Original Paper

Morphological Differences in Hippocampal Microglia in C57BL/6N Mice with Liver Injury and Depressive-Like Behavior

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ABSTRACT: Introduction: Microglia, one of the most important cells of the central nervous system, undergo specific changes depending on the pathology. It has been reported that both depressive disorders and liver diseases generate hippocampal changes and neuroinflammation. However, the combined effects of the two pathologies on microglia morphology in the hippocampus have not been sufficiently explored. Material and methods: In this study, we analyzed the morphological changes of the hippocampal microglia using confocal microscopy and a semi-manual method of quantification. We focused on total branch length, the branch number and the mean branch length. C57BL/6N mice were used and subjected to a methionine and choline deficient diet (MCD) to induce liver damage, and a chronic unpredictable mild stress (CUMS) procedure for depressive-like behavior. Results: We were able to show that CUMS protocol and MCD diet led to a reduction in total branch length, branch number and mean branch length. Also, CUMS alone was associated with a decrease in the number of secondary and terminal branches. Conclusion: Our study showed that depressive-like behavior and liver damage influence microglial morphology in the hippocampus, and it may be considered in future research of these intricate pathologies.

KEYWORDS: *Morphology, microglia, hippocampus, depression, NAFLD.*

Introduction

Microglia, one of the most important cells of the central nervous system (CNS), has a significant role in post-injury repair process and in regulating brain development [1,2].

These cells have the ability to adapt their morphology, being remarkable dynamic [3-5].

Changes in hippocampal microglial morphology, a brain region involved in emotion, memory and learning [6,7], were observed in neurological and psychiatric disorders, such as depression and neurodegenerative diseases [8-10].

Chronic stress represents a major risk factor for depression [11,12], and it seems to have a significant influence on microglial activity in the hippocampus [8,13].

The structural complexity of the microglia, especially the length and branching, provides information about their functional state, ranging from surveillance to activation depending on the pathology involved [14-16].

Understanding the mechanisms of the CNS disorders involving the microglial morphology in the hippocampus can bring additional information, especially as the chronic stress and liver disfunction are associated with changes in brain structure and function [17-20].

However, the combined effects of both on hippocampal microglial morphology are poorly explored.

Taking into account that the inflamation associated with liver diseases, such as nonalcoholic fatty liver disease (NAFLD), can exacerbate neuroinflammation and impair hippocampal function, even more in terms of depression, the investigation of the changes involved in the two pathologies is certainly necessary [21-23].

Chronic unpredictable mild stress (CUMS) protocol has been successfully used to induce depression-like behavior in rodents [24-26], and methionine choline deficient diet (MCD) is an effective murine model of NAFLD [27].

Using the CUMS protocol and the MCD diet, in this study we aimed to investigate the impact of depressive-like behavior and liver damage, analyzing the changes in microglia branching and complexity in order to provide an overview of how these conditions influence the hippocampal microglia morphology.

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Materials and Methods

Experimental Animals

In this study we used male C57BL/6N mice, age 16-18 weeks (n=12).

The animals were obtained from the Animal Facility of University of Medicine and Pharmacy of Craiova.

During the study, the mice were housed under controlled conditions, in ventilated cages and constant temperature.

No visitors or other experimental activities were allowed in the housing area.

All experimental procedures were approved by the Committee for Experimental Animals Wellbeing of the University of Medicine and Pharmacy of Craiova (2.1, 10.11.2022) and the Sanitary, Veterinary, and Food Safety Directorates (27, 18.10.2024).

Depressive-like behavior and non-alcoholic fatty liver disease/non-alcoholic steatohepatitis induction

Depressive-like behavior was induced using the CUMS protocol [25], following a previously established methodology [27].

Briefly, mice assigned to the CUMS group were exposed to 28 days of mild, unpredictable stressors, with no stressor repeated within a 3-day period, as previously described [27].

NAFLD was induced by substituting standard food pellets with a MCD diet (MP Biomedicals, Germany) [27-29].

Mice had ad libitum access to the MCD diet for 4 weeks, while control groups were provided with standard food pellets.

The animals were randomly assigned to one of four groups: WT (control group, n=3), MCD (MCD diet only, no stress, n=3), CUMS (CUMS protocol only, n=3), and CUMS+MCD (CUMS protocol combined with the MCD diet, n=3).

