Author’s response to reviews

Title: Peripheral blood lymphocytes immunophenotyping predicts disease activity in clinically isolated syndrome patients

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Author’s response to reviews:

Reviewer #1 – minor changes have been made directly in the text of the manuscript

Page 3:

1. Introduction:

Last paragraph: the control group is not apparent for the study

There was no control group in the study, the sentence has been amended to better clarify that.

2. Material and Methods:

2.1 Study population

Paragraph 1, Line 3: presence of > or = 2 T2-hyperintense lesions --> CIS can be recognized as one attack with suspected demyelinating lesion in brain, just one is also considered, if the study needed two or more T2 lesions, is it fulfilled with definition of CIS or it had already been diagnosed as Multiple sclerosis with lesions of DIS?

Only patients with CIS were included, the sentence has been amended to better clarify that.
Line 2-3: about the EDSS --> it is estimated as initial attack? or after treatment of steroid therapy to remission stage?

Paragraph 2: the number of Medical Ethics Committee lack

The Ethical Committee approves the grant as a whole, the number has been added.

Page 4:

2.2 Study outcomes:

Paragraph 1, Line 6: the definition of CDP based on an increase in EDSS by 1.0 point, if baseline EDSS > 0, or by 1.5, if baseline EDSS =0; basically the progression type MS should be relapsed before for several times then enter the stage of progression, if the patient had EDSS just 0 before and diagnosed as CIS, is it enough to define as progression type just during 6 months; besides, if the patient had severe attacks before, then EDSS increased significantly, but it should be considered as relapse but not progression.

We tried to use united criteria – definition according to Kaposi was used (reference 21)

Page 9:

4. Discussion

Paragraph 1, Line 2-3 --> I cannot recognize what the meaning the author want to express from the two lines

Paragraph 2, Line 3-4 --> how to explain the value of CD4+ T lymphocyte in your CIS patients at baseline were lower than MS?

Added:

Various immunological markers with a potential of predicting conversion to CDMS or a new relapse activity were not the only parameters analysed during long-term follow-up of the CIS patients, which complemented results of radiological investigation of the same cohort. [6]

In general, the levels of CD4+ cell population in MS patients (unless influenced by treatment (e.g. fingolimod)), are at the upper limit of normal range Values of CD4+ T lymphocytes in our CIS patients at baseline were lower than we commonly found in MS patients in our lab and so we assumed significant augmentation in this subset during the disease progression to CDMS
Paragraph 2, Line 4: Up regulation of naive CD45RA+ T-lymphocyte and parallel down regulation of memory CD45+RO+ cells ... --> what is the meaning for the Linomide to correlate with your study?

Added: Also in this study, the higher levels of naive CD4+ cells seem to be a positive prognostic marker.

Paragraph 3, Line 6: is the slight reduction of total CD45RA+ lymphocyte shown any statistically significant to clinical worsening?

Added: (4.1 % in relationship to baseline, statistically significant in the first, third and fourth year of follow-up)

Page 11:

Paragraph 1, Line 9-11: the lower levels of B cells means less production under stimulation of interferon or other factor? Traditionally, B cells is less considered as the major role to convert CIS to CDMS, how to explain the so-called lower levels of B cell, but more prominent conversion rate to MS?

Added: to interferon beta treatment and B cells analysis could predict need of treatment escalation so that relapse would be prevented.

Reviewer #2: In this study, the authors checked the immune-phenotype of peripheral leukocyte of multiple sclerosis patients at the different times. According to author, only individual differences between baseline values and values at the time of a new relapse were analyzed. The author stated that higher levels of B lymphocytes predicted relapse-free status, decrease of NK cells and the naive subset of cells (CD45RA+ in CD4+) were associated with disease progression. This is an interesting idea, which may apply to clinical practices. However, I think the methodology of statistical analysis is complicate and difficult to be understood by general reader. It is better to clarify the detailed statistical methods and simplify the figures.

For example, how to choose the threshold of 0.808 (ratio v.s. last year), 1.638 (ratio v.s. baseline), 9.050 (value) in Figure 1? Why choose those three indicators (value, ratio v.s. last year, and ratio v.s. baseline)? Please explain and compare which indicators is better.
Based on these curves, thresholds which maximised the sum of sensitivity and specificity for each measured parameter were defined.

It cannot be decided which of the indicators is better, they are therefore used together.

