD4	
Transcript	Spearman r	p-value	Spearman r	p-value
CCL2	-0.281	0.133	-0.193	0.283
CXCL5	0.0251	0.895	-0.0267	0.883
IL-6	-0.488	0.0062	-0.185	0.304
IL-8	-0.219	0.246	-0.143	0.427
NRGN	0.41	0.0244	0.314	0.0748

C.

	CD4 Count		Nadir CD4		Viral Load	
Transcript	Spearman r	p-value	Spearman r	p-value	Spearman r	p-value
CCL2	-0.0222	0.918	-0.0843	0.695	0.202	0.355
CXCL5	-0.277	0.190	-0.367	0.078	0.190	0.385
IL-6	-0.324	0.122	-0.414	0.0445	-0.199	0.364
IL-8	-0.495	0.0139	-0.609	0.0016	-0.085	0.670
NRGN	0.224	0.292	0.153	0.475	0.143	0.516

Spearman correlations between selected transcripts and CD4 count, Nadir CD4, and viral load (where applicable) within the control (n=37) **(A),** low viral load (n=30 for CD4 count, n=33 for Nadir CD4 count) **(B)**, and high viral load (n=24 for CD4 and Nadir CD4 count, n=23 for viral load) **(C)** obtained from GraphPad Prism set to produce a two-tailed p-value.

5.2.3 Biological validation of HIV-1 regulated factors

Although, an *in vivo* validation of serum cytokines and chemokines as well as other markers would be ideal, we first evaluated expression of the inflammatory factors using an *in vitro* experimental culture model. Normal human primary PBMCs infected with HIV-1 virus were assessed for CCL2, CCL8, IL-6, IL-8, and CXCL5 protein expression. Chemokines released from the infected culture post infection were assessed in the infected and uninfected culture using cells from multiple donors (Fig. 20). Results indicate that HIV-1 infection significantly increased the expression of inflammatory factors, and this expression was independent of viral tropism. Infection with either NL43 or YU2 significantly upregulated the level of IL-6 by 8-fold, CCL2 by 3-fold, and CXCL5 by 4-fold with a significance of $p<0.05$ in multiple donors. However, the upregulation was about 2.5-fold for CCL8 ($p>0.05$) and 8-fold with $p<0.05$ for IL-8 in NL43 infected PBMC, whereas 3-fold ($p>0.05$) in YU2 infected PBMC. These results exhibit a direct correlation with the mRNA transcript expression.

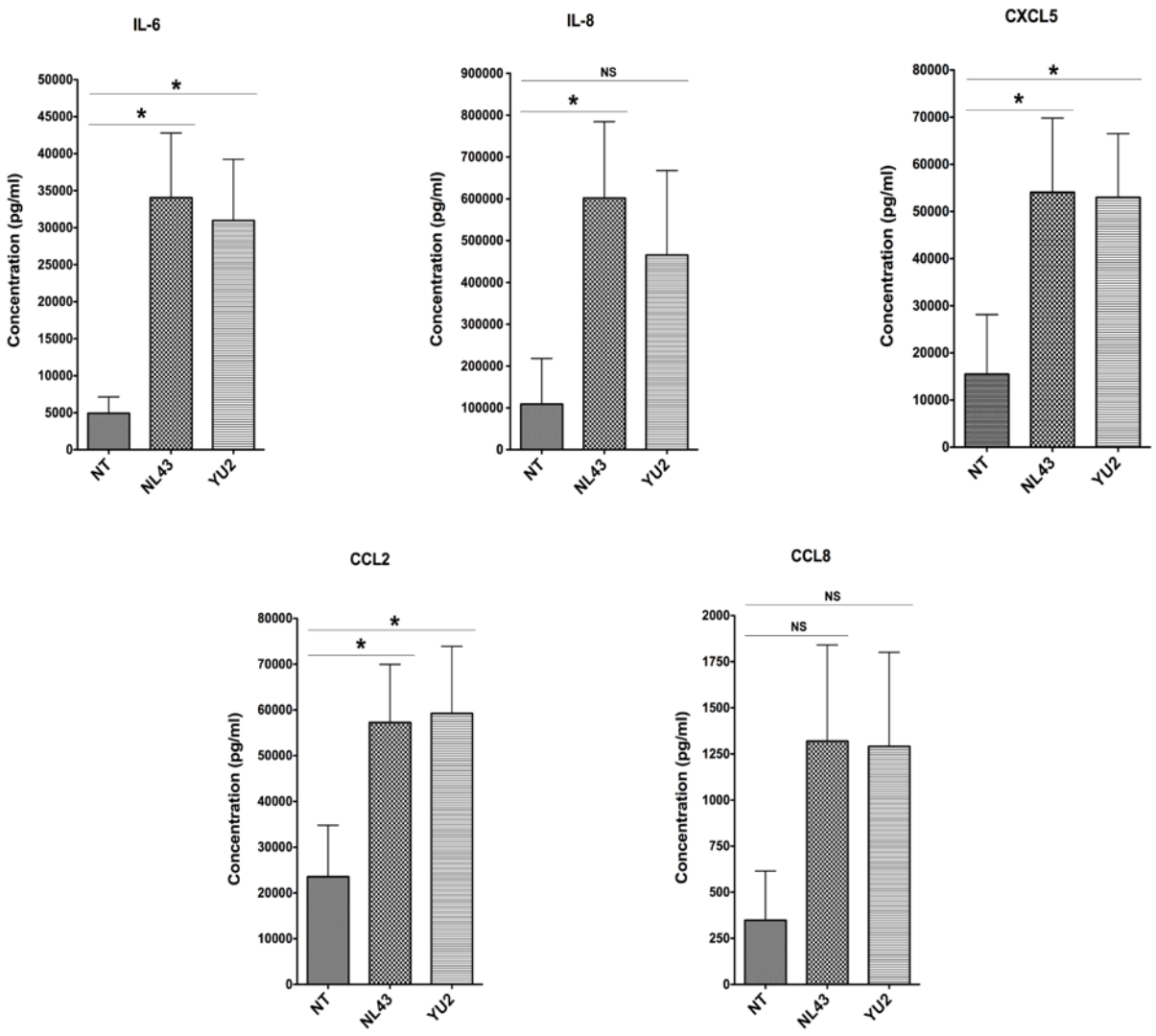

Figure 20. Biological validation of selected HIV-1 related factors by ELISA

Expression of CCL2, CCL8, CXCL5, IL-6 and IL-8 was monitored by ELISA in supernatants obtained from PBMCs infected with CXCR4-coreceptor virus (NL43), CCR5-coreceptor virus (YU2) or mock infected PBMCs (n=7). *=$p<0.05$, NS=not significant

We also tested the expression level of NRGN in the RNA of these samples by performing qRT PCR. Results indicate that PBMC infected with NL43 or YU2 showed significant downregulation of NRGN RNA transcript compared to mock or uninfected control (Fig. 21).

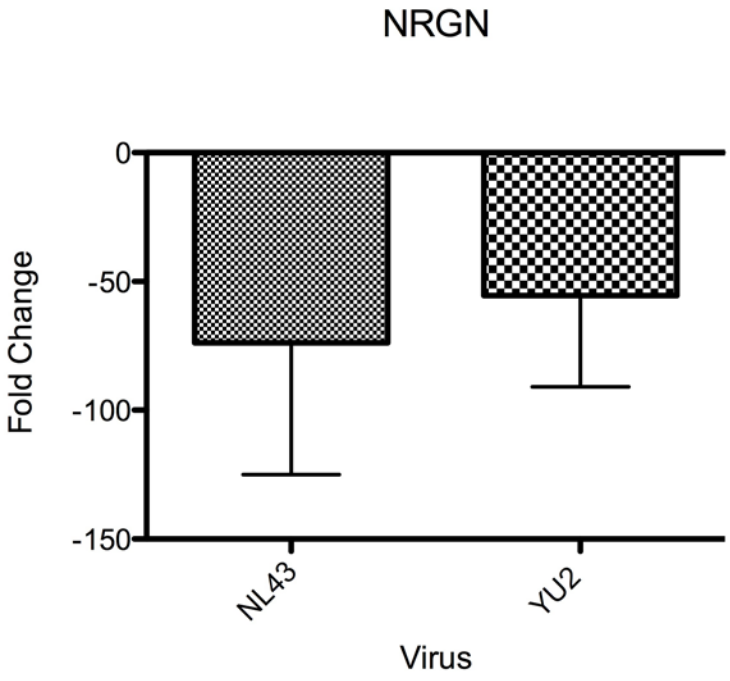

Figure 21. qRT-PCR expression of NRGN after normalization to RPLPO

qRT-PCR was used to assess the expression of NRGN in PBMCs infected with NL43 and YU2. Fold increase/decrease was calculated relative to uninfected PBMCs and based on normalization to RPLPO (n=2).

To further confirm the repression of NRGN at the protein level, expression of NRGN in cell lysates of PBMC infected with NL43, YU2 or mock was assessed by immunoblotting using anti-NRGN antibody (Fig. 22). Results suggest that PBMC infected with NL43 or YU2 showed significant down regulation of NRGN compared to uninfected or mock-infected control cells. Further quantitative analysis using densitometry scanning indicate that the reduction is ~55% in PBMC infected with NL43 virus, whereas a ~85% reduction was found in PBMC infected with

YU2 virus. Although it is not clear why the two different viruses exhibit different level of down regulation of NRGN, one of the possible explanations could be the infectivity level within these cultures, as both macrophages and CD4 cells are infected by YU2, whereas, NL43 infects only CD4+ T cells. Although it is not clear why the two different viruses exhibit different level of down regulation of NRGN, one of the possible explanations could be the infectivity level within these cultures, as both macrophages and CD4 cells are infected by YU2, whereas, NL43 infects only CD4+ T cells.

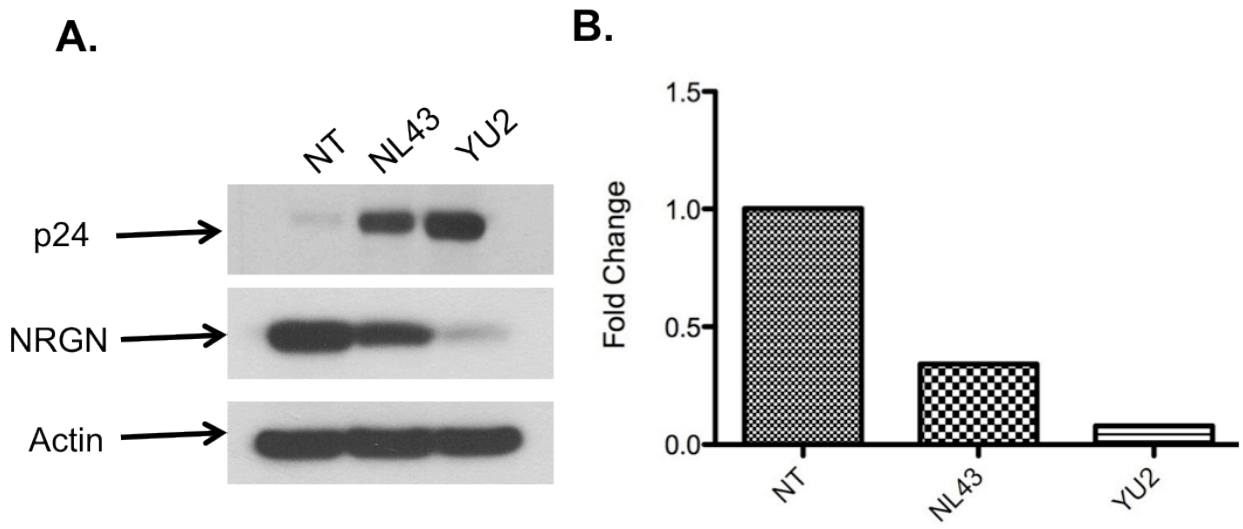

Figure 22. Western blot analysis of NRGN

NRGN expression of in vitro infected (NL43 and YU2) PBMCs was analyzed by Western blot and compared to mock infected **(A)**. Densidometry was performed on the blot **(B)**. Fold changes represent ratios of NRGN to Actin, normalized to mock infection.

5.2.4 Summary of AIM #2

Our computational analyses have predicted several miRNA-mRNA interactions. The comparison between HVL and control produced the largest number of predictions with several of

them having a confidence greater than 0.5. Even at this confidence, several miRNAs had multiple targets, with some targeting as many as 9 mRNAs. Similarly, the 3' UTR of a single mRNA contains many miRNA binding sites and, consequently, can be targeted by multiple miRNAs. Correlation studies of some of the mRNAs involved in these interactions showed no strong significant relationships to CD4 and Nadir CD4 counts, and no significant relationships to viral load. ELISA and qPCR results of selected HIV related transcripts tested in *in vitro* samples confirmed the expression patterns of the mRNA transcripts.

5.3 AIM #3 TO PERFORM MIRNA PROFILING IN CD4 AND CD14 CELLS OF LOW VIRAL LOAD, HIGH VIRAL LOAD, AND UNINFECTED CONTROL DONORS

The primary cell types targeted during HIV infection are CD4+ T cells and monocytes/macrophages. These cells play a major role in the immune system by recruiting neutrophils, eosinophils and basophils, inducing macrophages, and aiding B cells in the production of antibodies[75]. Macrophages, which phagocytoze pathogens and help fight off opportunistic infections[76]. Macrophages are thought to be the first infected cells following HIV entry into the body and the source of HIV following the depletion of CD4 T cells[77]. Both of these cell types express HIV coreceptors CCR5 and CXCR4[78], but in macrophages the CXCR4 coreceptor is thought to be nonfunctional[79].

We chose to assess if any of the significantly differentially regulated miRNAs found in the PBMC population were HIV-1 target cell type specific. CD4+ and CD14+ cells were isolated from a subset of control and low viral load donors, for which we had previously performed miRNA profiling in PBMCs.

