Author’s response to reviews

Title: MHC2TA mRNA levels and human herpesvirus 6 in multiple sclerosis patients treated with interferon beta along two-year follow-up

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Version: 2 Date: 24 August 2012

Author’s response to reviews: see over
Dear editor,

In relation to the manuscript 1673986457737013, entitled "MHC2TA mRNA LEVELS AND HUMAN HERPESVIRUS 6 IN MULTIPLE SCLEROSIS PATIENTS TREATED WITH INTERFERON BETA ALONG TWO-YEAR FOLLOW-UP ", we enclose the answers to the comments of the reviewers. Changes in the text are in red.

Sincerely yours

Roberto Alvarez-Lafuente, PhD
Reviewer 1:

1. Materials and Methods. Please provide detailed information on control (healthy) subjects studied. Normalization ratios (NR) in Figure 1 were calculated using gene expression data in both patients and control subjects. No information on healthy subjects (age, gender, other medical problems, medications taken) was provided in Material and Methods.

We have modified the text of the Materials and Methods. Subjects:

... for more than two years. A control group of 154 healthy Spanish individuals was included for comparative purposes in the expression study. RRMS patients and controls were paired by age and sex; none of the healthy controls had relatives of first or second degree with MS or other autoimmune diseases, and none of them had received antiviral medication for at least 6 months before the enrolment in the study. This study was approved...

2. Results. Please clarify which particular statistical test was used for each p value listed.

We have modified the text of the Materials and Methods (Statistical analysis):

… (SPSS Inc.). The chi-square test was used to compare qualitative variables. As MHC2TA expression levels (NR) were not normally distributed we used non-parametric tests for its analysis: Kruskal-Wallis method for comparison of more than two groups and U-Mann Whitney test to compare NRs between two groups. We considered…

3. Figure 1B. Please clarify if MHC2TA expression in patients with allele C was normalized against healthy subjects with allele C or the total population of healthy subjects. The control for MS patients with allele C shall be non-MS subjects with allele C. If information on allele C in control subjects is not available, relative gene expression instead of normalized ratio for MHC2TA shall be provided.

As you can see in previous publications of our group related to this paper (Martinez et al. Human Immunol 2007; 68: 685-689, and Alvarez-Lafuente et al. Eur J Neurol 2010; 17: 129-135), all the control subjects were also analyzed for the same polymorphism (rs4774C) that we analyzed in the group of RRMS patients. Then, the MS patients with allele C were normalized with healthy subjects with allele C, and MS patients with allele G were normalized with healthy subjects with allele G.

We have modified the text of the Results:
… minor allele C (Figure 1B); MS patients with allele C were normalized with healthy subjects with allele C, and MS patients with allele G were normalized with healthy subjects with allele G.

4. Figure 1C. Please clarify if MHC2TA expression in patients with active HHV-6 infection was normalized against control subjects with active HHV-6 infection or against the population of all control subjects. The control for patients with MS and active HHV-6 infection shall be non-MS subjects with active HHV-6 infection. If information on HHV-6 infection in control subjects is not available, relative gene expression instead of normalized ratio for MHC2TA shall be provided.

As you can see in our previous publications, we have analyzed the prevalence and viral load of HHV-6 in MS patients and healthy subjects in the last years. Then, although is not specified in the text of the article, the MS patients with HHV-6 infection were normalized with healthy subjects with HHV-6 infection, and MS patients without HHV-6 infection were normalized with healthy subjects without HHV-6 infection.

We have modified the text of the Results:

… under IFN-beta treatment (Figure 1C); MS patients with HHV-6 active infection were normalized with healthy subjects with HHV-6 active infection, and MS patients without HHV-6 active infection were normalized with healthy subjects without HHV-6 active infection. No statistical significant...

5. Figure 1C. Please provide information on MHC2TA expression in patients with active HHV-6 infection at each time point. For example, it would be important to know MHC2TA expression at the 12 month visit for those 19 patients who had active HHV-6 infection at the 12 month visit.