To maintain proper housing conditions during the study, mice in the WT and MCD groups were pair-housed (3 mice per cage), and mice undergoing the CUMS protocol were housed individually, as predetermined [30].

Histopathology and Immunohistochemistry

After anesthesia, animals were given an intracardiac perfusion consisting of 5 ml of saline followed by 5ml of 4% paraformaldehyde (PFA).

To prevent microglial activation, brains were then placed in 4% PFA at 4°C overnight [31].

Coronal 35µm brain sections, prepared with a vibratome and cut in 0.1 M phosphate-buffered saline (PBS), were used for immunohistochemistry.

Sections were initially incubated with 0.5% Triton X-100 and 5% horse serum in PBS at room temperature for 1h to permeabilize the tissues and prevent nonspecific binding.

Thereafter, they were incubated with the primary antibody rabbit anti-Iba1 (Wako, 019-19741, 1:1000) overnight at 4°C.

After washing with PBS (3x10min), the slices were incubated with the secondary antibody Alexa Fluor 647 donkey anti-rabbit (Invitrogen, A31573, 1:1000) for 2 hours at room temperature in the dark.

Finally, the stained brain sections were mounted using Fluoromount-G with DAPI (ThermoScientific, 00-4959-52).

This protocol guaranteed the optimal preservation and labeling of microglial cells, which facilitated the subsequent microscopic analysis.

Image analysis

For the analysis, Z-stack images of the hippocampal region were acquired using a 20×objective on a Zeiss LSM 900 Airyscan 2 confocal microscope, with Zen 3.5 software facilitating image capture (Figure 1A).

Microglial morphology was analyzed through a semi-manual quantification method as previously described [32,33].

Microglia exhibiting fully intact arborization within the Z-stack were manually selected for detailed analysis (Figure 1B, C).

To ensure accuracy and consistency, cells with processes extending beyond the Z-stack boundaries were excluded.

For each animal, a total of 10 microglial cells were analyzed.

Following the isolation of individual cells, skeletization techniques were applied to enable precise morphological quantification (Figure 1D, E).

Structural parameters were measured for each microglial cell, including the total length of the branching tree, the number of branches, the average branch length, and the distribution of branches across hierarchical levels [34].

These quantitative metrics were employed to identify and compare variations in microglial morphology between experimental groups.