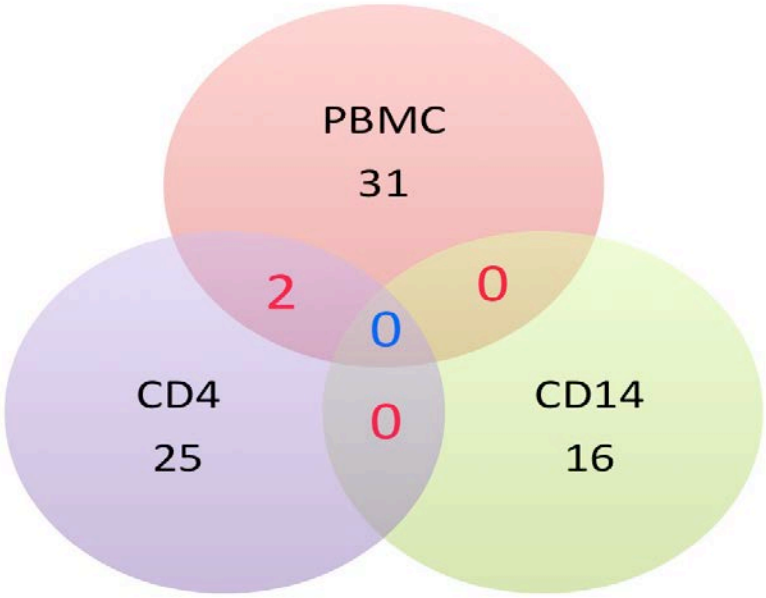

Figure 23. Venn diagram illustrating the number of significantly differentially regulated miRNAs between CD4, CD14, and PBMCs of subjects from uninfected and low viral load groups (N=5)

miRNA profiling was performed on PBMC, CD4+ T cells, and CD14+ T cells to identify the ____ of HIV target cells compared to PBMC (N=5). The Venn diagram displays the number and overlap of significantly differentially expressed miRNA ($p<0.05$) among the LVL group relative to the CT in CD4, CD14, and PBMCs of the same donors (CT: N=5, LVL: N=5).

Data (analyzed with RealTime StatMiner) from 5 subjects from the uninfected control and low viral load groups indicate that there were 25 miRNAs specific to CD4+ T cells and 16 miRNAs that were specific to CD14 cells. Analysis of PBMC data from the same donors showed a total of 33 miRNAs that were significantly dysregulated between the two groups. Only 2 of these miRNAs (miR-425* and miR-576-3p) were found in the CD4+ population, while none of these were found in the CD14 population (Fig. 23). There is currently no information relating these miRNAs to any disease or condition.

Furthermore, hierarchical clustering of miRNAs among the CD4 cells of low viral load and uninfected samples results in the formation of three clusters; the first is composed of low viral load, the second of uninfected control, and the third of both types of samples (Fig. 24). We also performed the clustering in CD14 cells of the same subjects and observed an occasional clustering of two samples from the same group (Fig. 25). Overall, however, the clustering separation was extremely variable. Although differentially regulated miRNAs in PBMCs separated between CT vs. LVL (with the exception of LVL30), similar clustering was not observed in CD4+T and CD14+ cells from the same donors, suggesting non-target cell types might be involved (Fig. 26). Alternatively, the number of samples may be too low to derive any meaningful conclusions; thus, the study needs additional work.

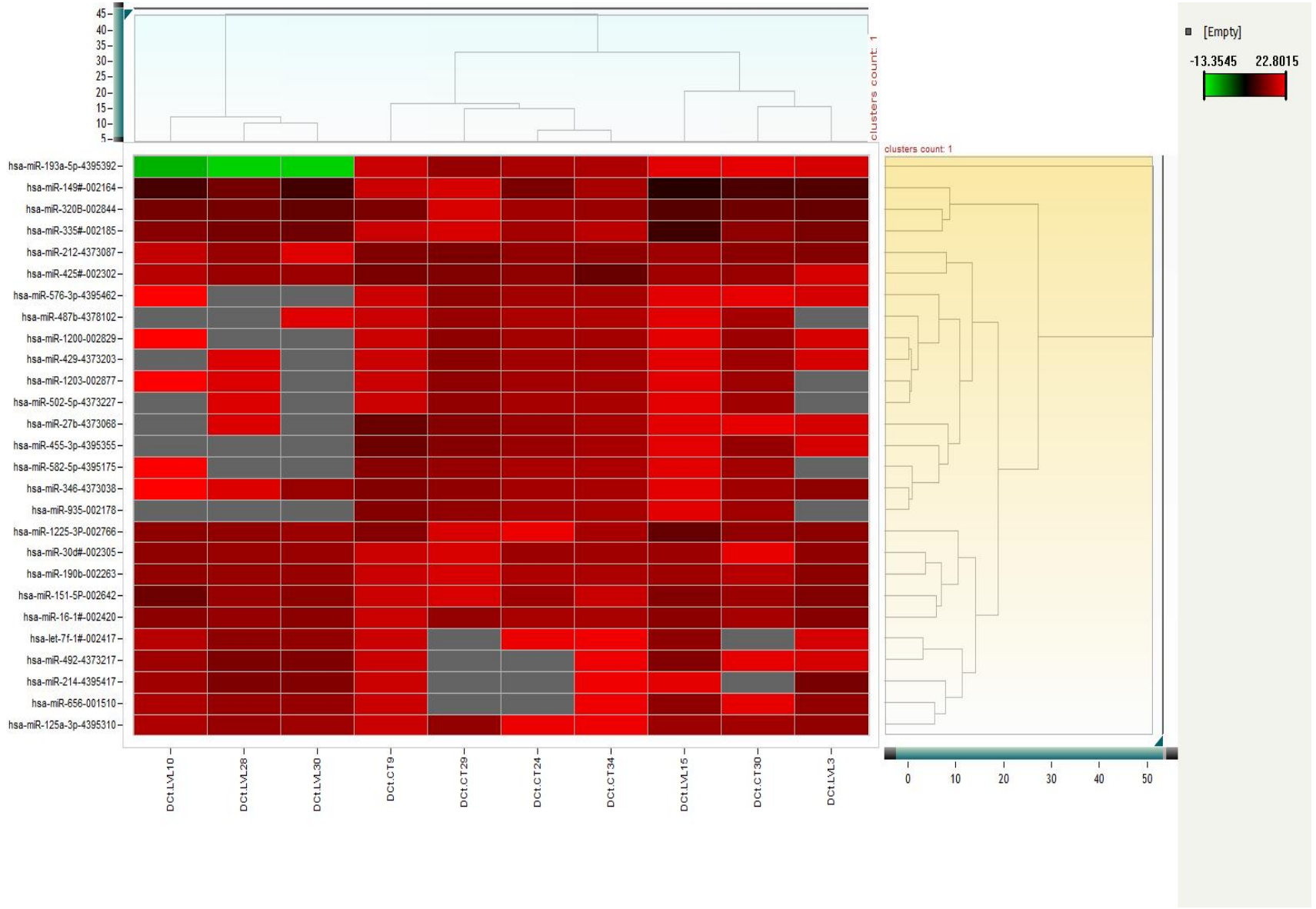

Figure 24. Hierarchical clustering of miRNAs in CD4 cells between low viral load and uninfected controls

Hierarchical clustering of miRNA in CD4 cells between CT and LVL (N=5, N=5). miRNA in the clustergram are dysregulated at a significance cutoff of $p<0.05$. The dendogram depicting the clustering of samples is calculated using Complete linkage with Euclidian distance measure values. Color ranging from green to red indicates minimum to maximum dCT. Gray indicates empty wells. Numbers on X axis represent subject group. CT, uninfected controls; LVL, low viral load subjects.

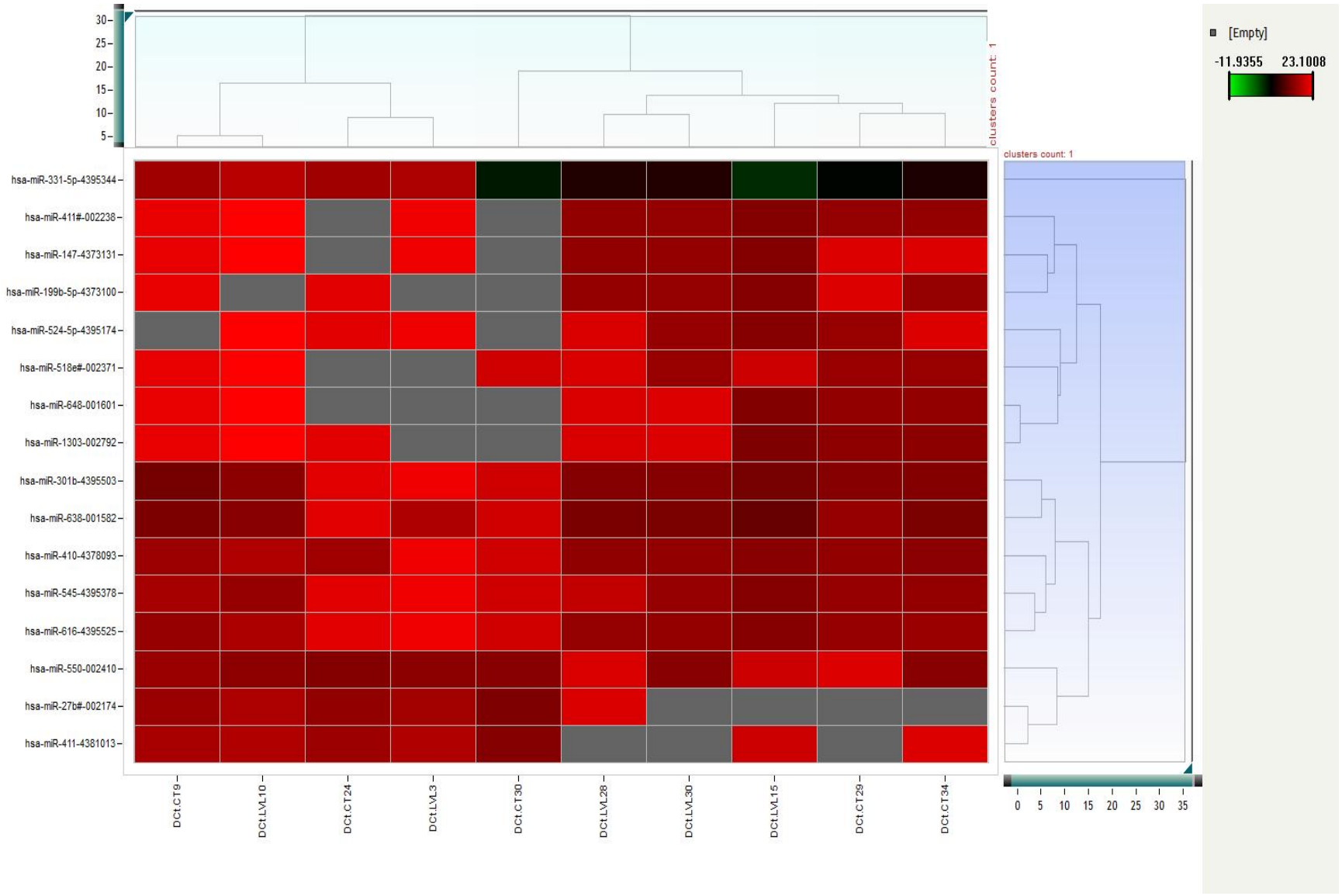

Figure 25. Hierarchical clustering of miRNA in CD14 cells between low viral load and uninfected controls

Hierarchical clustering of miRNA in CD14 cells between CT and LVL (N=5, N=5). miRNA in the clustergram are dysregulated at a significance cutoff of $p<0.05$. The dendogram depicting the clustering of samples is calculated using Complete linkage with Euclidian distance measure values. Color ranging from green to red indicates minimum to maximum dCT. Gray indicates empty wells. Numbers on X axis represent subject group. CT, uninfected controls; LVL, low viral load subjects.

