Peer Review File

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Reviewer: The paper titled “Cognitive impairment is related to a reduced count of T-lymphocytes in older patients diagnosed with non-small cell lung cancer” is interesting. The authors conclude that T-lymphocyte count is lower in patients diagnosed with lung cancer and cognitive impairment. However, there are several minor issues that if addressed would significantly improve the manuscript.

Comment 1: In this paper, it is best to supplement the mRNA and protein expression of surface molecules CD28 and CD95. This is more conducive to support the conclusion of this paper.
Reply 1: Our aim is to evaluate the possible association of lymphocyte counts (CD8-T cells, CD4-T lymphocytes, B and NK lymphocytes) on clinical parameters in a way that could be easily applied in clinical practice to help clinician evaluation of oncologic patients. Thus, we chose flow cytometry as the technique to evaluate lymphocyte counts because we know that it is available in almost every hospital in our environment.

Changes in the text 1: This is specified in the section 2.2 (Page 6, lines 19-21): “Flow cytometry is a widely used technique, available in most hospitals, which makes it the ideal procedure to evaluate immune system in daily clinical practice.”

Comment 2: Is cognitive impairment related to the clinical TNM staging, degree of cell differentiation, pathological type, and lymph node metastasis status of elderly lung cancer?
Reply 2: Cognitive impairment is a relatively common finding in elderly patients with lung cancer and it could be related to multiple factors (normal aging, inflammaging induced by cancer, oncologic treatments or paraneoplastic syndromes), and has been described in many different tumoral types and clinical stages without a clear association.

Changes in the text 2: This is further explained in section 1. Introduction: “Aging is a risk factor for both cancer and cognitive impairment [1, 2]. Furthermore, cognitive alterations have been widely studied in patients diagnosed with neoplasms, specially related to treatment with chemotherapy, as a condition globally known as chemobrain

or chemofog [3, 4]. However, the mechanisms that induce cognitive impairment in patients with cancer are not fully understood, especially in older patients, in which multiple risk factors coexist. Knowledge of associated factors to cognitive impairment in elderly patients with cancer appears to be of great importance given its higher prevalence in this population and the possible greater risk of treatments to induce or worsen cognitive damage [5]. During the last years, growing evidence of the relationship between immune alterations that occur with aging (known as immunosenescence) and neurodegenerative diseases has emerged [6, 7].” (Page4, lines 2-13); and 4. Discussion “As all the patients included in our study had been diagnosed with NSCLC, we hypothesized that advanced tumoral stage might also influence immunological status, as reported in a study by Onyema et al. [45]; however, we found no significant differences in lymphocyte populations depending on the extent of the disease. This could be due to the differences in the population included in their study, in which patients with small cell lung cancer and mesothelioma were also included, with a relatively low sample size (n=24 patients); this disease’s characteristics and evolution significantly differ from NSCLC and this could have acted as a confounding factor for the differences among lymphocyte subsets and warrants further investigation”. “Page 12, line 26 – Page 13, lines 1-8”.

Comment 3: Why the author only uses FCM to count lymphocyte? Why not combine with other methods to test, the results will be more reliable in that case.
Reply 3: As answered in question 1: we chose flow cytometry as the technique to evaluate lymphocyte counts because we know that it is available in almost every hospital in our environment.

Changes in the text 3: This is specified in the section 2.2 (Page 6, lines 19-21): “Flow cytometry is a widely used technique, available in most hospitals, which makes it the ideal procedure to evaluate immune system in daily clinical practice.”

Comment 4: In results sections, the authors reported “Among patients with cognitive impairment, 80% were male (8/10)”. The ratio of men to women is very different. How is the result convincing? Please explain the problem.
Reply 4: Although trends in lung cancer incidence have varied since the last few years, it is still a more prevalent disease in men. That is why, when studying a sample diagnosed with lung cancer, we found that 80% of patients with cognitive impairment were male (in our global population >75% patients were male).

Changes in the text 4: This is further explained in section 4. Discussion:

“Furthermore, not all the older patients diagnosed with NSCLC during the period of our analysis had been tested for their cognitive status until the comprehensive geriatric assessment was included in the diagnostic protocol of patients aged 70 years or more diagnosed with cancer in our Department, which explains the small size of the population analyzed. Also, although trends in lung cancer incidence have varied since the last few years, it is still a more prevalent disease in men [39]; and consequently, there was a low percentage of female population eligible for our analysis. This, added to the relatively reduced sample size, is why potential gender differences could not be assessed. However, this fact is not considered a major issue because although Alzheimer disease is more prevalent in women, mild cognitive impairment incidence is similar in both sexes [40]”. Page 13 lines 20-25; Page 14, lines 1-3.

Comment 5: There are still some weak points in this paper. It is suggested that the author increase the possible mechanism analysis. This is more conducive to support the conclusions of this study.
Reply 5: Possible mechanisms involved in the reduction of total T and CD8+ T-lymphocytes were explained more extensively and more examples were exposed.

