Peer Review File

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Reviewer A

This is an exhaustive paper, but it is difficult to follow due to of the numerous information often not well related together. In my opinion this paper couldn't accept in this form and requires major revision

Thank you for your feedback. We significantly revised the manuscript from the point of view of logical flow of information and divided the information into several subchapters.

Major revision

Plagiarism is observed in many sentences. The authors should be look at the work in the light of this and change the sentences

Thank you for the feedback, we revised the manuscript and changes the sentences of plagiarism.

Lines 438-440 this sentence is unclear. What means "indeterminate cases"? please explain better.

Thank you for the observation. We modified the sentence accordingly: "Flow cytometry immunophenotyping of CSF is a very reliable method to determine CNS leukemia in cases of low cellularity. The status of "indeterminate cases" is low, meaning 8.3% and it includes technical flaws such as very low number of cells, inappropriate stanning, insufficient normal population of cells for appropriate comparison."

Lines 441-464 the authors mentioned risk factors

We re-written this sentence: "The development of CNS leukemia involves a series of clinical characteristics at initial presentation or development of these characteristics over the course of the disease, in both ALL and AML." The details about risk factors are offered after this sentence.

Lines 578-580 I don't agree with this sentence. The modern diagnostic tools, as flow cytometry, are able to identify also few leukemic cells allowing the CNS involvement diagnosis. Furthermore, the authors didn't describe the prognostic significance of flow cytometry positivity.

Thank you for the observation. We added this paragraph in the subchapter:

"Flow cytometry immunophenotyping (FCI) is a very reliable alternative to cytomorphology and the combination between the two can significantly increase the chances of CNS detection. This is because FCI can detect leukemic blasts even in small number. Flow cytometry was used successfully in the detection of CNS involvement in ALL and can successfully distinct between low risk and high risk patients."

We also included this explanation in the Conclusion section: "Thus, a better, more targeted early diagnosis is crucial, for instance a combined use of flow cytometry and cytomorphology analysis of cerebrospinal fluid."

Minor revision

Line 435 the authors used acronyms FCM or FCI, please uniform

We replaced everywhere FCM with FCI. Thank you.

Line 443 the sentence starts with a number please change 68% with sixty-eight

We made the replacement. Thank you. "Sixty-eight of patients with elevated initial WBC count developed CNS leukemia,"

Reviewer B

Deak, Gorcea-Andronic and colleagues provides a review of CNS involvement in ALL and AML, covering biological mechanisms as well as clinical aspects of the topic. Additionally, a case story of CNS relapse after HSCT in a patient with B-cell precursor ALL is provided. The review covers a lot of ground and include interesting aspects of CNS involvement, such as the role of various cytokines and adhesion molecules and that of CD56. Thus, the manuscript can be a valuable contribution to the field.

However, I have some concerns regarding structure/balance and references. The manuscript would benefit from a thorough revision with particular focus on referencing. Further, some points in the manuscript are stated as though they were well-established, although in my opinion, these still have evidence pointing in various directions in the literature. Examples include:

Thank you for the observation. We did a detailed addition of reference in order to balance the text to references. We added more than 120 new references in the new form of our manuscript (from 151 references to 271 references)

LSCs (l 161) although well-established in AML, LSCs have been more of a topic of debate in ALL. This could be mentioned and/or reflected in the 8 references, which mainly address AML and solid cancers.

Answer: We reduced the number of citation and added more details in the text. "Acute leukemia is maintained by a pool of self-renewing cells denominated leukemic stem cells (LSCs), that express CD34+CD38– and increased aldehyde dehydrogenase activity [29] with properties similar to other cancer stem cells (CSC) [30-34]. The LSCs can originate from hematopoietic stem cells (HSCs) or from committed cells that have an inner higher self-renewal potential [35]. In ALL, there is a strong possibility that these stem cells originate from fully differentiated cells that during malignant transformation re-gain stem-like properties. The LSCs in pediatric ALL were discovered committed cells [36]. In AML LSCs are responsible for the maintenance of minimal residual disease which is responsible for treatment failure and disease relapse in AML. The reason behind the observed results is that the LSCs remain in G0 state and have high self-renewal capacity. Two pro-malignancy events have to happen in order for a malignant LSC to

develop. The LCSs then “mature” into leukemic progenitors, then leukemic blasts, which are the most often therapeutic targets. However, as long as the LSCs pool is maintained, the therapeutic death of leukemic blasts is insufficient [37].”

Regarding references: Many studies are mentioned throughout the manuscript without being referenced, making it hard to judge the balance of the review properly.

Examples include: l 201 (ref 63?), l 202, l 226-228, l 245, l 247-253, l 262-264, l 269, l 341 and others

We did a through correction on referencing and added more than … references.

More precisely:

L201: “ALL relapse can occur in bone marrow or extramedullary sites (less frequent), with higher incidence in ALL-blast sanctuaries and approximately 7.5-15% of relapses occurring in the CNS [99]”

Former l202: “As follows, the clonal origin of CNS leukemia is there ‘neurotrophic subclone’ [100-102].”

L 226-228: “VEGF synthesis was identified in leukemic cells of both AML and ALL [116]. In AML, high levels of VEGF-A are associated with disease diagnostic [117] with shorter survival, in vitro study assessment [118]. In patients with ALL, no correlation has been identified between plasma VEGF levels and CNS involvment, but the CSF VEGF level was significantly increased in patients with CNS involvement [119-122] .”