The data of the NR of MHC2TA expression at each time point in MS patients with and without HHV-6 infection are specified in the fourth paragraph of the results section:

“When we analyzed the MHC2TA mRNA levels in MS patients with HHV-6 active infection at the basal visit (28/154; NR=0.6, 1, 1.2, 1.5, 1.9, for the basal visit and 6, 12, 18 and 24 months later, respectively) and those without HHV-6 active infection at the basal visit (126/154; NR=1.6, 1.4, 1.6, 1.9, 2.2, for the basal visit and 6, 12, 18 and 24 months later, respectively), we found a statistical significant difference for the NRs at the basal visit (p=0.012), but not for the other scheduled visits, when MS patients were under IFN-beta treatment (Figure 1C)”.

6. Table 1. Please add information on the prevalence of HHV-6 DNA expression in the serum of control (“healthy”) subjects.

We have modified the text of the Results (fourth paragraph):
…18-month visit), while only 3/154 (1.9%) healthy subjects were positive for HHV-6 in their serum samples. When we…

7. Table 2. a) Two additional groups (“MHC2TA mRNA level increased + HHV-6 in serum” and “MHC2TA mRNA level decreased without HHV-6 in serum”) shall be added.

Those two “intermediate” groups (n=15 for MS patients with MHC2TA mRNA level increased and HHV-6 active infection, n=19 for MS patients with MHC2TA mRNA level decreased without HHV-6 in serum) were not included because we did not find any statistical significant difference between them and the other two “extreme” groups.

8. Table 2. b) The prevalence of IFN-beta neutralizing antibodies after two years of IFN-beta treatment for all four groups shall be provided. The abstract states, “no differences were found between patients with and without Nabs”. However, no data was provided in Results.

In the second paragraph of the results we state again that we did not find any statistical significant difference on the expression of MHC2TA gene when we compared MS patients with and without NAbs. Furthermore, we did not find any statistical difference when we analyzed the prevalence of NAbs in the four possible groups of the Table 2, and we decided not include them to highlight the statistical significant differences.

9. Table 2. c) As Betaseron and Rebif have higher clinical efficacy than Avonex (based on frequency of clinical relapses in patients with MS), the proportion of patients treated with each of the three drugs shall be provided for all four groups.

As proposed by the reviewer, it would be very interesting to perform a comparison of the clinical response to each IFN-beta formulation in relation to the expression level of MHC2TA and HHV-6 infection, but unfortunately it is not possible with our current MS population. Only 154 MS patients were analyzed and, as we can see in the Materials and methods section, 88 were treated with IFN-beta 1b, 51 with Rebif and only 15 with Avonex. For three of the four possible groups of the Table 2 we had less than 5 patients for each one of the IFN-beta formulations (zero in some cases), and it would be impossible to perform a statistical comparison. A higher number of MS patients would be needed to perform that interesting comparison.

10. Abstract. Please correct the error in the "results" section of the abstract. It shall read as follows: "...MHC2TA mRNA levels were significantly LOWER among MS patients with HHV-6 active infection..."
We have modified the text of the Abstract (results section):

… We found that MHC2TA mRNA levels were significantly lower among MS patients with HHV-6 active infection at the basal visit …

11. Table 2. It would be very interesting to know EDSS and Relapse rate in 23 patients who converted from HHV-6 DNA positive status at the basal visit to HHV-6 DNA negative status at the 6-month visit (described on page 9, lines 20-21). It may be reasonable to include this “HHV-6 converters” group in Table 2.

I am afraid that there is a little confusion. What is written in the text of the article on page 9 is the number of MS patients that had been positive for HHV-6 in their serum samples at least once: 28 MS patients were positive at the basal visit for the first time, other 5 different MS patients were positive at the 6-month visit for the first time (they were previously negative for HHV-6 detection at the basal visit), etc. If you compare these results with those on Table 1, you can see that the prevalence for HHV-6 at the basal visit was 28/154 (18.2%), and the prevalence at the 6-month visit was 22/154 (14.3%); then, from those 22 MS patients positive for HHV-6 at the 6-month visit, 5 of them were positive at this visit for the first time (previously negative at the basal visit), and 17 MS patients were also positive for HHV-6 at the basal visit. Then, only 11 MS patients converted from HHV-6 positive status to HHV-6 negative status from basal visit to the 6-month visit. Again, unfortunately, the number of patients is too small to perform any statistical study.
Reviewer 2:

1. The MHC2TA increase expression was significant only in the HHV6 negative patients or the significance for responders included the decrease in the HHV6 positive ones? Not clear how the analysis was performed: was the absolute level of expression used in analysis? (Correct also the abstract that provide wrong data (suggested that HHV6 positive have higher baseline levels)).