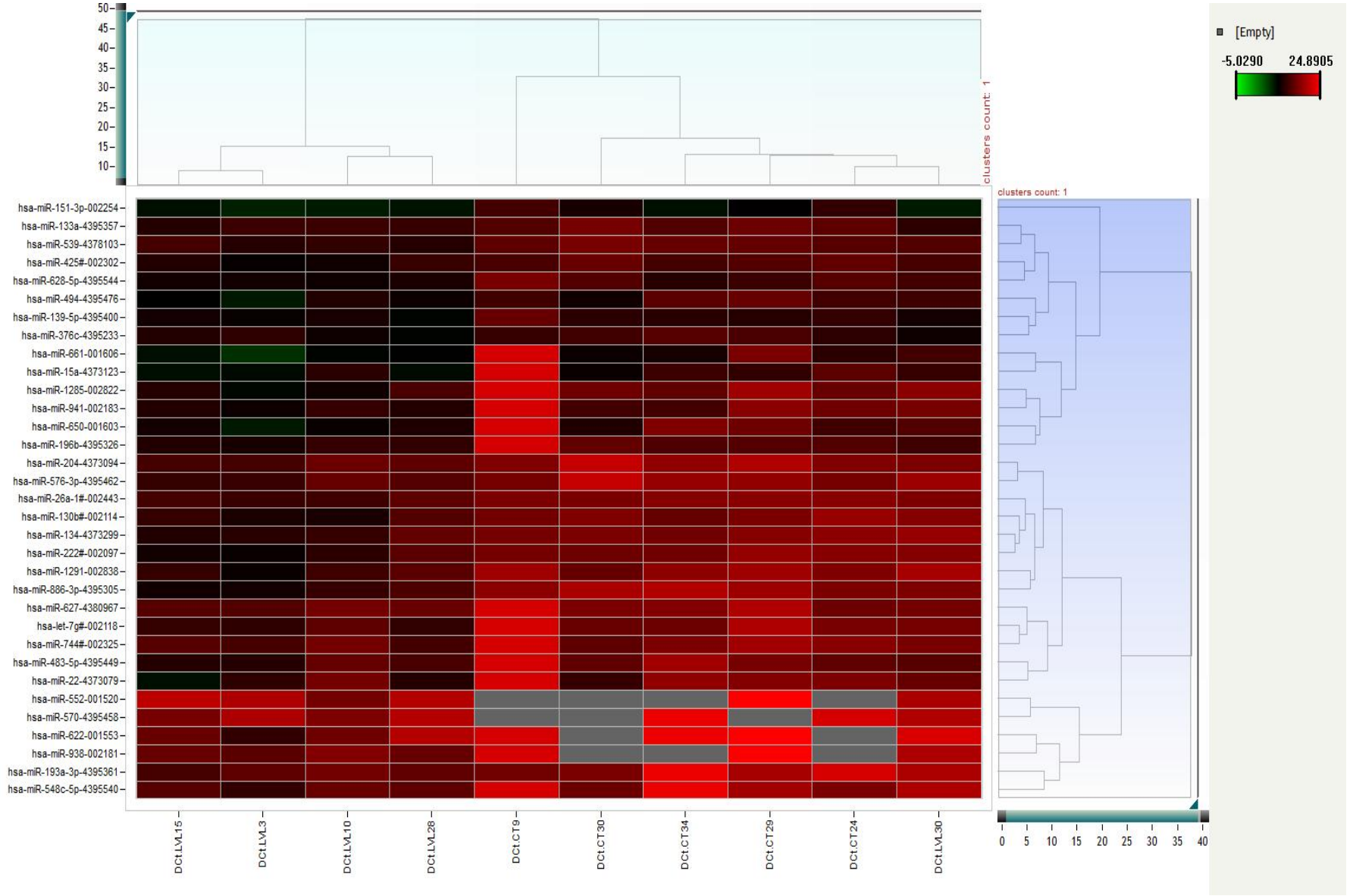

Figure 26. Hierarchical clustering of PBMCs between low viral load and uninfected controls

Hierarchical clustering of miRNA in PBMCs between CT and LVL (N=5, N=5). miRNA in the clustergram are dysregulated at a significance cutoff of $p<0.05$. The dendogram depicting the clustering of samples is calculated using Complete linkage with Euclidian distance measure values. Color ranging from green to red indicates minimum to maximum dCT. Gray indicates empty wells. Numbers on X axis represent subject group. CT, uninfected controls; LVL, low viral load subjects.

Since the majority of the subjects in our high viral load group were deceased or no longer participating in the study, we were unable to isolate the CD4 and CD14 cells from them. Additionally, because most of the current subjects in the Pitt MACS are on therapy and are, therefore, able to control their viral load, we did not have access to fresh blood of new high viral load donors. As a result, we isolated the cells from frozen PBMCs. These PBMCs, however, were several years old and did not yield high number of purified cell types. In order to perform the profiling in these samples, we would have to pre-amplify the cDNA. Pre-amplification results in higher expression of most miRNAs, including the endogenous controls. Samples that are pre-amplified, therefore, cannot be compared to those that are not pre-amplified.

As an alternative, we used an *in vitro* system as a model for the high viral load donors. CD4+ and CD14+ cells were isolated from PBMCs that were infected with either CXCR4 (NL43) or CCR5 (YU2) co-receptor utilizing virus. Post infection, RNA was isolated and miRNA profiling was performed. First, we examined the intersection between the differentially regulated donor PBMCs and a union of the *in vitro* infected PBMCs (NL43 vs. mock and YU2 vs. mock) (Fig 27).

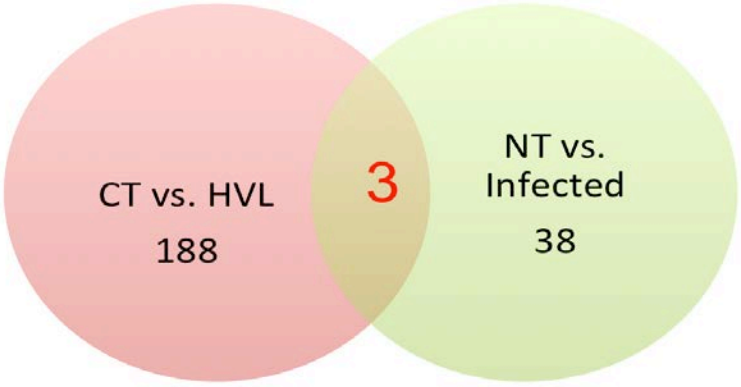

Figure 27. Venn diagrams illustrating the intersection of significantly differentially regulated miRNAs between HVL vs. CT donor PBMCs and in *in vitro* infected PBMC samples

The Venn diagram displays the number and overlap of significantly differentially expressed miRNA ($p<0.05$) among the HVL group relative to the CT in donor PBMCs and *in vitro* infected PBMCs relative to mock (CT: N=36, HVL: N=32, NT=4; YU2=4, NL43: N=4).

Since the sample size of the *in vitro* samples was rather small compared to our donor population, we obtained only 3 significant miRNAs (miR-518b, miR-639, miR-886-3p) in common between the two. Given that we did obtain miRNAs in common, however, we examined the expression of significantly differentially regulated miRNAs (determined by StatMiner and DataAssist) of PBMCs from the comparison of CT vs. HVL in these CD4+ and CD14+ *in vitro* infected samples. Results indicate that of the 191 miRNAs examined, 141 miRNAs had a common fold change pattern between the donor PBMCs and *in vitro* infected CD4 cells with NL43. Additionally, there were 146 miRNAs from the donor PBMCs that had a common fold change pattern with CD4 cells infected with YU2. Considering there were 112 miRNAs that were commonly regulated between the PBMCs and CD4 cells (regardless of the type of virus), 16 miRNAs from the 191 miRNAs in the PBMCS examined came from a cell type other than CD4 cells (Fig. 28A).

We also examined the intersection of these same 191 miRNAs in CD4 and CD14 cells infected with YU2. We discovered 115 miRNAs that had the same fold change pattern in the PBMCs and CD14 cells and 94 miRNAs that had the same fold change pattern between the PBMCs and CD4 and CD14 cells infected with YU2. Consequently, 24 out of the total 191 miRNAs did not come from the CD4 or CD14 cells infected with YU2 (Fig. 28B).

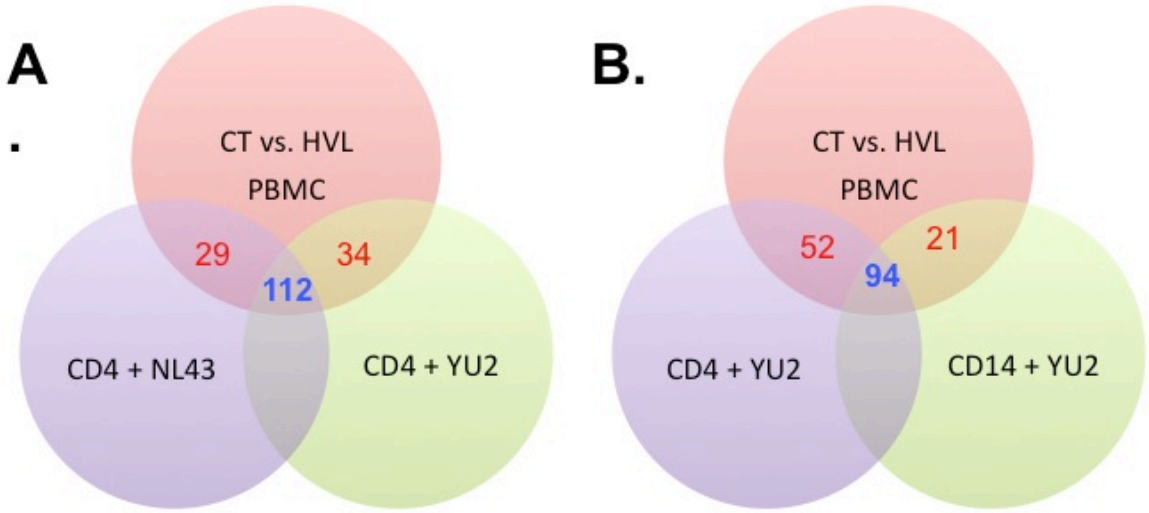

Figure 28. Venn diagrams illustrating the distribution of 191 significantly differentially regulated miRNAs from HVL in *in vitro* infected cell specific samples

A total of 191 PBMC miRNA were examined and their fold change pattern (up/down) was compared to *in vitro* data from CD4 and CD14 infected cells. The fold change for the cell specific data was generated with a comparison to untreated cells from the same donor. CT=uninfected controls, N=36; HVL=high viral load, N=31; CD4 + NL43=CD4 cells infected with NL43, N=3, CD4 + YU2=CD4 cells infected with YU2, N=3; CD14 + YU2= CD14 cells infected with YU2; N=3.

5.3.1 Summary of AIM #3

Since HIV infects both CD4+ T cells and monocytes/macrophages, we focused on studying the origin of the significantly differentially regulated miRNAs within the PBMCs. In our low viral

load comparison to uninfected controls, we discovered that most of the miRNAs come from cell types other than CD4 and CD14 cells. Our comparison of the donor high viral load PBMC miRNA data with cell specific samples isolated from *in vitro* infected PBMCs showed that most of the miRNAs originate from CD4+ and CD14+ cells.

6.0 DISCUSSION

Although it has been about 30 years since the first HIV/AIDS cases were reported, the virus continues to be a threat to the world population. Even with extensive research, it is not exactly clear how the virus interacts with the host immune system. Additionally, it is unclear why certain individuals progress through the disease stages faster than others and why some people are immune to infection despite repeated exposure. In this study, we concentrated on understanding the cellular transcription and its regulation to HIV infection in the form of microRNAs. Since miRNAs bind to the 3'UTR of their target mRNAs, we explored both miRNA and mRNA profiles between uninfected and infected individuals. Further, we studied the effect that viral load (low vs. high) may have on both of these profiles.

Several factors, including age, sex and infection/disease, may influence a miRNA profile[80, 81]. Consequently, to reduce the effect of these influences, we strived to match our subjects as closely as possible. All of our subjects were males and their median age placed them in the middle-aged category. Specifically, the median ages in our uninfected, low viral load, and high viral load groups were 50, 51.5, and 40, respectively. With respect to the viral load groupings, we classified viral load individuals as those who had mostly undetectable/low levels (~<40 copies/mL) and maintained a fairly low viral load for the duration of the past 5 years. High viral load individuals, on the other hand, were those whose viral load was high (>45,000 copies/mL) for at least 6 months prior to the date of collection.

To decrease the potential for variability of results due to software, we analyzed our data with two programs. Our results indicate that viral infection induces a differential regulation of microRNA compared to uninfected controls. Furthermore, viral load level has an additional effect. It is high viral load, in particular, that produces the greatest effect on altering the miRNA profile. This is further supported by our finding of fewer significantly dysregulated miRNAs between LVL and the control and the less distinctive clustering among these samples. We only found 21 miRNAs that were significantly differentially regulated between controls and LVL, compared to 191 miRNAs and 158 miRNAs that were significantly dysregulated between CT vs. HVL and LVL vs. HVL.

Witwer et al had recently published miRNA profiling data on PBMCs of uninfected, elite suppressors, and viremic donors[82]. Results from this study and ours differed in several aspects. First, our sample size of 99 was considerably larger than their 22 donor population. Additionally, all of our low viral load donors were on HAART and consequently, do not fit the definition of being elite suppressors. We found a total of 9 miRNAs in common with Witwer's data ($p<0.05$). Eight of these miRNAs exhibited the same fold change pattern. The only discrepancy was let-7g, which we found to be upregulated, while Witwer reported it as being downregulated. This difference could potentially be attributed to donor variability given that the fold change that Witwer reports for let-7g is not very high (-1.5 to -1.7).

As previously mentioned, Huang et al had found miR-28, miR-125b, miR-150, miR-223, and miR-382 to be downregulated in activated CD4+ T cells compared to resting CD4+ T cells[19]. Of these 5 miRNAs, we found three to be significantly differentially regulated in any of our analyses. Only, miR-223 was downregulated, however; while, miR-28-3p/5p, and miR-382 were upregulated. This suggests that profiling with multiple platforms would be

advantageous to determine which miRNAs are truly significant and to eliminate the possibility of platform related errors. Our independent validation with qRT PCR confirmed our high throughput data in 65-75% of the cases. Although this validation performance is standard, using multiple profiling platforms would certainly be more advantageous.

Similarly Bignami et al had performed a functional analysis of miRNAs dysregulated in CD4+ T lymphocytes. Several of our differentially regulated miRNAs fall into Bignami's categories. miR-34a is associated with viral replication, miR-21, miR-155, and miR-424 are linked to immune response, and miR-146b-3p is linked to both functions[83]. Of these, miR-34a and miR-424 were also significantly upregulated in the *in vitro* infected (NL43 and YU2, repsectively) CD4+ T cells. Although not significant, the remaining miRNAs also exhibited the same fold change pattern in the *in vitro* infected CD4+ T cells as in the donor PBMCs. Notably, the fold change of these miRNAs in the CD14 cells was either negative or close to 1, indicating that these miRNAs likely originated from the CD4 population.