The absolute number (or ratio?) of different subtypes of leukocyte seems didn't have significant difference between patients with and without disease progression at different time points (table 1 and 2). Is there any significant difference if we only directly compared the ratio (ratio v.s. last year or ratio v.s. baseline) of different subtypes of leukocyte between patients with and without disease progression at different time points? Can the author show the origin data?

Added: We aimed to test the possibility of finding a threshold value that would distinguish patients with higher or lower probability of relapse or CDP (the “value” in Figure 1-4). More importantly, we aimed to measure changes in lymphocyte subpopulations longitudinally in each patient separately so that disease course could be predicted. Therefore, we tested ratio of current values versus values obtained a year ago or versus baseline (Ratio vs. LY and Ratio vs. BL Figure 1-4).

For the assessment of lymphocyte subpopulations, relative percentage is usually used, but absolute counts can be also calculated. As interferon beta decreases the overall lymphocyte count, including absolute values of respective subpopulations, we selected relative values for the statistical analysis. Since only results obtained before a relapse or CDP were statistically assessed, no other treatment than interferon beta could have influenced the parameters, and treatment was the same in all patients. Also methylprednisolone pulses were the same in all patients and the treatment was given at least 30 days ahead of baseline.

Original data has been enclosed.

There are some minor suggestions:

In introduction: please add discussions about previous studies and applications of peripheral leukocyte immune-phenotype in multiple sclerosis in introduction.

The results of the studies dealing with peripheral blood were originally only mentioned in the Discussion so that the text was not too long.

Added: In this context, although peripheral blood is only a mirror of immune reaction in CNS, it is much easier to measure it longitudinally. CD4+ T cells and later CD4-Th1 cells were the most studied cell population in MS in the past [14], originally because of their potential role in the pathogenesis, whereas the effect of different therapies used in MS on this population was assessed more recently. Subsequently, attention was also paid to other regulatory subpopulations, e.g. Th2 cells, regulatory CD4+ T cells and NK cells, particularly to their relationship to disease
prognosis [15], or to radiologically confirmed MS activity, where changes in effector populations were also described [16]. Association of CD4+CD45RO+ IL-17A+ cells to clinical and radiological disease activity was reported. [17, 18] Differences in naive CD4 T-cell biology, notably in TCR and TLR signalling pathways, identified patients with MS with more rapid conversion to secondary progression [19].

In CIS patients, the most intriguing finding was published by Vilar et al [20], who showed possibility to use analysis of peripheral blood CD5+CD19+ subsets as a predictive factor for CDMS conversion.

2. In methods and materials (in the study population of CIS patients), may add the reference of inclusion criteria of CIS patients and "presence of >= two oligo clonal band". Did any patient have spinal cord lesions or optic neuritis?

Added: that could possibly be attributed to a neurological diseases other than MS (e.g. neuromyelitis optica).

3. In methods and materials (in study outcome), add reference of "Confirmed disability progression (CDP) was defined as an increase in EDSS by 1.0 point (if baseline EDSS > 0) or 1.5 points (if baseline EDSS = 0) confirmed after 6 months".

A reference has been added – Kaposi (21)

4. In methods and materials (in statistical methods), please clarify the statistical methods more detail as suggestions.

We used standard methods of statistical analysis which have been used in similar studies (e.g. reference Vilar (20)). For better understanding, we now amended the description of statistical methods (see also comments of the other reviewers).

5. In result: table 1 and 2 need to add the unit of results (ex: %?). may summarized positive findings in one table.

The units are listed below the table. We did not consider the results so substantial to provide more details.

Added: The results are shown for a better orientation and to complement the statistical analysis,
6. In result: how many patients have relapse or disease progression at 6, 12, 24, 36, 48 months, respectively? If any patient had 2 relapse during those periods?

Added:

During the first year of follow-up, the second disease relapse was observed in 55 patients, during the second year additional 25 patients relapsed, and 21 and 13 patients, respectively, relapsed during the third and the fourth year of follow-up. At 48 months, 114 (59.6%) patients experienced the second clinical attack (69 the third one) and 37 (19.3 %) had confirmed disability progression (CDP).

7. In results: all patients received IV steroid and interferon treatment. How many patients also received other treatments (fingolimod, copaxone)? If we can analyze the effect of different treatment to immune-phenotype of leukocyte?