Changes in the text 5: See modifications in section 4. Discussion “Our findings support the results of the publication by Magaki et al [21], including older patients with and without cognitive impairment. In this study, patients diagnosed with mild cognitive impairment had a significantly lower level of total lymphocytes and higher level of neutrophils. However, they were not able to find differences among lymphocyte subsets. The evidence of differential lymphocyte levels according to cognitive status, is also sustained by several studies that have correlated higher levels of neutrophil to lymphocyte ratio (NLR) to cognitive impairment [22, 23]. Although there is scarce data about specific lymphocyte subsets in patients with cognitive impairment, there is increasing evidence of the relationship between inflammatory status and cognition [24, 25]. Mechanisms by which cognitive impairment is related to inflammatory status are not fully understood, nevertheless, systemic inflammation could induce a similar level of central nervous system inflammatory status mediated by different mechanisms, including activation of peripheral nerves [26] and increased permeabilization of the blood-brain barrier, both inducing the production of proinflammatory cytokines by the microglia and astrocytes. Inflammation at this level would alter brain structures and could be associated with functional declining [27]. Nevertheless, the connection between cognitive impairment and the level of T lymphocytes is harder to elucidate. Older patients in our study with cognitive

impairment had significant lower levels of total, T and CD8 positive lymphocytes compared to elders with normal cognitive functioning, regardless of chronological age, which could indicate more extensive changes related to immunosenescence [28], or even sequestration of CD8+ T-lymphocytes in the central nervous system as postulated by Richartz-Salzburger et al. [29] in a study including patients with Alzheimer Disease with a similar lymphocyte distribution to the one reported in our analysis. Nevertheless, although a higher risk of cognitive impairment was also found in patients with lower levels of T-lymphocytes, due to the cross-sectional design of our study, no causal correlations could be stablished. Furthermore, in old healthy people, it has been observed that naïve T-lymphocytes decrease in number (mainly among the CD8+ subpopulation) and highly mature terminally differentiated cells increase in number [16, 30], including an increased number of autoreactive T lymphocytes that lead to higher levels of proinflammatory markers (such as IL-6 and CRP) and participating in neural degeneration [31]. That is why, lymphocyte distribution and differentiation and their correlation with cognitive impairment is being investigated including larger and prospective cohorts of patients diagnosed with lung cancer". Page 11, lines 5-26; page 12, lines 1-10.

Comment 6: What are the main changes or indicators of immunosenescence?
Reply 6: The hallmarks of immunosenescence are: reduced response to antigens and increased number of senescent cells associated with a chronic inflammatory status (inflammaging). The most studied area is the T cell subpopulation. In elderly healthy people, naïve T-lymphocytes decrease and highly mature terminally differentiated cells increase. However, to study these subpopulations would imply using more specific antibodies that are not currently used in clinical practice and that surpass the objective of this investigation. Furthermore, changes in the B-lymphocyte population (CD19+) have been described, and a decrease in their total number has been observed. Also, NK lymphocytes have been examined with a high interest because of its part in tumour cell destruction. During normal aging, the function of these cells reduces, but this is balanced because the number of these cells increases.

Changes in the text 6: This is further explained in section 1. Introduction: "The hallmarks of immunosenescence are reduced response to antigens and increased number of senescent cells associated with a chronic inflammatory status (inflammaging) [8] in a highly complex process that cannot only be explained by cellular aging, but is also influenced by antigenic exposure or nutritional status among other factors." (Page 4. Lines13-16), and 4. Discussion: "As described previously, immunosenescence is characterized by a reduced response capacity to new antigens

and increased number of memory and senescent cells associated with low grade of chronic inflammation, known as inflammaging [8, 9]. This changes have been deeply investigated regarding the distribution of T cell subpopulations: in elderly healthy people, naïve T-lymphocytes decrease (CD8+CD28+CD45RA+) and highly mature terminally differentiated cells increase (CD8+CD28-CD45RA+) [16, 17]. However, to study these subpopulations would imply using more specific antibodies that are not currently used in clinical practice and that surpass the objective of this investigation. Interestingly, results regarding total CD8+ and CD4+ lymphocytes have been conflicting in old healthy individuals, and higher and lower CD8+ total lymphocyte counts have been reported in healthy older individuals [18, 19]. Moreover, changes in the B-lymphocyte population (CD19+) have been described, and a decrease in their total number has been observed. Also, NK lymphocytes have been examined with a high interest because of its part in tumour cell destruction. During normal aging, the function of these cells reduces, but this is balanced because the number of these cells increases [20]. Although classically these changes were associated with a loss of functionality, their impact is still not completely understood and even to a lesser extent in patients with cancer". Page 10, lines 13-26, Page 11, lines 1-3.

Comment 7: The Introduction is too simple. Many researches on non-small cell lung cancer are not involved in the introduction of this paper. It is suggested to supplement relevant information and rewrite this part.
Reply 7: The introduction of the manuscript was rewritten to extend the background of the paper and fully explain the aim of the study.

Changes in the text 7: Introduction was edited. Pages 4 and 5.

Comment 8: The samples were too small. How to handle with the limitation. Such limitations should be addressed in the discussion.
Reply 8: We have completed the discussion as advised.

Changes in text 8: Section 4. Discussion was edited: "Furthermore, not all the older patients diagnosed with NSCLC during the period of our analysis had been tested for their cognitive status until the comprehensive geriatric assessment was included in the diagnostic protocol of patients aged 70 years or more diagnosed with cancer in our Department, which explains the small size of the population analyzed. Also, although trends in lung cancer incidence have varied since the last few years, it is still a more prevalent disease in men [39]; and consequently, there was a low percentage of female population eligible for our analysis. This, added to the relatively reduced

sample size, is why potential gender differences could not be assessed". Page 13, lines 20-26; page 14, line 1.

"Furthermore, despite the reduced number of patients diagnosed with cognitive impairment, statistically differences were found, and these results make our investigation a relevant hypothesis generating study". Page 14, lines 13-15.