Finally, we have also shown the notion that the LVL group is similar to the uninfected controls on the mRNA level by performing a STRING pathway analysis on the mRNA array data, which produced very few gene interactions in the comparison between the low viral load and uninfected controls; it did, however, produce many cytokine and chemokine interactions for the comparison between the high viral load and both uninfected controls and low viral load samples. Overall, we found only 41 significantly dysregulated mRNAs between CT and LVL and all of these miRNAs had a fold change<2. Meanwhile, the comparisons of CT vs. HVL and LVL vs. HVL yielded 309 and 182 significantly differentially regulated probes (Table 12). We found STAT1 to be upregulated in the high viral load (compared to low viral load), which is consistent with Rotger et al who reported this interferon receptor to be significantly upregulated

with increasing viral load[43]. We also found APOBEC3G, a cellular defense factor against retroviruses, to be upregulated in the presence of infection (CT vs. LVL) and high viral load (CT vs. HVL), which parallels Rotger et al and their finding that the gene is upregulated with increasing viral load[43]. The overall clustering of the samples is also similar in that the uninfected controls tended to cluster with treated subjects (similar to LVL group) and, for the most part, separate from the viremics[43]. Unfortunately, we have currently performed mRNA profiling only on PBMCs. It would certainly be interesting to perform it on HIV target cells (similar to what we have done on the miRNA level) to observe the effect that infection and viral load may have on the mRNA within the individual cell types.

To validate our mRNA data, we performed independent qRT PCR validations, which confirmed our high throughput data in 75% of the cases. The only mRNA, which did not yield the proper fold change pattern in the qRT PCR data was NRGN. The fold change that we have obtained for it in the qRT PCR data was not very high and was not significant, however. Our biological validations of NRGN using *in vitro* infected PBMCs indicate that NRGN is indeed downregulated following infection and that the downregulation is virus specific. Furthermore, our ELISA-based biological validations of HIV-1 related factors confirm the upregulation of these factors after infection.

To study the interaction between the miRNA and mRNA, we chose to not simply rely on online interaction databases like TargetScan. Instead, we chose to use our array data as a supplement to GenMiR++. GenMir++ is a Bayesian method that formulates miRNA-mRNA predictions using both experimentally measured data and a database of potential miRNA-mRNA sets[84]. In our analysis, we chose MicroCosm Targets as our database. Using two sets of filters (first set to the union of the significantly differentially regulated miRNAs and mRNAs, second

set to the significantly differentially regulated miRNAs and mRNAs from individual group analysis), we were able to come up with a number of predictions. Several of these predictions had confidence greater than 0.5 and would, therefore, be good targets for additional miRNA-mRNA related studies. Notably, our analysis has predicted targets for miRNAs associated with high viral load (miRNAs upregulated in CT vs. HVL and LVL vs. HVL: miR-564, miR-92a, and miR-130b) and those that are related to both viral load level and infection (miRNA upregulated in CT vs. LVL, CT vs. HVL, and LVL vs. HVL: miR-483-5p). It is these miRNAs that should be examined further in the future since they are the ones that hold the most value for possible clinical use.

We also performed correlation studies for selected transcripts to determine if other factors, besides the presence of high viral load, could have an effect on the differential mRNA expression. We found a significant negative correlation for IL-8 in HVL and CD4; this is consistent with data in the literature that associate infection (therefore a lower CD4 count) with elevated IL-8[73]. In this group, IL-6 and IL-8 also correlated with Nadir CD4. This was not surprising because the Nadir CD4 counts for some of the HVL subjects were quite low. Furthermore, this paralleled Stone et al's findings of elevated IL-6 in HIV patients after an immune restoration [85]. We also found significant correlations within LVL for IL-6 and NRGN and CD4. None of the observed correlations were strong (Spearman $r<0.5$). Interestingly, we found no significant correlations with the level of high viral load, indicating that the presence of high viral load has an effect on producing differential mRNA expression but the level of the high viral load makes no additional difference.

Our profiling was done on PBMCs, which are composed of several different cell types. Typically, PBMCs are composed of 15% NK cells, 10% B cells, 15% monocytes/macrophages

(CD14+), and 60% T cells[86]. Approximately 70% of the T cells are CD4+ [86]. Given that HIV infects both CD4+ T cells and macrophages, we chose to study miRNA profiles in these subsets of cells to determine the origins of the differentially regulated miRNAs found in PBMCs. Our results of the comparison between low viral load and uninfected control donors show that only two of the differentially regulated miRNAs in PBMCs come from CD4+ T cells, and none of them come from CD14+ cells, indicating that the miRNAs originate from non-target cell types. This is supported by our clustergram analyses. CD4 and CD14 clusterings showed an indistinctive cluster pattern, where as the PBMC miRNA clustergram showed a distinctive control cluster and a separate low viral load cluster.

Further analyses into the miRNAs that were differentially regulated in CD4+ cells indicates that miR-30d* and miR-656 may be particularly important. This is because these miRNAs were found to be significantly differentially regulated by both of our software programs, and they exhibited similar expression patterns in *in vitro* infected and uninfected samples. Similarly, of the miRNAs significantly differentially regulated in CD14 cells, miR-301b, miR-550, and miR-616 may be the key players since their expression was similar in our *in vitro* samples. It is possible that in the PBMC population, these miRNAs become less differentially regulated due to the effect of other cell types and that is why they are not significant there.

Although we were unable to perform CD4 and CD14 profiling in high viral load donors, we were able to compare the results of the high viral load PBMC profiling to those of *in vitro* infected samples. Of the 191 miRNAs that were differentially regulated in PBMCs, all but 16 originated from cells other than the HIV target cells. This suggests that the presence of high viral load causes the dysregulation of several miRNAs in the CD4 and CD14 cells and that the

expression of these miRNAs cannot be brought back to normal levels by the non-target cells, leading to those miRNAs still being dysregulated in the PBMC population.

Our study of miRNA and mRNA profiles in individuals infected with low and high levels of viral load has significant public health relevance. The finding of several miRNAs that are specific to a particular viral load and infection could lead to the use of these miRNAs as biomarkers for the determination of prognosis of the disease. In addition, these miRNAs could aid in the determination of the infected individuals' ability to respond to antiviral treatment. Our identification of additional cellular molecules, namely Neurogranin, that are dysregulated by HIV-1 infection may also lead to new potential targets for antiviral development.

APPENDIX A

SIGNIFICANTLY DIFFERENTIALLY REGULATED MIRNA

A.1 CONTROL VS. LVL

CT vs. LVL				
	RealTime Statminer		DataAssist	
	p-value	Fold Change	p-value	Fold Change
hsa-miR-1183	2.80E-02	4.57	0.0255	5.42
hsa-miR-1260	3.99E-02	5.66	0.0359	6.55
hsa-miR-1262	4.13E-04	-1117.81	0.0016	-909.09
hsa-miR-1275	2.84E-02	6.26	0.0255	7.55
hsa-miR-1291	1.46E-02	5.13	0.0366	5.05
hsa-miR-148b*	1.30E-02	3.53	0.0135	3.89
hsa-miR-18b*	2.58E-02	-27.60	0.035	-95.24
hsa-miR-191*	1.63E-02	4.49	0.0255	5.85
hsa-miR-21*	2.27E-03	6.66	0.002	7.59
hsa-miR-454*	1.82E-03	3.18	0.0016	3.57
hsa-miR-483-5p	4.10E-03	5.97	0.0048	6.56
hsa-miR-542-5p	2.47E-02	4.64	0.0255	6.13
hsa-miR-550	4.10E-03	4.06	0.0016	4.65
hsa-miR-584	6.72E-03	13.78	0.0016	22.48
hsa-miR-650	3.15E-02	5.90	0.0359	6.91
hsa-miR-744*	1.46E-03	5.01	<9E-05	8.24
hsa-miR-93*	2.84E-02	3.10	0.0255	3.44
hsa-miR-938	2.60E-04	20.48	0.0048	14.21
hsa-miR-941	4.59E-02	3.97	0.0447	4.46
rno-miR-29c*	4.00E-02	3.97	0.0255	5.41

Continued Control vs. LVL

rno-miR-7*	3.02E-02	5.60	0.0434	5.67

A.2 CONTROL VS. HVL

CT vs. HVL				
	Realtime StatMiner		DataAssist	
	p-value	Fold Change	p-value	Fold Change
dme-miR-7	1.26E-07	13.87	<9E-05	14.28
has-miR-1305	5.30E-11	4078.26	<9E-05	4098.64
has-miR-155	1.03E-04	16.78	1.00E-04	16.86
has-let-7d	2.03E-02	4.09	0.0131	4.41
has-let-7g	1.19E-02	8.76	0.01	8.81
has-miR-106b*	3.86E-02	2.75	0.0309	2.76
has-miR-10a	1.58E-03	4.58	8.00E-04	4.88
has-miR-10b*	4.80E-02	2.53	0.0151	3.07
has-miR-1180	1.59E-02	3.43	0.0036	5.31
has-miR-1183	2.17E-06	12.67	<9E-05	12.73
has-miR-1201	1.12E-03	5.40	4.00E-04	6.55
has-miR-1225-3P	4.67E-13	26.45	<9E-05	26.59
has-miR-1227	1.49E-02	3.84	0.0118	3.86
has-miR-124	1.58E-10	63.94	<9E-055	83.40
has-miR-1247	5.57E-04	49.54	6.00E-04	49.79
has-miR-1253	1.45E-07	17.03	<9E-05	18.30
has-miR-1254	1.21E-04	4.64	1.00E-04	5.91
has-miR-1255B	8.44E-04	4.92	6.00E-04	4.95
has-miR-125b-1*	2.22E-02	4.46	4.00E-04	10.09
has-miR-1260	9.72E-03	5.88	0.0078	5.91
has-miR-1262	2.66E-02	-60.30	0.0288	-59.88
has-miR-1267	2.62E-02	-20.06	0.0316	-19.96
has-miR-127-3p	2.89E-02	3.18	0.0153	3.73
has-miR-1270-	3.39E-03	5.80	0.0012	6.88
has-miR-1271	1.18E-04	3.04	<9E-05	3.75
has-miR-1274A	6.05E-05	4.47	<9E-05	4.49
has-miR-1275	7.47E-05	17.07	1.00E-04	17.16

Continued Control vs. HVL

hsa-miR-1276	1.96E-06	10.48	<9E-05	13.10
hsa-miR-1282	6.59E-04	4.78	7.00E-04	4.82
hsa-miR-1285	5.15E-03	10.10	0.0036	12.68
hsa-miR-1290	4.19E-05	5.12	<9E-05	5.15
hsa-miR-1291	4.33E-04	4.35	4.00E-04	4.29
hsa-miR-1298	1.78E-03	18.84	0.0025	18.93
hsa-miR-1300	1.50E-03	4.71	5.00E-04	5.85
hsa-miR-1303	4.67E-13	137.21	<9E-05	252.19
hsa-miR-130b*	1.05E-04	-7.37	0.0016	-5.49
hsa-miR-130b	1.48E-02	2.55	0.0039	3.25
hsa-miR-132	3.14E-07	12.92	<9E-05	15.52
hsa-miR-135b	5.43E-03	4.19	0.001	6.59
hsa-miR-138-2*	7.47E-05	14.76	<9E-05	21.88
hsa-miR-140-5p	1.19E-02	3.04	0.0085	3.06
hsa-miR-142-3p	6.03E-03	10.84	0.005	10.90
hsa-miR-144	5.08E-06	-25.71	1.00E-04	-23.31
hsa-miR-146a*	4.63E-02	-6.13	0.021	-9.88
hsa-miR-146a	9.83E-03	10.81	0.0084	11.62
hsa-miR-146b-3p	2.31E-05	6.03	<9E-05	6.23
hsa-miR-148a*	2.41E-02	-89.82	0.0316	-106.38
hsa-miR-148b*	7.62E-03	3.11	0.0047	3.23
hsa-miR-151-5P	1.50E-03	6.11	7.00E-04	7.07
hsa-miR-152	1.90E-03	5.26	0.0011	5.83
hsa-miR-155*	1.78E-05	7.63	<9E-05	11.64
hsa-miR-15a	2.23E-02	4.92	0.0151	5.37
hsa-miR-16	1.20E-02	16.03	0.0106	16.11
hsa-miR-181a	2.26E-03	7.42	0.0015	8.49
hsa-miR-181c	1.30E-02	2.90	0.0039	3.54
hsa-miR-1826	1.08E-02	-39.77	0.0494	-5.58
hsa-miR-185	1.01E-02	4.10	0.0056	4.81
hsa-miR-186	1.24E-02	3.86	0.0096	3.88
hsa-miR-18a*	2.02E-02	2.41	0.0037	3.35
hsa-miR-18b*	2.14E-02	-15.81	0.023	-140.85
hsa-miR-191*	3.49E-03	5.96	0.0025	7.77
hsa-miR-193a-5p	2.17E-06	13.71	<9E-05	16.28
hsa-miR-193b	1.50E-03	6.81	0.0011	6.84
hsa-miR-194	2.31E-03	4.87	0.0016	4.89
hsa-miR-195	2.46E-02	3.99	0.0183	4.40
hsa-miR-19a	2.37E-02	7.68	0.0209	7.71
hsa-miR-19b-1*	1.00E-02	2.76	0.0073	3.10