Added: The results at 48 months of following should be influenced by treatment (natalizumab n=18 , fingolimod n=3, copaxone n=6).

The effect of treatment on peripheral lymphocytes has been greatest in fingolimod (decrease of CD4+CD3+ population), we did not examine that in detail. The results are shown in the Table to display complete results.

Reviewer #3: I reviewed article 'Peripheral blood lymphocytes immunophenotyping predicts disease activity in clinically isolated syndrome patients' by Posová Helena, Horáková Dana, Čapek Václav, Uher Tomáš, Hrušková Zdenka, Havrdová Eva.

I must admit that authors provided thorough longitudinal data, when they followed-up 181 patients with CIS (151 for four years). This longitudinal long term approach is from my point of view the most useful way how to reach convincing conclusion in MS. Authors try to identify blood marker of neurological progression in MS patients. Statistics level seems sufficient according to patients' population needs. Language quality is suitable for publication.

Authors formulated some hypotheses and their data in many ways supported already published studies. I believe that paper needs some changes to be more understandable and less contradictory.

Patients were very early in disease course started on DMT (INF Beat 1a), which is admirable but from the context of table annotations ('The results at 48 months of follow-up should be influenced by treatment (natalizumab,fingolimod, copaxone)’ I understood that variable switches
between treatments were made over the period of 4 years. This seems to be major downfall. All treatment mentioned above would inevitably influence levels of lymphocyte subpopulations. I believe that authors should be paying attention mostly to first year or two as I believe that population of CIS patient was at that point the most homogeneous from treatment point of view. This subgroup would be the best to study differences between future "MS converters" or "CIS remainers". Authors also admit that steroid treatment before baseline (which I believe was used in all of patients to treat initial CIS?) skewed FBC (full blood count results) and subpopulation results.

We are aware that subsequent therapy changes could, particularly in the case of fingolimod, influence the results. Results of these patients were, therefore, not included in the statistical analysis, they are just listed in the Table to show complete data of 48 month follow-up. This has been mentioned below the table.

Added:

Since only results obtained before a relapse or CDP were statistically assessed, no other treatment than interferon beta could have influenced the parameters, and treatment was the same in all patients. Also methylprednisolone pulses were the same in all patients and the treatment was given at least 30 days ahead of baseline.

Authors are mentioning role of NK cells which were assumed to play rather positive role in MS parthenogenesis (authors mentioned that 'MS relapses and new brain lesions detected by magnetic resonance imaging are often preceded by a reduction in NK cell functional activity') however authors mention in Results section that 'The decrease of NK cells below 10.8 % increased the Clinically defined progression (CDP)…..but increase (of more than 28.7%) led to EDSS worsening'. These statements are contradictory or authors did not explain their conclusions properly.

An explanation has been added: There was a slight gradual decrease of NK cells in our study during whole 4 years and the value of 10.8 % could be considered as a critical point. In contrast with this, an increase at year one (and three) should predict CDP. This inconsistent result could be related to changes in NK subpopulation – CD56bright NK cells - with the capacity to control activated T cells. [40] This, within NK cells minor subpopulation, seems to decrease during follow-up, which leads to a lower control of T lymphocytes. Inter-annual increase of NK cell preceding CDP could then be caused by transient increase of major CD56low population, which can be a simple sign of chronic inflammatory process.

Nevertheless, in our study, we only assessed total population of CD3-CD16+56+ NK cells as the representation of CD56bright NK cells was low in our patients, and we therefore did not consider
this population suitable for long-term assessment and statistical analysis. However, in recent years, some publications described the necessity of examination of this subpopulation.

Authors should put accent on naive cells (including helpers (CD45RA+ in CD4)) as those seem to be more stable and statistically related to clinical course (CDP)

Other contradiction was identified in Discussion section as authors mentioned 'increase of CD5+ memory B cells in remitting stage of the disease...other authors concluded that increased percentages of blood CD5+ B cells were associated with further elevated risk of conversion to MS and increase in relapse'.

Added: 4.1 % in relationship to baseline, statistically significant in the first, third and fourth year of follow-up

CD5+ B lymphocytes form only a minor part of B lymphocytes but the population behaves differently.

An explanation has been added:

Completely different results were found in one of the B lymphocyte subpopulations – CD5+. Earlier, this subpopulation was thought to be the potential source of autoantibodies, which was later not confirmed, but its role in autoimmunity is still studied.