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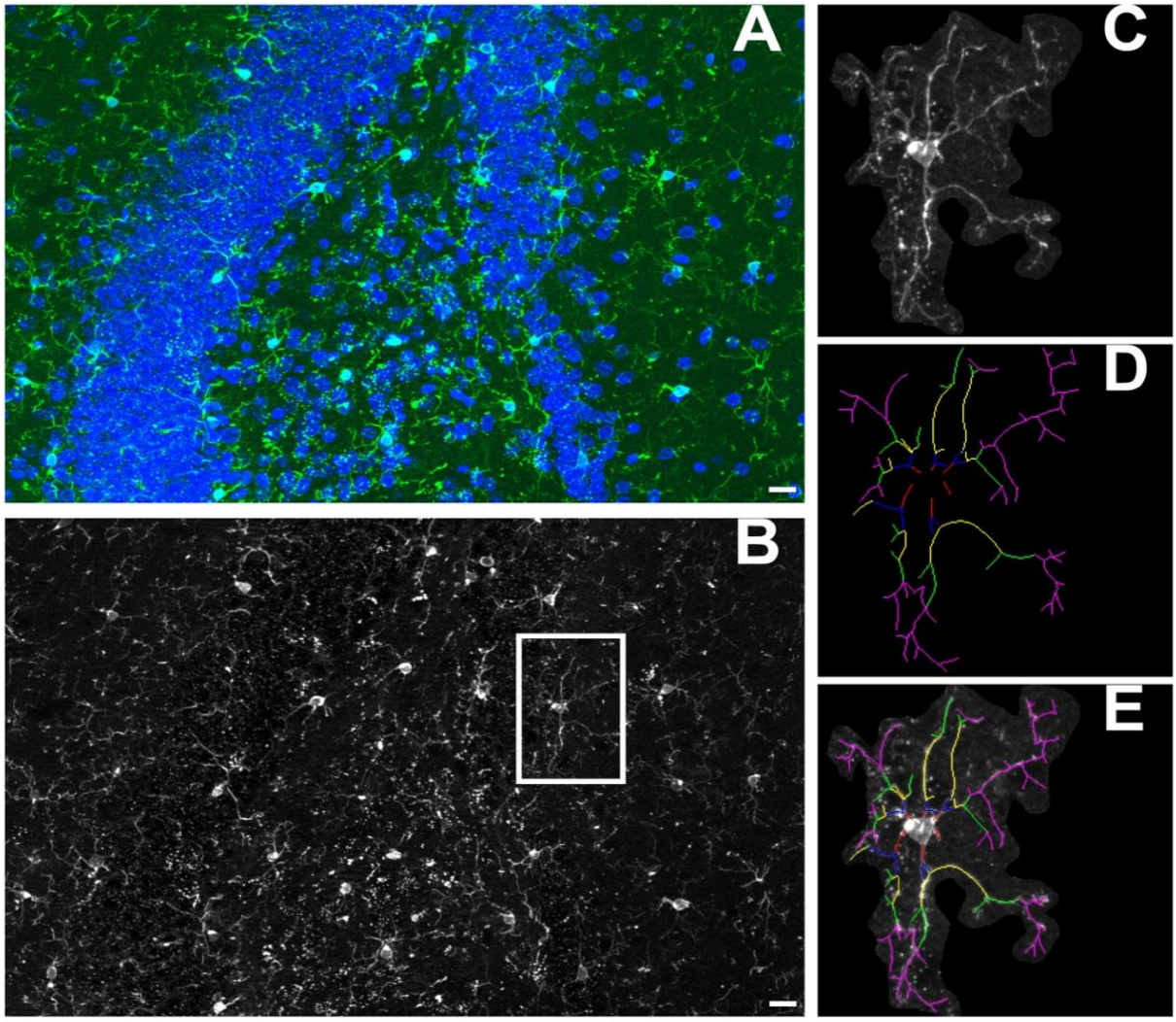

Figure 1. Schematic overview of the methodology used.
(A) Imaging of microglial cells in the mouse hippocampus. (B) A representative microglial cell (within the white square), illustrating the soma and fine processes. Each analyzed cell was (C) isolated, (D) manually traced, and (E) subsequent verified. Scale bar: 20µm.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 10.3.1 and Microsoft Excel.

Data normality was assessed using D'Agostino&Pearson test to ensure the appropriateness of parametric methods.

Group mean differences were analyzed using one-way ANOVA (Tukey's multiple comparisons test), with Column Factor defined as Protocols.

All data are presented as mean±standard deviation (SD) in the figures, with statistical significance indicated as follows: *p<0.05, ***p<0.001, and ****p<0.0001.

Results

Analysis of hippocampal microglia reveals that liver damage and depressive-like behavior induce notable morphological changes

Analysis of total branch length using one-way ANOVA showed significant differences between the Protocols ($F_{3,36}$=15.60, p<0.0001).

Depressive-like behavior and liver injury led to a decrease in total branch length in MCD (270.5±57.06µm, p<0.0001), CUMS (235.4±58.55µm, p<0.0001) and CUMS+MCD (265.5±56.77µm, p<0.0001) mice, compared to the WT group (409.4±74.72µm) (Figure 2A).

One-way ANOVA revealed differences among Protocols in the number of branches analysis ($F_{3,36}$=4.107, p=0.0132), with CUMS

animals displaying a decreased number (78.10±18.85), compared to WT mice (105.50±15.72) (p=0.0121) (Figure 2B).

Assessment of mean branch length using one-way ANOVA revealed differences between Protocols ($F_{3,36}$=15.19, p<0.0001).

Mice subjected to MCD (2.96±0.30µm, p=0.0001), CUMS (3.03±0.40µm, p=0.0003), and CUMS+MCD (2.67±0.31µm, p<0.0001) protocols exhibited decreased mean branch length, compared to WT animals (3.91±0.63µm) (Figure 2C).

Images of representative microglia from each experimental group are presented as follows: WT (Figure 2D), MCD (Figure 2E), CUMS (Figure 2F), and CUMS+MCD (Figure 2G).