Continued Control vs. HVL

hsa-miR-19b	1.28E-02	13.55	0.0113	13.62
hsa-miR-200b	4.10E-03	4.74	0.0021	5.12
hsa-miR-200c	3.66E-02	3.79	0.0248	4.10
hsa-miR-202	9.92E-04	5.24	7.00E-04	5.26
hsa-miR-206	1.16E-03	3.75	6.00E-04	3.77
hsa-miR-20a	1.08E-02	10.19	0.0095	10.24
hsa-miR-20b	1.97E-02	3.32	0.0152	3.34
hsa-miR-21*	4.03E-05	7.89	<9E-05	8.24
hsa-miR-21	3.51E-06	6.15	<9E-05	6.18
hsa-miR-210	1.21E-04	10.54	1.00E-04	11.15
hsa-miR-211	1.67E-02	3.03	0.0106	4.51
hsa-miR-212	7.74E-08	7.20	<1E-05	9.46
hsa-miR-219-5p	3.22E-02	5.07	6.00E-04	13.44
hsa-miR-22*	4.08E-02	1.77	0.0051	2.17
hsa-miR-22	4.47E-05	15.41	<9E-05	18.00
hsa-miR-222	1.70E-06	4.89	<9E-05	4.91
hsa-miR-223	5.58E-03	-2.05	0.0028	-2.04
hsa-miR-24-2*	1.70E-02	2.79	0.0137	2.94
hsa-miR-25	1.17E-03	2.89	1.00E-04	3.59
hsa-miR-27a*	1.13E-03	2.03	1.00E-04	2.04
hsa-miR-27a	4.32E-02	2.43	0.0213	2.98
hsa-miR-28-3p	7.98E-04	1.96	1.00E-04	1.97
hsa-miR-28-5p	8.00E-03	3.89	0.0036	4.81
hsa-miR-29a*	2.66E-02	2.86	0.0099	3.64
hsa-miR-29a	3.98E-02	2.91	0.032	2.92
hsa-miR-29b-1*	1.19E-04	7.59	1.00E-04	8.17
hsa-miR-29b-2*	6.75E-03	6.47	0.0014	9.77
hsa-miR-29b	2.07E-02	3.56	0.01	4.24
hsa-miR-302a*	5.15E-03	5.62	1.00E-04	10.27
hsa-miR-30a-5p	4.08E-02	3.79	0.0247	4.58
hsa-miR-30b	1.83E-02	7.28	0.0155	7.32
hsa-miR-31*	1.97E-02	-2.72	0.0272	-2.76
hsa-miR-320B	4.38E-03	6.78	0.0034	6.82
hsa-miR-324-3p-	5.61E-04	5.66	4.00E-04	6.85
hsa-miR-324-5p	2.28E-03	5.17	0.0012	5.56
hsa-miR-330-3p	4.43E-03	3.60	0.0016	4.19
hsa-miR-331-3p	3.19E-02	4.60	0.0227	5.24
hsa-miR-335*	3.04E-03	4.62	0.002	4.64
hsa-miR-337-5p	2.37E-02	3.10	0.0108	3.74
hsa-miR-338-5P	2.41E-04	136.49	3.00E-04	137.17

Continued Control vs. HVL

hsa-miR-339-5p	3.72E-03	3.32	0.0016	4.22
hsa-miR-33a*	1.81E-02	2.47	0.0052	3.12
hsa-miR-342-3p	4.08E-02	6.91	0.0319	7.61
hsa-miR-342-5p	9.42E-05	9.71	1.00E-04	11.30
hsa-miR-346	1.70E-06	22.98	<9E-05	39.30
hsa-miR-34a*	4.02E-02	3.04	0.0435	3.07
hsa-miR-34a	1.53E-05	20.68	<9E-05	20.78
hsa-miR-34b	4.29E-04	3.24	3.00E-04	3.26
hsa-miR-361-3p	1.30E-02	3.75	0.0097	4.53
hsa-miR-361-5p	2.62E-02	2.81	0.0062	3.61
hsa-miR-362-5p	4.09E-02	2.87	0.0256	3.08
hsa-miR-363	4.91E-03	3.35	0.0028	3.69
hsa-miR-378	1.21E-04	6.37	1.00E-04	6.40
hsa-miR-380-5p	1.90E-03	7.67	0.0016	8.23
hsa-miR-381	9.03E-05	7.34	<9E-05	9.05
hsa-miR-382	5.82E-04	7.74	4.00E-04	8.25
hsa-miR-424	1.45E-03	4.82	3.00E-04	5.73
hsa-miR-425	7.25E-03	6.52	0.0057	6.56
hsa-miR-433	1.70E-06	14.28	<1E-05	16.55
hsa-miR-451	1.19E-02	-4.36	0.0118	-4.34
hsa-miR-483-5p	2.05E-07	17.09	<1E-05	18.24
hsa-miR-486-3p	4.08E-02	3.36	0.0352	3.38
hsa-miR-487a	4.02E-02	2.51	0.0357	2.52
hsa-miR-490-3p	5.64E-06	18.24	<9E-05	21.19
hsa-miR-491-5p	2.77E-03	3.05	0.0014	3.14
hsa-miR-500	1.76E-04	3.93	1.00E-04	4.12
hsa-miR-501-5p	3.32E-03	5.66	0.0018	6.13
hsa-miR-502-5p	1.85E-03	3.47	5.00E-04	4.74
hsa-miR-505	3.36E-02	3.31	0.024	3.64
hsa-miR-509-5p	5.87E-03	5.46	0.0022	7.30
hsa-miR-511	1.38E-04	8.24	<9E-05	14.85
hsa-miR-512-3p	2.98E-02	3.94	8.00E-04	11.35
hsa-miR-516-3p	3.38E-02	2.95	0.0438	2.86
hsa-miR-516b	1.75E-06	10.10	<9E-05	9.22
hsa-miR-518b	1.54E-11	32.73	<9E-05	37.28
hsa-miR-518d-3p	4.91E-03	4.87	0.0039	5.32
hsa-miR-518e	8.69E-03	4.28	0.0083	4.31
hsa-miR-541	8.65E-03	6.52	0.0117	5.49
hsa-miR-551b*	2.25E-02	2.37	0.0053	3.25
hsa-miR-564	1.70E-06	11.67	<9E-05	11.73

Continued Control vs. HVL

hsa-miR-571	1.45E-07	23.50	<9E-05	23.62
hsa-miR-572	2.28E-03	5.38	5.00E-04	8.54
hsa-miR-575	6.71E-03	6.34	0.0119	6.11
hsa-miR-579	1.81E-02	2.87	0.0093	3.83
hsa-miR-584	5.32E-05	34.84	1.00E-04	35.01
hsa-miR-589	9.02E-03	3.41	0.0039	3.81
hsa-miR-596	3.95E-05	4.74	<9E-05	4.76
hsa-miR-598	1.19E-02	4.06	0.0094	4.08
hsa-miR-605	1.27E-08	29.06	<9E-05	32.75
hsa-miR-615-3p	2.79E-02	4.81	<9E-05	51.71
hsa-miR-622	1.82E-02	6.32	4.00E-04	18.54
hsa-miR-623	3.78E-08	35.24	<9E-05	35.41
hsa-miR-636	3.25E-02	3.05	0.0271	3.06
hsa-miR-638	1.84E-05	14.71	<9E-05	21.19
hsa-miR-639	1.53E-03	5.45	0.0011	5.48
hsa-miR-642	7.47E-05	3.65	<9E-05	4.90
hsa-miR-643	1.95E-03	6.81	0.0015	6.85
hsa-miR-644	1.61E-02	4.02	0.0083	6.16
hsa-miR-650	1.47E-05	18.04	<9E-05	20.29
hsa-miR-652	5.30E-04	6.07	4.00E-04	6.10
hsa-miR-654-3p	3.17E-02	3.46	0.0047	5.29
hsa-miR-655	1.58E-02	6.57	0.0271	6.69
hsa-miR-659	1.97E-04	25.33	2.00E-04	27.59
hsa-miR-660	7.78E-03	6.45	0.0062	6.48
hsa-miR-661	2.09E-05	12.97	<9E-05	17.85
hsa-miR-668	4.32E-03	-4.47	0.048	-3.34
hsa-miR-671-3p	4.08E-02	2.61	0.0152	3.43
hsa-miR-672	4.47E-07	21.24	<9E-05	22.23
hsa-miR-744*	1.58E-03	4.24	5.00E-04	5.20
hsa-miR-766	2.14E-02	2.02	0.0114	2.03
hsa-miR-769-5p	1.08E-02	3.04	0.0038	3.82
hsa-miR-770-5p	1.27E-03	7.69	1.00E-04	19.19
hsa-miR-886-3p	7.68E-03	4.26	0.0118	5.17
hsa-miR-9*	4.49E-02	1.99	0.0118	2.48
hsa-miR-9	9.42E-05	10.66	<9E-05	12.40
hsa-miR-92a	4.29E-03	6.82	0.0034	6.85
hsa-miR-93*	5.51E-03	3.27	0.0036	3.29
hsa-miR-93	1.00E-02	4.21	0.0077	4.23
hsa-miR-935	1.79E-08	-22.43	<9E-05	-75.76
hsa-miR-938	2.19E-04	10.36	0.0204	5.46

Continued Control vs. HVL

hsa-miR-939	2.57E-05	37.01	<9E-05	37.20
hsa-miR-99b*	3.46E-04	5.69	3.00E-04	5.72
rno-miR-29c*	1.52E-02	3.09	0.0062	3.62
rno-miR-7*	9.81E-03	5.92	0.008	5.95

A.3 LVL VS. HVL

LVL vs. HVL				
	Realtime StatMiner		DataAssist	
	p-value	Fold Change	p-value	Fold Change
dme-miR-7	3.62E-03	4.10	0.006	4.95
has-miR-1305	7.97E-12	6460.41	<9E-05	6811.67
has-miR-155	8.08E-07	3.24	<9E-05	3.25
hsa-miR-103	1.85E-02	2.53	0.02	2.54
hsa-miR-106b	2.01E-02	2.43	0.0209	2.44
hsa-miR-107	1.95E-02	3.36	0.0204	3.51
hsa-miR-10a	1.59E-04	5.46	2.00E-04	5.47
hsa-miR-120	8.73E-03	2.96	0.0099	2.97
hsa-miR-1225-3P	3.09E-09	18.30	<9E-05	18.36
hsa-miR-1227	2.01E-05	4.86	<9E-05	4.87
hsa-miR-1233	1.22E-02	3.21	0.0137	3.22
hsa-miR-124	5.07E-03	10.59	0.0085	10.62
hsa-miR-1247	3.31E-04	37.27	5.00E-04	40.96
hsa-miR-1253	8.31E-03	4.80	0.0113	4.81
hsa-miR-1254	1.39E-02	3.19	0.016	3.20
hsa-miR-1262	3.40E-02	18.54	0.0446	18.60
hsa-miR-1267	1.25E-02	-69.08	0.0172	-68.97
hsa-miR-127-3p	1.93E-02	3.69	0.0191	3.99
hsa-miR-1270	3.25E-04	8.97	3.00E-04	9.77
hsa-miR-1271	1.65E-02	2.14	0.0117	2.26
hsa-miR-1274B	9.93E-03	2.52	0.0105	2.52
hsa-miR-1275	1.82E-02	2.73	0.0192	2.74
hsa-miR-1276	2.70E-02	3.23	0.0318	3.24
hsa-miR-1282	3.53E-05	11.33	<9E-05	17.03

Continued LVL vs. HVL

hsa-miR-1303	1.18E-04	18.47	0.0014	28.20
hsa-miR-130a	1.52E-02	3.46	0.0185	3.47
hsa-miR-130b*	6.47E-04	-9.05	9.00E-04	-9.02
hsa-miR-130b	1.52E-02	2.32	0.0117	2.44
hsa-miR-132	8.80E-09	7.86	<9E-05	8.66
hsa-miR-134	1.09E-03	-7.85	0.0074	-6.43
hsa-miR-135b	4.00E-03	3.27	0.0035	3.58
hsa-miR-140-5p	3.38E-02	2.24	0.035	2.25
hsa-miR-142-3p	2.20E-03	4.10	0.0026	4.11
hsa-miR-144*	1.22E-02	-3.96	0.0157	-3.94
hsa-miR-144	2.41E-04	-21.77	3.00E-04	-25.97
hsa-miR-146a	2.28E-02	1.84	0.018	1.85
hsa-miR-146b-3p	9.63E-04	5.38	8.00E-04	6.56
hsa-miR-148b	1.64E-02	2.34	0.0146	2.43
hsa-miR-151-5P	4.22E-02	1.93	0.0394	1.93
hsa-miR-152	5.44E-04	4.23	7.00E-04	4.25
hsa-miR-15a*	4.78E-03	-3.23	0.0024	-3.70
hsa-miR-181a	1.53E-03	3.16	0.0017	3.17
hsa-miR-181c	4.27E-02	2.29	0.0463	2.30
hsa-miR-1825	1.22E-02	18.09	0.0161	18.15
hsa-miR-1826	3.40E-02	-2.76	0.0188	-2.75
hsa-miR-191	5.81E-05	-2.35	<9E-05	-2.35
hsa-miR-193a-5p	2.56E-04	9.87	6.00E-04	10.89
hsa-miR-193b	1.22E-02	1.91	0.0088	1.91
hsa-miR-194	3.96E-02	2.51	0.0441	2.51
hsa-miR-196b	4.78E-03	-2.32	0.0036	-2.31
hsa-miR-202*	3.40E-02	-2.57	0.0182	-2.56
hsa-miR-202	2.89E-02	3.97	0.0363	3.98
hsa-miR-21	2.99E-05	5.82	<9E-05	5.84
hsa-miR-210	2.76E-03	3.60	0.0028	3.61
hsa-miR-211	4.27E-02	2.92	0.0028	7.27
hsa-miR-212	1.19E-05	5.36	<9E-05	5.82
hsa-miR-219-5p	4.58E-03	11.46	0.0054	9.45
hsa-miR-22	2.12E-04	10.53	5.00E-04	10.41
hsa-miR-221	1.56E-02	3.94	0.0199	3.96
hsa-miR-222	1.29E-04	2.86	1.00E-04	2.87
hsa-miR-223	2.66E-02	-2.15	0.0279	-2.15
hsa-miR-224	3.71E-02	3.27	0.0403	3.54
hsa-miR-24-2*	1.95E-02	2.79	0.0411	2.71
hsa-miR-24	4.48E-02	1.73	0.0377	1.73

