Author's response to reviews

Title: MicroRNA-29 family expression and its relation to antiviral immune response and viro-immunological markers in untreated HIV-1-infected patients

Authors:

Katia Monteleone (katia.monteleone@uniroma1.it)
Carla Selvaggi (carla.selvaggi@uniroma1.it)
Giulia Cacciotti (giulia.cacciotti@uniroma1.it)
Francesca Falasca (francesca.falasca@uniroma1.it)
Ivano Mezzaroma (ivano.mezzaroma@uniroma1.it)
Gabriella D'Ettorre (gabriella.dettorre@uniroma1.it)
Ombretta Turriziani (ombretta.turriziani@uniroma1.it)
Vincenzo Vullo (vincenzo.vullo@uniroma1.it)
Guido Antonelli (guido.antonelli@uniroma1.it)
Carolina Scagnolari (carolina.scagnolari@uniroma1.it)

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Author's response to reviews: see over
Thank you very much for your email of November 28, 2014 giving us the opportunity to revise this manuscript. We have attended to all of the Reviewers comments and revised the paper accordingly. As requested by one of the Reviewer, we have demonstrated by performing additional experiments that a similar miRNA-29a/b/c signatures can be recorded in key cellular subsets important in HIV-1 pathogenesis, such as CD4+ T cells and CD14+ monocytes. Interestingly, we found that some HIV-1 patients expressed higher levels of miRNA29a-c in T CD4+ lymphocytes compared to CD14+ monocytes while others manifested an opposite miRNA29a-c pattern in these cellular subsets. Thus, these findings suggest that the relationship observed between miRNA29 and the immuno-virological markers of HIV-1 infection cannot be explained solely by CD4+ T-cell variation recorded in HIV-1 patients. In agreement to our observations, the work of Kenneth W Witwer et al. published in Retrovirology, suggest that CD4+ T-cell decline alone does not explain all of the observed variation in miRNA expression during HIV-1 infections. In the attached file you will find the revised paper; only the main changes from the original are highlighted in red.

I would like to thank you and the reviewers for your positive comments on the manuscript and for the constructive criticism. I trust that the revised manuscript is now suitable for publication in BMC Infectious Diseases.

I look forward to hearing from you.

yours

Carolina Scagnolari
Response to Reviewers

Reviewer 1:

This is an original manuscript which contains interesting data about the contribution of the miRNA-29 family on the clinical progression of HIV-1 infection (e.g. the negative influence of miRNA 29 levels on proviral load) and on the activation of antiviral immune response. Results of the manuscript are well presented and methods well described. The manuscript can be considered for publication upon addressing only minor points mentioned below:

• Minor Essential Revisions:

1) Method section: MiRNA should be corrected in mRNA (page 4);

Response. We have corrected the sentence in the text as requested by the Reviewer. We apologize for this mistake. See page 6 line 18.

2) #-glucuronidase primers/probe sequence or relative reference, should be included in the context of RT/Real Time assay (page 5);

Response. We thank the Reviewer for this need of clarification. The beta glucuronidase primers/probe sequence has been added to the text. See page 7 lines 1-4.

3) mRNA should be removed from the text in the discussion section (page 9)

Response. We have removed mRNA from the text as requested by the Reviewer. We apologize for this mistake. See page 14 line 4.

• Major Compulsory Revisions:

1) Authors should discuss whether the expression of miRNA29 in different cell subtypes could influence the impact of miRNA29 on the integrated HIV-DNA levels;

Response. We thank the Reviewer for this comment. We have now discussed that we quantified both miRNA-29a-c and HIV-1 DNA integrated levels using total PBMCs of naïve HIV-1 infected patients. Thus, we may have missed differences in various cell types, such as resting central memory T cells and translational memory T cells, which serve as major sites for HIV-1 latency. See page 14 lines 22-25.
2) Since the study evaluated the impact of miRNA29 expression on IL-32/MxA pathway, it is advisable a discussion about the effect of miRNA29 levels on IL-32 and type I and III IFN subtypes as well as the possible effect of IL-32 or IFN on the expression of miRNA29 members;

**Response.** We thank the Reviewer for this comment. We have now discussed the relationship between miRNA29 levels, IL-32 and IFN. Furthermore, we have mentioned in the discussion section the cellular pathways affected by both miRNA29 and IFN to underline the complexity of the phenomenon analyzed in this study. See page 16 lines 9-12; 15-24.

3) Several studies have associated a negative effect of IFN alpha/beta levels on the clinical outcome in patients with a chronic HIV infection. Thus, the Authors should clarify the importance of the presence of a negative correlation between miRNA29 levels and those of IL-32/MxA in the context of the high.

**Response.** We thank the Reviewer for this comment. Indeed, considering that IFN-α/β play a complex and to some extent controversial role in HIV-1 disease, the effects of miRNA-29b on the rate of IFN activation during HIV-1 infection have been now discussed. See page 16 lines 9-12; 15-24.
Reviewer 2:

The aim of this paper is to explore the role of the miR-29 members (29a, 29b and 29c) in HIV infection. Whilst there were few positive findings, one of the main findings was that miR-29b levels were higher in PBMCs from HIV-1 infected individuals compared to healthy donors. However, there was no correlation noted between miR-29 and either HIV viral load or CD4+ T cell counts.

Major Compulsory Revisions:

There has been a number of papers which have shown that distinct cellular subsets harbour unique miRNA profiles. The reliance of looking at PBMCs ignores the fact that key subsets are altered in HIV-1 infection, such as CD4+ T cell subsets. The claim, for example that miR-29c levels were higher in HIV-1 infected individuals with low viral loads (Fig 2A) may have been true but it is very likely that these patients also had an elevated T cell count which may have accounted for this.