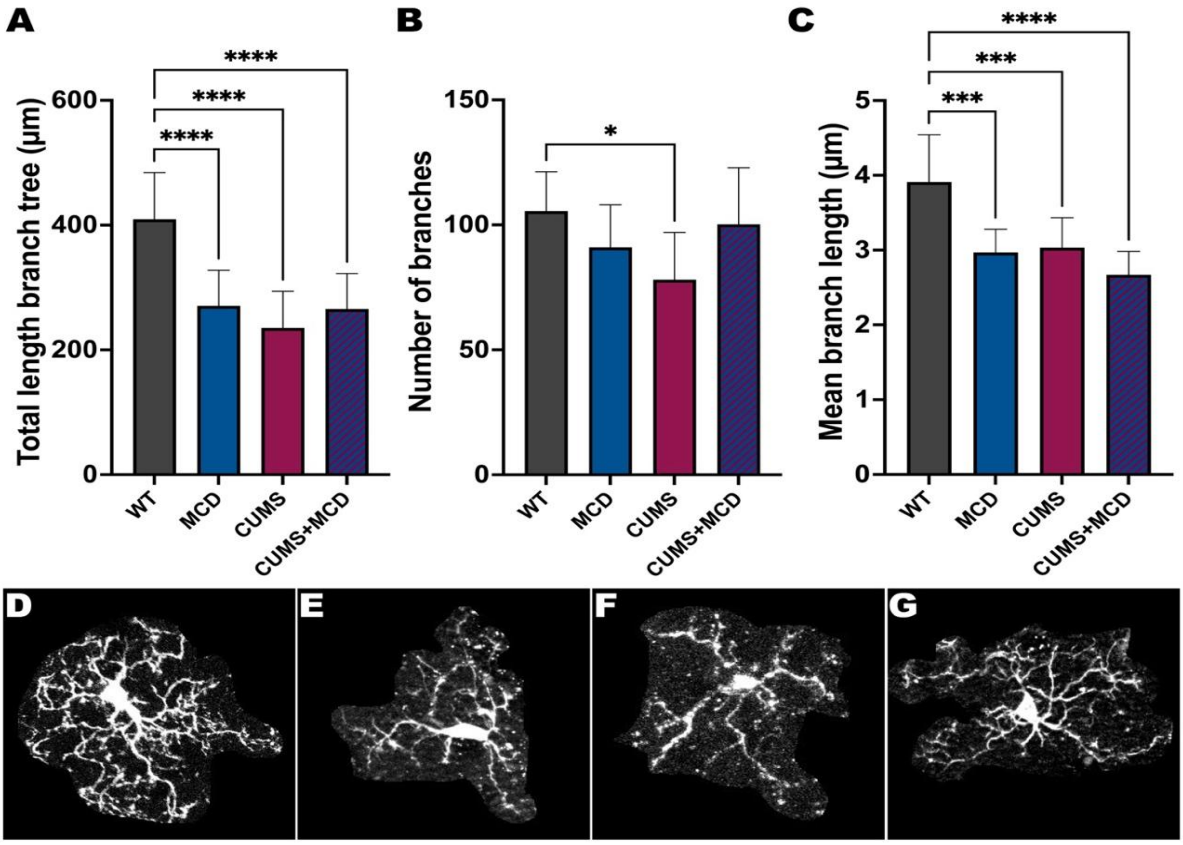

Figure 2. Microglial arbor morphology in hippocampus: The detailed morphology of microglia was assessed through a systematic analysis of individual branches, focusing on key parameters, including (A) total arbor length, (B) branch count, and (C) mean branch length. Representative images of microglia from each experimental group: (D) WT, (E) MCD, (F) CUMS, and (G) CUMS+MCD. Data are shown as mean±SD, with statistical significance represented as *p<0.05, ***p<0.001, and ****p<0.0001.

Application of the CUMS protocol results in alterations in the number of secondary and terminal branches in the hippocampus

Detailed evaluation of branch order revealed no difference between Protocols when looking at the number of primary branches ($F_{3,36}$=1.172, p=0.3339) (Figure 3A).