Continued LVL vs. HVL

hsa-miR-25	7.81E-03	3.17	0.0085	3.18
hsa-miR-27a*	4.43E-03	2.09	0.0024	2.09
hsa-miR-27a	1.19E-02	2.90	0.0131	2.91
hsa-miR-28-3p	9.74E-03	1.96	0.0066	1.97
hsa-miR-28-5p	8.08E-03	2.94	0.0095	2.95
hsa-miR-298	2.81E-02	9.82	0.016	8.35
hsa-miR-29b-1*	1.38E-03	5.83	1.00E-04	10.01
hsa-miR-29b-2*	8.73E-03	8.44	9.00E-04	16.92
hsa-miR-29b	1.82E-02	3.67	0.0221	3.68
hsa-miR-302a*	3.69E-02	3.47	0.0265	4.25
hsa-miR-302c*	1.32E-04	46.73	1.00E-04	70.20
hsa-miR-30b	1.74E-02	2.55	0.0191	2.56
hsa-miR-30c	1.82E-02	2.38	0.0195	2.38
hsa-miR-31*	3.55E-03	-3.60	0.0064	-3.66
hsa-miR-320B	3.27E-02	3.66	0.0403	3.67
hsa-miR-324-3p	1.34E-03	3.80	0.0017	3.81
hsa-miR-324-5p	3.27E-03	4.81	0.0039	5.12
hsa-miR-328	1.10E-02	3.46	0.0133	3.47
hsa-miR-330-3p	7.46E-04	3.50	8.00E-04	3.52
hsa-miR-331-3p	1.16E-02	2.28	0.0117	2.29
hsa-miR-339-3p	1.42E-02	1.78	0.0085	1.79
hsa-miR-339-5p	4.57E-06	5.43	<9E-05	5.45
hsa-miR-33a*	3.27E-02	2.79	0.0249	3.11
hsa-miR-33a	1.22E-02	-4.88	0.016	-4.86
hsa-miR-342-5p	5.61E-06	8.08	<9E-05	8.11
hsa-miR-346	8.74E-06	19.77	<9E-05	37.83
hsa-miR-34a	5.76E-04	10.68	8.00E-04	12.59
hsa-miR-34b	1.82E-02	3.00	0.0182	3.24
hsa-miR-361-3p	6.85E-03	5.24	0.0121	4.88
hsa-miR-363	4.34E-02	2.25	0.0376	2.40
hsa-miR-381	1.02E-03	5.95	0.0013	5.97
hsa-miR-382	2.83E-04	10.04	4.00E-04	10.08
hsa-miR-422a	2.99E-02	-1.86	0.0267	-1.85
hsa-miR-425	4.44E-02	1.87	0.0411	1.88
hsa-miR-433	4.58E-05	12.25	<9E-05	15.91
hsa-miR-454*	1.82E-02	-2.01	0.0307	-1.86
hsa-miR-483-3p	2.59E-03	8.69	0.0013	26.74
hsa-miR-483-5p	1.25E-02	2.86	0.0136	2.87
hsa-miR-490-3p	2.04E-04	10.47	1.00E-04	12.39
hsa-miR-491-5p	1.10E-02	3.40	0.0135	3.41

Continued LVL vs. HVL

hsa-miR-500	9.74E-03	2.39	0.0065	2.58
hsa-miR-501-5p	2.32E-02	3.24	0.0283	3.25
hsa-miR-502-5p	1.82E-02	2.53	0.0102	3.44
hsa-miR-503	7.88E-03	-5.29	0.018	-10.30
hsa-miR-505	3.25E-04	9.95	1.00E-04	15.80
hsa-miR-509-5p	1.16E-02	6.19	0.0058	8.64
hsa-miR-511	1.21E-03	7.18	<9E-05	17.56
hsa-miR-512-3p	8.29E-03	5.95	<9E-05	18.66
hsa-miR-516-3p	1.17E-06	9.90	<9E-05	9.93
hsa-miR-518b	2.01E-05	14.99	<9E-05	15.04
hsa-miR-518d-3p	3.67E-03	5.47	0.0035	7.71
hsa-miR-518e	1.45E-03	6.34	0.0018	6.36
hsa-miR-519b-3p	1.18E-03	198.48	0.0021	199.13
hsa-miR-520D-3P	3.73E-02	-51.72	0.0471	-51.55
hsa-miR-523	1.25E-02	-8.74	0.0207	-8.58
hsa-miR-532-3p	3.88E-02	2.04	0.0393	2.04
hsa-miR-541*	2.67E-03	13.56	8.00E-04	12.52
hsa-miR-545*	1.16E-02	-3.40	0.02	-3.08
hsa-miR-550	7.80E-03	-2.73	0.0084	-2.72
hsa-miR-564	3.06E-03	4.73	0.004	4.75
hsa-miR-571	5.01E-05	26.35	1.00E-04	26.43
hsa-miR-572	4.17E-02	4.21	0.0449	4.81
hsa-miR-579	7.93E-03	4.45	0.005	6.86
hsa-miR-589	4.49E-02	2.25	0.0468	2.26
hsa-miR-590-3P	1.12E-06	-6.15	<9E-05	-6.13
hsa-miR-596	3.78E-03	3.48	0.0043	3.49
hsa-miR-598	3.46E-02	2.42	0.0392	2.43
hsa-miR-605	5.54E-08	24.96	<9E-05	28.35
hsa-miR-623	2.15E-05	18.51	<9E-05	19.76
hsa-miR-624	2.81E-02	-3.15	0.0036	-3.16
hsa-miR-628-5p	1.85E-02	-4.08	0.0229	-4.06
hsa-miR-636	1.45E-04	4.89	1.00E-04	4.91
hsa-miR-638	3.53E-05	18.77	1.00E-04	18.83
hsa-miR-639	1.45E-02	5.35	0.0188	5.37
hsa-miR-642	3.53E-05	3.96	<9E-05	4.41
hsa-miR-643	5.61E-06	21.78	<9E-05	24.22
hsa-miR-648	1.64E-02	5.85	0.0112	8.46
hsa-miR-649	2.85E-03	-9.87	0.0419	-6.74
hsa-miR-650	1.85E-02	3.06	0.0205	3.07
hsa-miR-652	5.76E-04	4.91	8.00E-04	4.93

Continued LVL vs. HVL

hsa-miR-659	5.61E-06	29.90	<9E-05	31.51
hsa-miR-660	2.33E-02	2.83	0.0267	2.84
hsa-miR-661	1.95E-02	3.95	0.0242	3.97
hsa-miR-672	8.02E-07	17.80	<9E-05	19.98
hsa-miR-720	3.45E-04	-3.00	4.00E-04	-2.99
hsa-miR-769-5p	2.42E-02	2.73	0.0282	2.74
hsa-miR-770-5p	2.58E-02	4.60	0.0169	6.91
hsa-miR-9	1.74E-04	7.41	1.00E-04	9.26
hsa-miR-922	5.98E-05	-5.13	0.0205	-7.91
hsa-miR-92a	2.89E-02	2.30	0.0315	2.31
hsa-miR-93	3.46E-02	2.62	0.0402	2.62
hsa-miR-935	1.35E-05	-13.14	<9E-05	-64.10
hsa-miR-939	1.29E-03	5.37	0.0017	5.39
hsa-miR-95	1.10E-02	2.71	0.0117	2.72

APPENDIX B

SIGNIFCANTLY DIFFERENTIALLY REGULATED MRNA

B.1 CONTROL VS. LVL

Gene Symbol	Fold change	p-value	Gene Symbol	Fold change	p-value
LAG3	1.03	3.16E-02	PDE5A	-0.77	4.71E-02
CD8A	0.95	2.10E-02	KRT73	-0.72	2.10E-02
CD8B	0.84	1.59E-02	COLQ	-0.65	2.81E-02
CCL4L2	0.83	4.42E-02	RNF165	-0.62	1.59E-02
APOBEC3G	0.81	1.82E-02	HGD	-0.57	3.16E-02
APOBEC3H	0.76	1.59E-02	LTB4R	-0.50	3.46E-02
PPP2R5C	0.63	4.42E-02	COLQ	-0.49	4.26E-02
SCD	0.61	4.27E-02	MLST8	-0.36	3.71E-02
APOL6	0.58	4.26E-02	FASTK	-0.31	7.51E-03
KIAA1671	0.56	4.42E-02	DDX28	-0.26	4.71E-02
HDGFRP3	0.52	2.95E-02			
RAB27A	0.50	4.42E-02			
CDCA7	0.49	1.98E-02			
SERPINB9	0.49	4.42E-02			
UEVLD	0.48	3.46E-02			
B3GNT2	0.48	3.86E-02			
MAPK14	0.48	2.10E-02			
RAB28	0.47	4.26E-02			
TNFRSF9	0.45	1.59E-02			
GALK2	0.44	4.16E-02			
RPL29	0.44	4.42E-02			
PCGF6	0.43	2.10E-02			
UBE2G1	0.43	4.27E-02			

Continued Control vs. LVL

REEP3	0.35	3.46E-02
HDLBP	0.34	3.68E-02
GCLM	0.34	4.16E-02
TMEM110	0.33	4.16E-02
RCAN1	0.32	1.59E-02
PPP2R3C	0.31	3.72E-02
IL12A	0.30	4.16E-02
NIPA2	0.29	4.42E-02
DDX28	-0.26	4.71E-02
FASTK	-0.31	7.51E-03
MLST8	-0.36	3.71E-02
COLQ	-0.49	4.26E-02
LTB4R	-0.50	3.46E-02
HGD	-0.57	3.16E-02
RNF165	-0.62	1.59E-02
COLQ	-0.65	2.81E-02
KRT73	-0.72	2.10E-02
PDE5A	-0.77	4.71E-02

B.2 CONTROL VS. HVL

Gene Symbol	Fold change	p-value	Gene Symbol	Fold change	p-value
INDO	5.18	1.44E-23	DEFA1	-5.60	2.22E-16
IFI27	5.17	4.61E-21	DEFA3	-5.28	5.88E-14
CCL8	5.11	4.31E-15	CPVL	-4.91	1.15E-21
MT1G	4.93	2.01E-16	FCN1	-4.84	4.11E-24
SERPINB2	4.76	3.04E-19	FCER1A	-4.70	6.28E-30
CXCL5	4.58	6.33E-15	CSF3R	-4.47	3.09E-31
C15orf48	4.55	1.34E-17	SEPT5	-4.40	3.55E-27
CCL2	4.53	6.48E-11	FOSB	-4.20	2.55E-22
CXCL5	4.53	1.11E-15	MS4A6A	-4.15	2.55E-22
SERPINB2	4.42	2.21E-19	CFD	-4.08	1.19E-18
MT1H	4.39	4.27E-14	DEFA1B	-4.06	1.20E-13
ADAMDEC1	4.07	6.88E-21	NRGN	-4.03	1.70E-29
IL-6	4.01	2.24E-14	TUBB1	-4.00	1.16E-27
CTSL1	3.98	1.42E-16	LST1	-4.00	2.82E-19

Continued Control vs. HVL

IL1A	3.95	2.73E-17	DEFA1B	-3.97	6.83E-13
OLR1	3.80	2.00E-12	F13A1	-3.90	1.64E-27
CTSL1	3.74	8.40E-17	NFE2	-3.88	6.53E-26
PLA2G7	3.62	9.92E-18	S100P	-3.86	5.31E-18
CTSL1	3.62	2.52E-14	CD1D	-3.86	1.11E-27
CCL7	3.61	3.01E-07	CAMP	-3.82	4.19E-19
IFI44L	3.51	1.08E-17	ASGR1	-3.81	3.89E-19
CCL22	3.46	3.42E-26	FOS	-3.77	5.28E-16
RGL1	3.38	3.02E-18	CLC	-3.76	6.17E-13
CCL3	3.36	4.11E-16	MS4A6A	-3.69	7.80E-16
C15orf48	3.36	5.81E-18	ZNF467	-3.60	1.59E-21
MT1M	3.36	1.22E-10	FLJ22662	-3.56	4.68E-22
RSAD2	3.34	4.21E-20	SDPR	-3.54	9.46E-25
PMP22	3.27	1.01E-11	FCGR3B	-3.51	2.16E-14
CXCL10	3.22	3.71E-12	C10orf54	-3.49	1.25E-19
SLC7A11	3.21	5.39E-22	GP9	-3.43	2.74E-26
CCL3L1	3.16	7.48E-15	PADI4	-3.41	8.34E-27
IL1B	3.11	2.84E-11	CMTM2	-3.39	8.79E-15
CCL3L1	3.06	5.14E-12	FOLR3	-3.36	2.70E-19
LAMP3	3.04	1.85E-27	SIGLEC10	-3.33	8.65E-22
ADAMDEC1	3.01	5.11E-18	FPR1	-3.32	1.49E-17
CCL20	2.97	2.90E-10	PRAM1	-3.29	6.78E-27
CXCL2	2.97	2.99E-12	ASGR2	-3.29	6.48E-25
MMP9	2.96	1.01E-06	KLF4	-3.21	6.53E-26
HSPA1B	2.91	9.26E-22	MS4A6A	-3.19	6.53E-16
TNFAIP6	2.89	2.03E-12	GPBAR1	-3.19	7.85E-25
CCL3L3	2.86	8.83E-14	LST1	-3.16	4.83E-19
EPSTI1	2.81	1.21E-18	SULF2	-3.15	6.82E-24
GPNMB	2.79	1.01E-08	CD36	-3.08	4.08E-11
DFNA5	2.76	1.26E-19	LYZ	-3.07	2.33E-14
KIAA1199	2.74	1.44E-10	SLC40A1	-3.00	5.16E-26
IFNG	2.73	6.28E-08	CD302	-2.95	6.26E-22
MT1E	2.73	1.97E-11	ALDH2	-2.92	3.27E-18
IL24	2.72	7.88E-10	S100A12	-2.90	2.24E-16
GPR68	2.69	9.42E-23	UBE2L3	-2.89	1.19E-21
GBP1	2.68	1.76E-16	ASGR2	-2.88	4.23E-24
KYNU	2.67	6.25E-18	GNG11	-2.84	1.45E-18
FPR3	2.67	7.83E-08	ACRBP	-2.83	3.68E-21
EBI3	2.66	4.44E-14	CLEC7A	-2.82	1.17E-13
PMP22	2.65	1.70E-16	DYSF	-2.80	6.59E-20