Response. We thank the Reviewer for this comment. Then, we have evaluated the expression of microRNA29a/b/c separately in CD14+ monocytes and CD4+ T lymphocytes collected from treated HIV-1 patients with detectable viremia. We observed that the expression profile of miRNA-29 a-c were similar in CD14+ monocytes and CD4+ T lymphocytes to that observed in the previous analysis performed in total PBMC of naïve HIV-1 patients. In addition, we found that the differences in the amount of miRNA-29 between CD14+ monocytes and T CD4+ lymphocytes were highly variable. In this regards, some HIV-1 patients expressed higher levels of miRNA29a-c in T CD4+ lymphocytes compared to those in CD14+ monocytes while others manifested an opposite pattern of miRNA29a-c expression in these cellular subsets. These findings underline the complexity of the phenomenon analyzed, but more importantly, suggest that the influence of some miRNA29 components on the immuno-virological markers of HIV-1 infection cannot be entirely explained by an alteration of different T CD4 cell counts recorded in HIV-1 patients. In agreement to our observations, the work of Witwer KW et al. published in Retrovirology, suggests that CD4+ T lymphocyte decline alone does not explain all of the observed variation in cellular miRNA expression during HIV-1 infections. See page 2 lines (9-11; 20-23); page 4 (14-16, 22-23); page 5 (21-24); page 8 (1-2; 12-13); page 10 (22-26); page 11 (1-12); page 15 (1-13); 25 (1-13); Fig.3.
I also had difficulty understanding how the relative gene expression was calculated. The usual method is to use relative quantification \( RQ = 2^{-(\#Ct)} \) for each miRNA using the \#Ct method as suggested by the manufacturer. I could not figure out why there was such a huge dynamic range of miRNA expression noted for each of the groups that were shown in each of the figures.

**Response.** We thank the Reviewer for this need of clarification. We have added in the text the equation used to analyze data on miRNA expression obtained from RT/Real time PCR assays. In particular, we have explained that the data were analyzed using the widely used equation 2−\( \Delta CT \), where \( \Delta CT=(CT \text{ of target miRNA}−CT \text{ of internal control}) \). The same explanation has been also added for IL-32 and MxA mRNAs analysis. Furthermore, the results obtained from the analysis of miRNA expression performed on CD4+ T lymphocytes and CD14+ monocytes confirmed the existence of a huge dynamic range of miRNA29 family expression in HIV-1 infected patients (see new fig 3). Furthermore, it must underline that Ct values of the internal control (RNAU6B) showed a limited variation (mean ± dvs: 31.58 ± 0.5) compared to miRNA29a-c in HIV-1 patients. See page 6 lines 12-14; page 7 (3-4).

I also found the link between miR-29b and IL-32 (and MxA) less than convincing. Whist the link between miR-29b potentially modulating IL-32 may have been published, the weak correlation observed in this cohort was not that impressive.

**Response.** We thank the Reviewer for this comment. We agree with the Reviewer that the correlation between miRNA29b and IL-32 was weak. Then, we have now added in the text that the correlation between these two parameters was weak. Moreover, we have mentioned in the discussion section that future studies performed with a larger sample size of HIV-1 patients will be needed to confirm the existence of the negative correlation between miRNA-29b and IL-32. See page 11 line 19; page 15 (23-24).

**Minor Essential Revisions:**

The way that data was presented in the Table (Table 1 for example) was difficult to interpret. Was the T cell count the mean or median? I found the legend confusing in this table.

**Response.** We have modified the table 1 and its legend as requested by the Reviewer to simplified data interpretation. See the revised Table 1.
Also, how many years post diagnosis were the samples taken (or are all of these patients newly diagnosed?)

**Response.** We have added in the table 1 the information about “years post HIV-1 diagnosis” in the table 1 as requested by the Reviewer. See the revised Table 1.

Do any of the patients have Hepatitis B/C co-infection and what is the primary mode of acquisition of HIV in these patients? Some of this information would have been useful.

**Response.** We thank the Reviewer for this comment. We have examined the medical records of all HIV + patients included in this study and we found that none patients had mycobacterium tuberculosis, cytomegalovirus disease and HCV or HBV infections. A sentence has been added in the text to indicate that HIV-1 patients analyzed had none infectious diseases. Unfortunately, we don’t know the primary mode of acquisition of HIV in our patients due to the difficult arises to collect this information from the medical records. However, if the Editor retains essential for publication to insert this data we are ready to modify the paper accordingly but we need more time to collect this information from the medical records.
See page 4 line 26; page 5 (1-2).

**RNA quality is very important in miRNA studies. There is no description of the quality control that was used to assess whether high quality RNA was being used in this study or not (such as the use of Bioanalyzer, 260/230 or 260/280 ratios) etc. Were the PBMC pellets stored for any extended period of time before RNA extraction?**

**Response.** We apologize that we didn’t mention in the previous version of the manuscript how we assessed the RNA quality extracted from blood samples collected from HIV-1 infected patients. However, it is understood that we evaluated the cellular RNA purity by measuring the absorbance at 230, 260 and 280 nm with a spectrophotometer. We have now added in this revised version of the manuscript a sentence on this technical aspect. See page 6 lines 5-7, 19-21.

Also there is no description of how HIV viral load or CD4+ T cell count was measured
Response. The assays used to measure HIV-1 viral load or CD4+ T cell count have been added in the method section. See page 5 lines 10-14.

DIScretionary Revisions:

The language is somewhat loose at times. For example, on page 6, line 167, the sentence begins, "MiRNA levels of IL-32#, IL-32non#...." when surely the authors mean mRNA levels of IL-32#, IL-32non#...."

Response. The whole manuscript has been checked for the presence of mistakes.