However, one-way ANOVA identified significant variations among Protocols in the number of secondary branches ($F_{3,36}$=2.994, p=0.0434), with CUMS mice displaying a reduced number of branches (8.70±1.25), compared to WT group (11.90±3.31) (p=0.0276) (Figure 3B).

No differences were observed between Protocols when applying one-way ANOVA on tertiary ($F_{3,36}$=2.393, p=0.0845) (Figure 3C) and quaternary ($F_{3,36}$=0.2987, p=0.8261) (Figure 3D) branches.

One-way ANOVA revealed differences among Protocols in the number of terminal branches ($F_{3,36}$=3.185, p=0.0353).

Animals subjected to CUMS protocol showed a decreased number of terminal branches (37.90±17.18), compared to CUMS+MCD mice (59.10±22.87) (p=0.0334) (Figure 3E).

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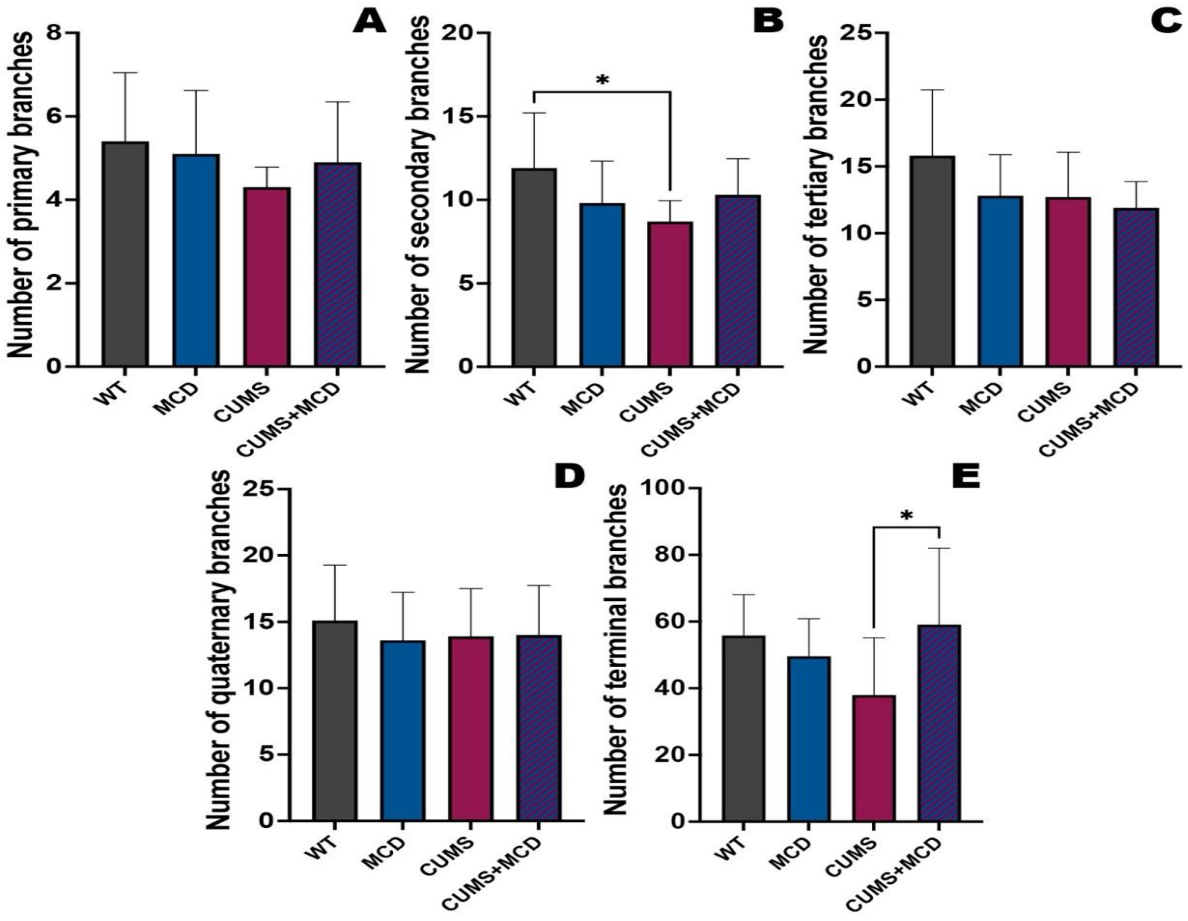

Figure 3. Detailed morphological analysis comparing differences between the number of (A) primary, (B) secondary, (C) tertiary, (D) quaternary and (E) terminal branches. Data are shown as mean±SD, with statistical significance represented as *p<0.05.