Line 245: “Further in vitro assays showed that PBX1 is upregulated in co-culture models of preB-ALL cells with BBB epithelial cells and choroid plexus epithelial cells, these LCs having increased cytarabine and methotrexate chemoresistance. Despite chemoresistance, inducing PBX1 overexpression leads to increased CNS infiltration in vivo [127].”

l 247-253: “CNS is one of the ALL-blast sanctuaries and once LSCs get there, they are protected from chemotherapy [99] and the natural killer cells from the patient’s immune system thus LSCs can persist as MRD in CNS or can rapidly develop into CNS leukemia [128]. Meninges also appear to have protective effects on leukemic cells. In vitro studies show that adherence of ALL blasts to primary meningeal cells leads to increased chemoresistance, decreased apoptosis and quiescence [82].”

l 262-264 “MER positive ALL-blasts enter G0/G1 phase when co-cultured with CNS derived cells [82]. In ALL with t(1;19) translocation is found the highly encountered E2A-PBX1–rearrangement [131], with increased IL7R expression on the blasts surface and CNS leukemia incidence [132].”

l 269 “When cells undergo malignant transformation, they can express CD56 and show an aggressive behavior [141]. Apart from carcinomas with neuroendocrine phenotype [142] or tumors of neuroectodermal origin [143], well known for CD56 positivity, hematological malignancies can also express CD56 in various proportions, including NK leukemias [144], T-cell leukemias/lymphomas [145], acute myeloid leukemia [146, 147].”

l 341 “CD56 positivity in acute leukemias was also found to increase the tendency to develop extramedullary disease or relapse [179]. Extramedullary disease (EMD) show high frequency in M4 and M5 types of AML [180] and in AML with t(8;21) translocation [181].”

Likewise, the paragraph on CD56 includes a very thorough review on the role of the molecule in NK cells and other immune cells as well as CD56 in leukemias as such, but the evidence for a role for CD56 in CNS involvement is basically lacking. The only reference mentioned in this paragraph is ref 93, which concerns CD56 on mesenchymal stem cells. I am aware that several studies have suggested an association between CD56 in both AML and ALL, but the authors should include these (preferably for both AML, T-ALL and BCP-ALL), so the readers have a chance to assess the evidence properly.

Thank you, we extended this section, provided more details and added more references:

" ALL patients with CD56+ cells developed ALL more rapidly and was more severe, considering that the overall survival of ALL patients with CNS involvement was lower for CD56+ cases [133]. CD56 presence was associated with CNS leukemia at initial presentation with 19% of cases in CD56+ ALL patients versus 4% in CD56- ALL patients. Even in the case of treatment with 8 consecutive cycles of cyclophosphamide the CNS leukemia developed more often in CD56+ ALL cases. However, it should be considered that there were only 16 CD56+ cases versus 184 CD56 – case [184]. A study analyzing the role of CD56+ in T-ALL concluded that there was no difference in CNS leukemia between CD56+ or CD56- T-ALL cases [145]. A case presentation also reported the expression of CD56, along with CD34, CD19, CD79s in B cell precursor ALL (BCP-ALL) who had KMT2A-AFF1 gene rearrangement [185].

In AML, as stated above, there are less frequent cases of CNS involvement and the role of CD56 status is still debatable. However, CD56 positivity in AML leukemic blasts was associated with higher incidence of extramedullary involvement, including CNS infiltration [182]. CNS relapse rate, and overall relapse rate too, are higher in CD56+ AML patients [176], especially second remission [186], including acute promyelocytic leukemia, especially if associated with high levels of WBC [187]. According to a case report, acute promyelocytic leukemia immunophenotype after CNS relapse switched to CD34 and CD56 expression, and a thorough reanalysis of the bone marrow aspirate at the initial diagnosis identified a small subpopulation of CD34 and CD56 positive blasts [188]."

On this note, since the authors have chosen to address CNS involvement in both AML and ALL, it is important to assess evidence for both conditions when discussing biological mechanisms and clinical features. Oftentimes the two conditions are mixed up, and it makes the line of thought hard to follow. For instance in line 133, the authors discuss pediatric AML patients, but the references (15,16) concern two ALL studies.

We apologize for the mistake, we corrected the reference accordingly. And separated throughout the text AML from ALL.

Minor comments:

There are several places where sentences seem to lack words or endings, or phrases are not logical, which diminishes readability of the manuscript. A second reading of the manuscript would be of benefit, perhaps by a native English-speaker.

It would be valuable to touch upon the issue of traumatic LBP in the setting of assessment of CNS involvement at diagnosis and later risk of relapse

We introduced the following paragraph where we talked about this issue: ” The established way of assessing the CNS leukemia is lumbar puncture (LP). However, practitioners have to pay attention to the fact that in 10-30% of LPs there is a danger of blood entering into CSF due to the trauma caused by the needle, this is defined as traumatic LP [24]. Some studies concluded that traumatic LP increases the chances of disease recurrence in ALL [25], especially in high-risk patients [26]. The choice of during an LP only if symptoms are present in ALL is also a strategy to decrease the risk of recurrence. If LP should be introduced as routine check-up in adult ALL, then the procedures from childhood ALL or AML should be introduced, such as intrathecal cytarabine given from the beginning [27].”

L 430: the use of transfix increases this time to up to 72 hours

We replaced it with 72 hours.

Regarding the case story:

Was flow cytometry performed on CSF at initial diagnosis?

CSF analysis, both by microscopy analysis as well as by flow was carried out, with no evidence of leukemia involvement.

Do the authors believe that the delayed diagnosis of CNS relapse (as opposed to infection) contributed to the fatal outcome?

Yes, we believe it did.