The error of the Abstract (results section) has been corrected:

… We found that MHC2TA mRNA levels were significantly lower among MS patients with HHV-6 active infection at the basal visit …

The MHC2TA expression level was calculated by the 2e-ΔΔCt relative method (Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3: 1101-1108).

To clarify how the normalization ratios were calculated, we have modified the text of the Results:

… minor allele C (Figure 1B); MS patients with allele C were normalized with healthy subjects with allele C, and MS patients with allele G were normalized with healthy subjects with allele G.

… under IFN-beta treatment (Figure 1C); MS patients with HHV-6 active infection were normalized with healthy subjects with HHV-6 active infection, and MS patients without HHV-6 active infection were normalized with healthy subjects without HHV-6 active infection. No statistical significant...

We have also modified the text of the Materials and Methods (Statistical analysis) to clarify how the analysis was performed:

… (SPSS Inc.). The chi-square test was used to compare qualitative variables. As MHC2TA expression levels (NR) were not normally distributed we used non-parametric tests for its analysis: Kruskal-Wallis method for comparison of more than two groups and U-Mann Whitney test to compare NRs between two groups. We considered…

Regarding the increase of the MHC2TA expression levels, as you can see in Figure 1, it was found along the interferon beta treatment (Fig. 1A), and it was also found when we analyzed MS patients with and without allele C of MHC2TA along two years of interferon beta treatment (Fig. 1B), and it was also found when we analyzed MS patients with and without HHV-6 active infection along two years of interferon beta treatment (Fig. 1C). However, the combination of the increasing of MHC2TA expression levels and the absence of HHV-6 in serum was a marker of good response when we analyzed the clinical response after two years of interferon beta treatment; meanwhile, those MS patients with a decrease of the MHC2TA expression levels and with HHV-6 active
infection along the two years of interferon-beta treatment had a worst clinical response, with an increase in the EDSS score and with a higher relapse rate (Table 2).

2. Was the responsiveness to IFN therapy controlled based on clinical or also MRI findings?

The response to the IFN therapy was only controlled by clinical data.

3. From the fig C it appears that although a lower MHC2TA expression at baseline in the HHV6 positive patients, the trend for increase continued during the therapy. The decrease in the responsive group is difficult to explain.

As you can see in Fig 1C, the MHC2TA expression in MS patients without HHV-6 active infection only decrease –not significantly- from basal visit to 6-month visit, and then, the MHC2TA expression increase at 12-month, 18-month and 24-month visit. Not all the MS patients that are considered HHV-6 negative at the basal visit were clinical responders after two years of treatment. As you can see in Results (fourth paragraph), 5 MS patients were positive for HHV-6 at the 6-month visit for the first time (they were previously negative at the basal visit), 2 at the 12-month visit and 1 at the 18-month visit.

I am sorry if the reviewer is a little bit confused with the results of the Figure 1C and the Table 2, but they are different. As you can see, on Figure 1C we only compared MHC2TA expression from MS patients with and without HHV-6 at the basal visit. On Table 2 we compared those MS patients that were positive for HHV-6 (at least once among the five programmed visits, not only at the basal visit) with those MS patients without HHV-6 at the five programmed visits.

4. Were the HHV6 patients responsive to therapy positive at baseline or become positive later? Were their levels higher than the non-responders?

As you can see in Table 1, the prevalence of HHV-6 in serum samples decreased along the two-years interferon beta treatment, and as you can see in Table 2 the absence of HHV-6 in serum samples was related to a better clinical response. Those MS patients with HHV-6 at baseline that were negative in the following visits were good clinical responders; however, those MS patients that were negative at baseline and positive in one or more of the following programmed visits were worse clinical responders. These results agree with previous studies of our group (Garcia-Montojo M, De Las Heras V, Dominguez-Mozo M, Bartolome M, Garcia-Martinez MA, Arroyo R, Alvarez-Lafuente R; On behalf of the HHV-6 and Multiple Sclerosis Study Group. Human herpesvirus 6 and effectiveness of interferon beta 1b in multiple sclerosis patients. Eur J Neurol. 2011 Aug;18(8):1027-35.)

The viral loads of HHV-6 in all the serum samples that were positive to HHV-6 were very similar.
5. Were the patients with C allele also the ones negative at baseline for HHV6 infection?