Discussion

Microglial morphology is a direct reflection of their functional state [35], ranging from the surveillant phenotype in homeostasis [36] to the amoeboid, activated phenotype during inflammation or injury [37].

In response to inflammatory stimuli or injury, microglia undergo a transformation characterized by retraction of their processes and enlargement of the soma, adopting an amoeboid shape [38].

This activated phenotype is associated with increased phagocytic activity, secretion of pro-inflammatory cytokines, and engagement in repair mechanisms [38].

The transition between these states is not binary but exists along a spectrum, with intermediate morphologies reflecting the graded nature of microglial responses [15].

The structural shifts observed in microglia are tightly coupled with their functional roles, making morphological analysis a powerful tool for understanding their state and behavior under various pathological conditions [15,39].

Chronic stress is a well-established risk factor for depression and has been shown to induce microglial changes in the hippocampus [40].

The hippocampus is particularly vulnerable to neuroinflammatory processes, given its role in stress regulation and cognitive function [41].

In the present study, mice exposed to CUMS protocol exhibited significant simplification of microglial arbors, characterized by shorter total branch length and fewer branches, confirming previous research stating that chronic stress model increased the presence of less branched $Iba1^{+}$ cells in the hippocampus [8], or that the complexity of microglia is significantly decreased after rodents are exposed to chronic stress [16].

Our results demonstrate that CUMS significantly reduces both secondary and terminal branches of microglia.

These findings align with reports that chronic stress increases the production of

pro-inflammatory cytokines, such as IL-1β and TNF-α, disrupting hippocampal synaptic plasticity and neurogenesis [8,42].

Furthermore, microglial pruning of synaptic connections may become excessive under stress conditions, contributing to the observed morphological simplifications [43].

Systemic inflammation and metabolic disturbances associated with liver injury exacerbate neuroinflammatory processes in the CNS [44].

NAFLD and related liver conditions are increasingly recognized for their contribution to cognitive dysfunction [21,22,45], depressive and anxiety disorders [46-48] and hippocampal damage [23,49].

Mice fed the MCD diet showed reduced total branch length and mean branch length.

Interestingly, the combination of CUMS and the MCD diet did not produce additive effects on microglial morphology, suggesting that both conditions may converge on similar inflammatory pathways.

Our findings support the hypothesis that microglial dysfunction contributes to the pathophysiology of depression and systemic disorders such as NAFLD [50-52].

The reductions in microglial arbor complexity observed in this study may impair the cells' ability to survey their microenvironment, prune synapses appropriately, and support neuronal health.

These dysfunctions could exacerbate the structural and functional deficits observed in depressive disorders and cognitive impairments linked to liver injury.

This study has some limitations that warrant further investigation.

First, the small sample size may limit the generalizability of the findings.

Second, while morphological analysis provides insights into microglial state, it does not capture the full spectrum of functional changes, such as cytokine production or phagocytic activity.

Future studies should incorporate functional assays and transcriptomic analyses to better understand the molecular pathways involved.

Conclusion

This research reveals that both depressive-like behavior and liver injury independently and synergistically affect microglial morphology, with significant reductions in total branch length, number of branches, and mean branch length compared to controls.

Furthermore, detailed analysis of branch order highlights specific alterations in secondary and terminal branches, suggesting that these pathological states disrupt microglial arborization patterns in a hierarchical manner.

These results not only advance our understanding of microglial responses to systemic and neuropsychiatric stressors but also underscore the hippocampus as a critical site of neuroinflammation in the context of liver damage and depression.

Data Availability Statement

The corresponding author can provide the data described in this study upon request.

Authors' contribution

Gabriel Nedelea and Mădălina Iuliana Mușat contributed equally to the present manuscript and therefore share the first authorship.

Conflict of interests

The authors declare that they have no conflict of interests.

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