Continued Control vs. HVL

EMP1	2.62	1.09E-10	RNASE2	-2.80	7.62E-19
KYNU	2.62	1.59E-14	LGALS2	-2.79	4.65E-17
TXN	2.59	3.57E-15	CSF3R	-2.79	3.15E-21
RN7SK	2.59	7.79E-24	CD36	-2.78	6.56E-10
ANKRD22	2.58	5.37E-13	CD4	-2.77	3.00E-25
CCL4L2	2.55	6.24E-16	KBTBD11	-2.76	6.47E-21
EOMES	2.53	5.23E-16	IMPA2	-2.72	7.50E-20
GBP1	2.50	3.71E-15	EGR1	-2.71	4.35E-21
TNIP3	2.49	9.72E-16	MTMR11	-2.71	3.69E-21
CYP1B1	2.47	7.42E-12	LILRA2	-2.71	1.49E-12
SLC16A10	2.45	7.84E-11	VCAN	-2.70	2.73E-09
IL4I1	2.45	5.52E-15	MMP25	-2.69	7.56E-20
SLC39A8	2.40	4.54E-16	HDC	-2.68	1.78E-17
MT1A	2.39	7.66E-12	AIF1	-2.67	7.62E-12
IL19	2.36	2.73E-10	CLEC7A	-2.65	3.39E-12
MT2A	2.34	1.45E-14	CD33	-2.64	2.56E-16
TXN	2.33	3.85E-21	CST3	-2.62	6.91E-14
TBC1D2B	2.33	1.27E-22	AIF1	-2.61	3.24E-11
OAS2	2.31	8.35E-18	IL8RB	-2.61	3.71E-12
CLK1	2.31	7.05E-23	ALDH1A1	-2.60	3.43E-17
FASLG	2.31	4.57E-22	P2RY13	-2.60	1.05E-19
LAG3	2.29	1.76E-12	TSPAN32	-2.58	1.34E-15
THBS1	2.29	2.20E-06	PI3	-2.57	3.90E-09
GK	2.28	2.43E-15	LOC338758	-2.56	2.59E-20
OASL	2.27	1.06E-13	PTCRA	-2.56	1.49E-20
CCL13	2.27	3.68E-07	CLEC12A	-2.55	5.52E-11
TMEM51	2.26	5.11E-12	KLF11	-2.55	1.42E-16
IL19	2.26	3.33E-09	GARNL4	-2.55	2.63E-20
IFI44	2.26	2.08E-19	CEBPD	-2.54	4.76E-22
PTGR1	2.26	1.01E-16	PTGS1	-2.53	1.15E-18
FLJ14213	2.24	2.06E-17	LY86	-2.53	8.47E-21
LOC730249	2.22	5.67E-08	LILRA5	-2.53	2.24E-17
GK	2.22	5.28E-16	RGS18	-2.52	8.94E-11
CCL3L1	2.22	1.98E-07	CDA	-2.52	3.59E-24
PLSCR1	2.21	7.11E-16	LOC653600	-2.51	7.43E-09
MTE	2.21	2.23E-11	TSPAN18	-2.51	1.53E-25
CCL24	2.19	3.88E-09	CLEC1B	-2.49	1.93E-14
STAT1	2.15	1.41E-14	VENTX	-2.48	8.99E-24
HSPA1A	2.13	2.31E-17	MAL	-2.48	4.71E-15
ACTA2	2.12	7.45E-16	GAS7	-2.46	3.11E-13

Continued Control vs. HVL

SLC39A8	2.11	1.98E-07	TCEA3	-2.45	1.32E-19
RNASE1	2.09	4.50E-05	LILRA5	-2.45	1.40E-18
IFIH1	2.08	4.01E-21	TESC	-2.44	6.61E-21
ISG15	2.07	1.55E-12	RRP12	-2.42	3.01E-17
IFIT1	2.07	2.03E-08	TREM1	-2.42	1.18E-11
APOBEC3G	2.06	8.25E-18	CRIP2	-2.39	3.92E-21
TIGIT	2.06	4.59E-17	G0S2	-2.38	1.29E-10
APOBEC3G	2.03	3.02E-18	CD300LF	-2.37	1.81E-13
CD8A	2.03	2.45E-14	MME	-2.37	4.34E-10
NPC1	2.03	1.98E-19	PELI2	-2.36	1.52E-19
GBP4	2.03	5.22E-18	ZNF185	-2.35	5.24E-27
SC4MOL	2.03	1.43E-28	MPL	-2.35	2.64E-16
VCAM1	2.01	1.82E-15	RBP7	-2.34	6.78E-27
MT1X	2.01	1.91E-09	CRISPLD2	-2.34	1.62E-17
IL-8	2.00	1.08E-06	ALAS2	-2.34	5.67E-08
BCL11A	-2.01	1.98E-19	TSPAN9	-2.33	3.78E-16
UBXN11	-2.01	3.04E-17	MGST2	-2.33	1.13E-14
PYGL	-2.01	3.49E-12	PLEKHG3	-2.33	1.34E-15
EMR3	-2.03	1.09E-12	MGC13057	-2.33	2.31E-18
FCER2	-2.03	4.66E-14	MGC33556	-2.32	2.80E-20
VIPR1	-2.03	1.31E-21	H3F3B	-2.32	4.57E-22
CD93	-2.03	1.48E-06	MNDA	-2.31	5.05E-10
RASGRP2	-2.04	3.43E-14	CTTN	-2.31	8.21E-19
CACNA2D3	-2.04	8.30E-17	NCR3	-2.30	8.18E-22
NLRP12	-2.04	5.26E-19	DEFA4	-2.29	1.77E-08
GPBAR1	-2.04	1.02E-17	CDH23	-2.29	9.14E-24
PROK2	-2.05	5.29E-08	ZNF101	-2.27	2.13E-18
SERPINA1	-2.05	4.39E-07	LOC255809	-2.27	3.00E-25
OAF	-2.06	2.53E-16	C22orf32	-2.26	3.75E-23
SELL	-2.06	4.63E-11	TMEM154	-2.24	8.83E-16
CTSS	-2.07	4.07E-11	NACC2	-2.24	6.30E-19
CREB5	-2.08	8.29E-23	ABL1	-2.24	5.88E-26
BST1	-2.08	2.28E-15	PGLYRP1	-2.24	5.85E-12
PECAM1	-2.09	1.27E-16	GRASP	-2.24	2.19E-16
KRT72	-2.10	4.23E-10	SERPINA1	-2.23	1.15E-10
HK3	-2.10	1.78E-14	TCN1	-2.23	2.07E-10
S100A4	-2.10	1.08E-16	CLEC12A	-2.22	2.23E-12
ALDH1A1	-2.11	9.11E-19	LCN2	-2.22	3.17E-17
IRS2	-2.12	1.55E-22	PTGDS	-2.22	2.72E-19
C1orf162	-2.13	3.21E-16	WWP2	-2.22	8.84E-16

Continued Control vs. HVL

MS4A3	-2.13	1.21E-14	ETNK1	-2.21	1.79E-17
TOP1MT	-2.13	5.12E-19	FGL2	-2.21	5.66E-09
ESAM	-2.14	1.72E-19	VNN2	-2.20	4.24E-06
ADAM15	-2.17	9.82E-22	ITGAM	-2.20	4.81E-15
SULF2	-2.18	2.54E-26	S100A8	-2.20	6.05E-14
TMEM91	-2.19	1.03E-18	SH3BGRL2	-2.20	1.58E-22
SH3BGRL2	-2.20	1.58E-22	TMEM91	-2.19	1.03E-18
S100A8	-2.20	6.05E-14	SULF2	-2.18	2.54E-26
ITGAM	-2.20	4.81E-15	ADAM15	-2.17	9.82E-22
VNN2	-2.20	4.24E-06	ESAM	-2.14	1.72E-19
FGL2	-2.21	5.66E-09	TOP1MT	-2.13	5.12E-19
ETNK1	-2.21	1.79E-17	MS4A3	-2.13	1.21E-14
WWP2	-2.22	8.84E-16	C1orf162	-2.13	3.21E-16
PTGDS	-2.22	2.72E-19	IRS2	-2.12	1.55E-22
LCN2	-2.22	3.17E-17	ALDH1A1	-2.11	9.11E-19
CLEC12A	-2.22	2.23E-12	S100A4	-2.10	1.08E-16
TCN1	-2.23	2.07E-10	HK3	-2.10	1.78E-14
SERPINA1	-2.23	1.15E-10	KRT72	-2.10	4.23E-10
GRASP	-2.24	2.19E-16	PECAM1	-2.09	1.27E-16
PGLYRP1	-2.24	5.85E-12	BST1	-2.08	2.28E-15
ABL1	-2.24	5.88E-26	CREB5	-2.08	8.29E-23
NACC2	-2.24	6.30E-19	CTSS	-2.07	4.07E-11
TMEM154	-2.24	8.83E-16	SELL	-2.06	4.63E-11
C22orf32	-2.26	3.75E-23	OAF	-2.06	2.53E-16
LOC255809	-2.27	3.00E-25	SERPINA1	-2.05	4.39E-07
ZNF101	-2.27	2.13E-18	PROK2	-2.05	5.29E-08
CDH23	-2.29	9.14E-24	GPBAR1	-2.04	1.02E-17
DEFA4	-2.29	1.77E-08	NLRP12	-2.04	5.26E-19
NCR3	-2.30	8.18E-22	CACNA2D3	-2.04	8.30E-17
CTTN	-2.31	8.21E-19	RASGRP2	-2.04	3.43E-14
MNDA	-2.31	5.05E-10	CD93	-2.03	1.48E-06
H3F3B	-2.32	4.57E-22	VIPR1	-2.03	1.31E-21
MGC33556	-2.32	2.80E-20	FCER2	-2.03	4.66E-14
MGC13057	-2.33	2.31E-18	EMR3	-2.03	1.09E-12
PLEKHG3	-2.33	1.34E-15	PYGL	-2.01	3.49E-12
MGST2	-2.33	1.13E-14	UBXN11	-2.01	3.04E-17
TSPAN9	-2.33	3.78E-16	BCL11A	-2.01	1.98E-19
ALAS2	-2.34	5.67E-08			
CRISPLD2	-2.34	1.62E-17			
RBP7	-2.34	6.78E-27			

Continued Control vs. HVL

MPL	-2.35	2.64E-16
ZNF185	-2.35	5.24E-27
PELI2	-2.36	1.52E-19
MME	-2.37	4.34E-10
CD300LF	-2.37	1.81E-13
G0S2	-2.38	1.29E-10
CRIP2	-2.39	3.92E-21
TREM1	-2.42	1.18E-11
RRP12	-2.42	3.01E-17
TESC	-2.44	6.61E-21
LILRA5	-2.45	1.40E-18
TCEA3	-2.45	1.32E-19
GAS7	-2.46	3.11E-13
MAL	-2.48	4.71E-15
VENTX	-2.48	8.99E-24
CLEC1B	-2.49	1.93E-14
TSPAN18	-2.51	1.53E-25
LOC653600	-2.51	7.43E-09
CDA	-2.52	3.59E-24
RGS18	-2.52	8.94E-11
LILRA5	-2.53	2.24E-17
LY86	-2.53	8.47E-21
PTGS1	-2.53	1.15E-18
CEBPD	-2.54	4.76E-22
GARNL4	-2.55	2.63E-20
KLF11	-2.55	1.42E-16
CLEC12A	-2.55	5.52E-11
PTCRA	-2.56	1.49E-20
LOC338758	-2.56	2.59E-20
PI3	-2.57	3.90E-09
TSPAN32	-2.58	1.34E-15
P2RY13	-2.60	1.05E-19
ALDH1A1	-2.60	3.43E-17
IL8RB	-2.61	3.71E-12
AIF1	-2.61	3.24E-11
CST3	-2.62	6.91E-14
CD33	-2.64	2.56E-16
CLEC7A	-2.65	3.39E-12
AIF1	-2.67	7.62E-12
HDC	-2.68	1.78E-17

Continued Control vs. HVL

MMP25	-2.69	7.56E-20
VCAN	-2.70	2.73E-09
LILRA2	-2.71	1.49E-12
MTMR11	-2.71	3.69E-21
EGR1	-2.71	4.35E-21
IMPA2	-2.72	7.50E-20
KBTBD11	-2.76	6.47E-21
CD4	-2.77	3.00E-25
CD36	-2.78	6.56E-10
CSF3R	-2.79	3.15E-21
LGALS2	-2.79	4.65E-17
RNASE2	-2.80	7.62E-19
DYSF	-2.80	6.59E-20
CLEC7A	-2.82	1.17E-13
ACRBP	-2.83	3.68E-21
GNG11	-2.84	1.45E-18
ASGR2	-2.88	4.23E-24
UBE2L3	-2.89	1.19E-21
S100A12	-2.90	2.24E-16
ALDH2	-2.92	3.27E-18
CD302	-2.95	6.26E-22
SLC40A1	-3.00	5.16E-26
LYZ	-3.07	2.33E-14
CD36	-3.08	4.08E-11
SULF2	-3.15	6.82E-24
LST1	-3.16	4.83E-19
GPBAR1	-3.19	7.85E-25
MS4A6A	-3.19	6.53E-16
KLF4	-3.21	6.53E-26
ASGR2	-3.29	6.48E-25
PRAM1	-3.29	6.78E-27
FPR1	-3.32	1.49E-17
SIGLEC10	-3.33	8.65E-22
FOLR3	-3.36	2.70E-19
CMTM2	-3.39	8.79E-15
PADI4	-3.41	8.34E-27
GP9	-3.43	2.74E-26
C10orf54	-3.49	1.25E-19
FCGR3B	-3.51	2.16E-14
SDPR	-3.54	9.46E-25

Continued Control vs. HVL

FLJ22662	-3.56	4.68E-22
ZNF467	-3.60	1.59E-21
MS4A6A	-3.69	7.80E-16
CLC	-3.76	6.17E-13
FOS	-3.77	5.28E-16
ASGR1	-3.81	3.89E-19
CAMP	-3.82	4.19E-19
CD1D	-3.86	1.11E-27
S100P	-3.86	5.31E-18
NFE2	-3.88	6.53E-26
F13A1	-3.90	1.64E-27
DEFA1B	-3.97	6.83E-13
LST1	-4.00	2.82E-19
TUBB1	-4.00	1.16E-27
NRGN	-4.03	1.70E-29
DEFA1B	-4.06	1.20E-13
CFD	-4.08	1.19E-18
MS4A6A	-4.15	2.55E-22
FOSB	-4.20	2.55E-22
SEPT5	-4.40	3.55E-27
CSF3R	-4.47	3.09E-31
FCER1A	-4.70	6.28E-30
FCN1	-4.84	4.11E-24
CPVL	-4.91	1.15E-21
DEFA3	-5.28	5.88E-14
DEFA1	-5.60	2.22E-16

B.3 LVL VS. HVL

Gene Symbol	Fold change	p-value	Gene Symbol	Fold change	p-value
IFI27	4.12	9.49E-11	DEFA1	-5.13	1.28E-07
CXCL5	4.07	2.43E-08	DEFA3	-5.12	9.67E-08
CXCL5	4.04	1.72E-08	CPVL	-4.61	1.08E-15
SERPINB2	3.96	8.28E-10	FCN1	-4.43	4.74E-14
CCL2	3.95	1.42E-05	DEFA1B	-4.07	2.99E-07
CCL8	3.89	5.18E-05	DEFA1B	-3.96	3.24E-07
MT1G	3.87	1.78E-06	FCER1A	-3.71	1.56E-11

Continued LVL vs. HVL

C15orf48	3.87	4.50E-08	MS4A6A	-3.71	1.38E-12
INDO	3.85	8.14E-07	LST1	-3.61	7.06E-14
SERPINB2	3.66	7.66E-10	CSF3R	-3.56	1.35E-13
IL1A	3.39	4.17E-09	FOSB	-3.56	1.39E-10
MT1H	3.29	2.15E-05	CFD	-3.55	3.42E-11
IL-6	3.28	1.71E-07	MS4A6A	-3.51	2.16E-11
CTSL1	3.20	9.18E-08	CD1D	-3.47	1.09E-13
ADAMDEC1	3.16	3.26E-08	ASGR1	-3.46	1.57E-13
CTSL1	3.01	1.45E-07	S100P	-3.41	5.84E-11
OLR1	3.00	6.66E-06	SEPT5	-3.37	4.37E-10
PLA2G7	2.84	5.02E-08	CAMP	-3.34	1.77E-08
CCL7	2.80	2.50E-03	FOS	-3.34	4.56E-10
RSAD2	2.78	1.71E-09	NFE2	-3.25	3.60E-12
CTSL1	2.75	4.21E-06	FLJ22662	-3.23	8.43E-13
SLC7A11	2.75	1.37E-10	ZNF467	-3.23	1.33E-14
C15orf48	2.75	1.42E-07	F13A1	-3.19	3.48E-13
CXCL2	2.70	2.24E-08	C10orf54	-3.09	2.33E-13
CCL20	2.68	2.05E-07	GPBAR1	-3.07	1.42E-14
CCL22	2.67	6.57E-07	NRGN	-3.06	5.42E-12
IFI44L	2.66	1.94E-08	KLF4	-3.04	1.91E-13
IL24	2.63	6.22E-08	FPR1	-3.02	8.09E-13
MT1M	2.61	1.62E-04	LOC653600	-3.00	9.45E-07
RGL1	2.57	1.47E-06	MS4A6A	-2.98	2.86E-09
RN7SK	2.55	5.75E-19	FOLR3	-2.97	2.80E-10
PMP22	2.51	7.10E-05	TUBB1	-2.97	1.27E-10
THBS1	2.50	2.58E-05	SIGLEC10	-2.96	6.92E-13
CCL3L1	2.49	1.30E-06	LYZ	-2.95	1.11E-11
CCL3L3	2.47	1.54E-07	S100A12	-2.91	1.97E-13
IL1B	2.47	4.20E-06	CLC	-2.91	3.52E-07
TNFAIP6	2.45	2.49E-07	DEFA4	-2.86	1.11E-07
CCL3	2.42	1.39E-07	CD36	-2.85	2.44E-07
CCL3L1	2.42	1.74E-07	FCGR3B	-2.84	3.52E-08
ADAMDEC1	2.39	9.90E-08	ASGR2	-2.83	1.03E-14
EMP1	2.38	1.02E-06	PADI4	-2.82	1.23E-11
LAMP3	2.38	6.62E-08	LST1	-2.81	7.33E-13
HSPA1B	2.33	6.78E-12	RNASE2	-2.77	1.55E-10
DFNA5	2.25	2.64E-09	PRAM1	-2.68	1.10E-12
IL19	2.17	1.80E-07	CD36	-2.64	4.17E-07
CYP1B1	2.14	8.13E-08	UBE2L3	-2.64	5.17E-12
MMP9	2.12	5.38E-03	CLEC12A	-2.64	4.94E-08

Continued LVL vs. HVL

KYNU	2.11	9.32E-10	GP9	-2.63	2.15E-10
EBI3	2.11	3.19E-06	ALDH2	-2.60	6.65E-13
IL19	2.11	5.37E-07	SULF2	-2.60	3.80E-13
PTGR1	2.11	3.10E-12	AIF1	-2.59	1.38E-10
KYNU	2.07	2.39E-07	LILRA2	-2.59	7.66E-11
PMP22	2.05	1.57E-06	CMTM2	-2.56	6.97E-07
TNIP3	2.05	3.84E-08	CD33	-2.54	8.47E-12
SLC16A10	2.03	7.50E-05	AIF1	-2.53	4.19E-11
GPNMB	2.03	6.82E-04	CD302	-2.53	2.59E-11
TXN	2.01	5.31E-09	SDPR	-2.51	8.07E-11
GPR68	2.01	3.97E-08	LGALS2	-2.51	2.33E-10
MGST2	-2.00	1.11E-08	DYSF	-2.49	1.32E-11
CLEC12A	-2.01	1.42E-06	P2RY13	-2.48	2.43E-14
IL8RB	-2.01	1.42E-06	CLEC7A	-2.48	9.44E-10
PGLYRP1	-2.02	1.81E-06	LILRA5	-2.45	3.15E-15
KLF10	-2.02	1.92E-09	ALDH1A1	-2.45	3.71E-09
GPBAR1	-2.03	3.79E-11	MTMR11	-2.40	8.52E-11
MGC33556	-2.04	3.15E-15	MNDA	-2.40	1.71E-09
LOC338758	-2.04	1.08E-08	CST3	-2.39	7.62E-10
GAS7	-2.04	1.11E-08	KBTBD11	-2.37	2.58E-14
ALDH1A1	-2.04	1.41E-08	CLEC7A	-2.35	3.86E-09
S100A8	-2.06	2.06E-10	CD4	-2.33	9.37E-14
PTCRA	-2.06	1.56E-11	SLC40A1	-2.33	2.64E-13
LY86	-2.07	1.05E-09	CRISPLD2	-2.28	6.31E-14
ACRBP	-2.07	3.35E-10	RNASE3	-2.26	1.62E-08
LOC255809	-2.08	5.42E-12	ASGR2	-2.25	9.40E-13
GNG11	-2.08	4.75E-08	PLEKHG3	-2.25	1.60E-13
PI3	-2.10	4.85E-06	VNN2	-2.25	3.10E-06
TSPAN32	-2.10	5.85E-09	LILRA5	-2.25	3.07E-13
KLF11	-2.11	1.19E-09	FGL2	-2.24	1.16E-07
G0S2	-2.12	9.91E-06	CDA	-2.23	4.84E-12
SERPINA1	-2.15	2.59E-11	FCGR1B	-2.22	7.04E-07
SERPINA1	-2.16	5.04E-09	VCAN	-2.19	1.66E-05
IMPA2	-2.17	1.16E-08	GARNL4	-2.19	8.15E-12
CSF3R	-2.17	4.48E-11	CD300LF	-2.19	2.39E-10
EGR1	-2.18	4.51E-09	TESC	-2.19	1.58E-11
TESC	-2.19	1.58E-11	EGR1	-2.18	4.51E-09
CD300LF	-2.19	2.39E-10	CSF3R	-2.17	4.48E-11
GARNL4	-2.19	8.15E-12	IMPA2	-2.17	1.16E-08
VCAN	-2.19	1.66E-05	SERPINA1	-2.16	5.04E-09

Continued LVL vs. HVL

FCGR1B	-2.22	7.04E-07	SERPINA1	-2.15	2.59E-11
CDA	-2.23	4.84E-12	G0S2	-2.12	9.91E-06
FGL2	-2.24	1.16E-07	KLF11	-2.11	1.19E-09
LILRA5	-2.25	3.07E-13	TSPAN32	-2.10	5.85E-09
VNN2	-2.25	3.10E-06	PI3	-2.10	4.85E-06
PLEKHG3	-2.25	1.60E-13	GNG11	-2.08	4.75E-08
ASGR2	-2.25	9.40E-13	LOC255809	-2.08	5.42E-12
RNASE3	-2.26	1.62E-08	ACRBP	-2.07	3.35E-10
CRISPLD2	-2.28	6.31E-14	LY86	-2.07	1.05E-09
SLC40A1	-2.33	2.64E-13	PTCRA	-2.06	1.56E-11
CD4	-2.33	9.37E-14	S100A8	-2.06	2.06E-10
CLEC7A	-2.35	3.86E-09	ALDH1A1	-2.04	1.41E-08
KBTBD11	-2.37	2.58E-14	GAS7	-2.04	1.11E-08
CST3	-2.39	7.62E-10	LOC338758	-2.04	1.08E-08
MNDA	-2.40	1.71E-09	MGC33556	-2.04	3.15E-15
MTMR11	-2.40	8.52E-11	GPBAR1	-2.03	3.79E-11
ALDH1A1	-2.45	3.71E-09	KLF10	-2.02	1.92E-09
LILRA5	-2.45	3.15E-15	PGLYRP1	-2.02	1.81E-06
CLEC7A	-2.48	9.44E-10	IL8RB	-2.01	1.42E-06
P2RY13	-2.48	2.43E-14	CLEC12A	-2.01	1.42E-06
DYSF	-2.49	1.32E-11	MGST2	-2.00	1.11E-08
LGALS2	-2.51	2.33E-10			
SDPR	-2.51	8.07E-11			
CD302	-2.53	2.59E-11			
AIF1	-2.53	4.19E-11			
CD33	-2.54	8.47E-12			
CMTM2	-2.56	6.97E-07			
LILRA2	-2.59	7.66E-11			
AIF1	-2.59	1.38E-10			
SULF2	-2.60	3.80E-13			
ALDH2	-2.60	6.65E-13			
GP9	-2.63	2.15E-10			
CLEC12A	-2.64	4.94E-08			
UBE2L3	-2.64	5.17E-12			
CD36	-2.64	4.17E-07			
PRAM1	-2.68	1.10E-12			
RNASE2	-2.77	1.55E-10			
LST1	-2.81	7.33E-13			
PADI4	-2.82	1.23E-11			
ASGR2	-2.83	1.03E-14			

Continued LVL vs. HVL

FCGR3B	-2.84	3.52E-08
CD36	-2.85	2.44E-07
DEFA4	-2.86	1.11E-07
CLC	-2.91	3.52E-07
S100A12	-2.91	1.97E-13
LYZ	-2.95	1.11E-11
SIGLEC10	-2.96	6.92E-13
TUBB1	-2.97	1.27E-10
FOLR3	-2.97	2.80E-10
MS4A6A	-2.98	2.86E-09
LOC653600	-3.00	9.45E-07
FPR1	-3.02	8.09E-13
KLF4	-3.04	1.91E-13
NRGN	-3.06	5.42E-12
GPBAR1	-3.07	1.42E-14
C10orf54	-3.09	2.33E-13
F13A1	-3.19	3.48E-13
ZNF467	-3.23	1.33E-14
FLJ22662	-3.23	8.43E-13
NFE2	-3.25	3.60E-12
FOS	-3.34	4.56E-10
CAMP	-3.34	1.77E-08
SEPT5	-3.37	4.37E-10
S100P	-3.41	5.84E-11
ASGR1	-3.46	1.57E-13
CD1D	-3.47	1.09E-13
MS4A6A	-3.51	2.16E-11
CFD	-3.55	3.42E-11
FOSB	-3.56	1.39E-10
CSF3R	-3.56	1.35E-13
LST1	-3.61	7.06E-14
MS4A6A	-3.71	1.38E-12
FCER1A	-3.71	1.56E-11
DEFA1B	-3.96	3.24E-07
DEFA1B	-4.07	2.99E-07
FCN1	-4.43	4.74E-14
CPVL	-4.61	1.08E-15
DEFA3	-5.12	9.67E-08
DEFA1	-5.13	1.28E-07

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