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TITLE: Potential role of the microbiome-endocannabinoidome connection in the gut-brain axis after traumatic brain injury and its association with Alzheimer's disease

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8. PERFORMING ORGANIZATION REPORT NUMBER

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U.S. Army Medical Research and Development Command
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14. ABSTRACT

Based on published preclinical, clinical, and epidemiological data, we hypotesised that the gut-brain axis and the connection with the endocannabinoidome may peripherally influence the physiopathology of TBI and the subsequent risk of latent neurodegenerative diseases. Therefore, the objective of this research is to investigate the effects of a mild TBI on the subsequent development of Alzheimer's disease-related neuropathology and cognitive impairments in an APP/PS1 mice, the role of inflammation, the potential perturbation of the gut microbiome and how the potential alteration in gut microbiota composition may determine the severity of these disorders by regulating the activity of endocannabinoid and related mediators using a multidisciplinary approach. To date, our data in control mice confirm previous studies showing that the mTBI induces a induces a characteristic dual behavioral phenotype (aggressive/depressive) in mice In addition, we demonstrate that mTBI causes significant impairments in the discriminative and spatial memory tasks.

15. SUBJECT TERMS Microbiome, TBI, Alzheimer, endocannabinoids, gut-brain					
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1. INTRODUCTION:

Background: Traumatic brain injury (TBI) is the leading cause of death under the age 45 in the Western World and is followed by secondary brain damage leading to long-term consequences, such as increased prevalence of dementia, and Alzheimer's disease (AD). Recent evidence suggested that both TBI and AD have an alteration in the brain-gut microbiota axis that may significantly contribute to their pathogenesis and could be the missed link to understand their association. Furthermore, accumulating evidences in literature have showed that the endocannabinoid (eCB) system with the accompanying "endocannabinoidome" (eCBome), have a key role in numerous physiological and pathological conditions, including neuroprotection. The endocannabinoidome is increasingly emerging as a system of lipid mediators of the health-disease continuum and its strong connection with the gut microbiome has been so far suggested only in the context of inflammatory metabolic and intestinal disorders and has never been investigated in other disorders. **Hypothesis**: Based on published preclinical, clinical, and epidemiological data, we propose a theoretical framework that highlights the potential mechanisms by which the gut-brain axis and the connection with the eCBome may peripherally influence the physiopathology of TBI and the subsequent risk of latent neurodegenerative diseases. **Specific aims**: Therefore, the objective of this research is to investigate the effects of a mild TBI on the subsequent development of AD-related neuropathology and cognitive impairments in an APP/PS1 mice, the role of inflammation, the potential perturbation of the gut microbiome and how the potential alteration in gut microbiota composition may determine the severity of these disorders by regulating the activity of endocannabinoid and related mediators. **Research strategy:** We plan to analyse the microbiome of amyloid precursor protein (APP)/PS1 mice after mTBI and their healthy controls. Feces and intestinal tissues from these animals will be used to compare the taxonomic composition, genome, transcriptome, proteome and metabolome of the gut microbiome. The endocannabinoidome will be profiled in the gut and in other target tissues, with particular emphasis on brain. Microbiome analyses will be related to the biochemical characterization of the endocannabinoidome in key target tissues. To accomplish this aim we will take advantage to be part of the Joint International Research Unit (UMI) that is a bilateral research unit between the Italian National Research Council (CNR) and the Université Laval of Quebec that has among its proposed ambitious goals the development of research projects, and the innovation, education and knowledge transfer in the emerging field of the biomolecular study of the intestinal microbiome. **Innovation and impact:**

This represents an unique opportunity to carry out this pilot study that could open new perspectives for the development of novel microbiome-based interventions for neurodegenerative diseases and to prevent long-term consequences of TBI.

2. KEYWORDS:

mTBI, behavior, cognition, Alzheimer, endocannabinoids, microbiome, gut-brain axis

3. ACCOMPLISHMENTS:

What were the major goals of the project?

SOW Task	Timeline for completion	Progress
Major Task 1 "Characterization of behavioral impairments mTBI-induced in mice and identification of novel biomarkers.**"** Subtask 1: Institutional and ACURO approvals. Italian Ministry of Health approval	**Y1Q1Q2**	Complete
Subtask 2: Inducing mTBI in wild type mice and behavioral characterization	Y1Q3	Completed
Subtask 3: Specific markers analysis, brain immunohistochemistry and molecular biology analyses	Y1Q4	In progress

What was accomplished under these goals?

To achieve the specific aim 1, institutional and ACURO approvals were obtained. We have also obtained approval from Italian Ministry of Health with the authorization number 1185/2020-PR.

Moreover, we have performed in vivo experiments as indicated in the subtask 2. Specifically, we induced the mild traumatic brain jury (m-TBI) in wild type mice for the behavioral characterization. Based on our previous studies, behavioral tasks have been performed at different time points, as follows:

- Aggressiveness at day 15 (Resident intruder).
- Exploratory activity at days 30 and 60 (Open field).
- Cognitive performance and memory at 30 and 60 days (Y-maze, Novel object recognition test)
- Depressive-like behavior at 15, 30, 45 and 60 days (Tail suspension Test)
- Social interaction at 60 days (Three chamber sociability).

As compared with controls, mTBI mice showed an aggressive behavior, as indicated by the increased numbers of attacks and duration of total fighting and the reduced latency to the first attack (fig.1).

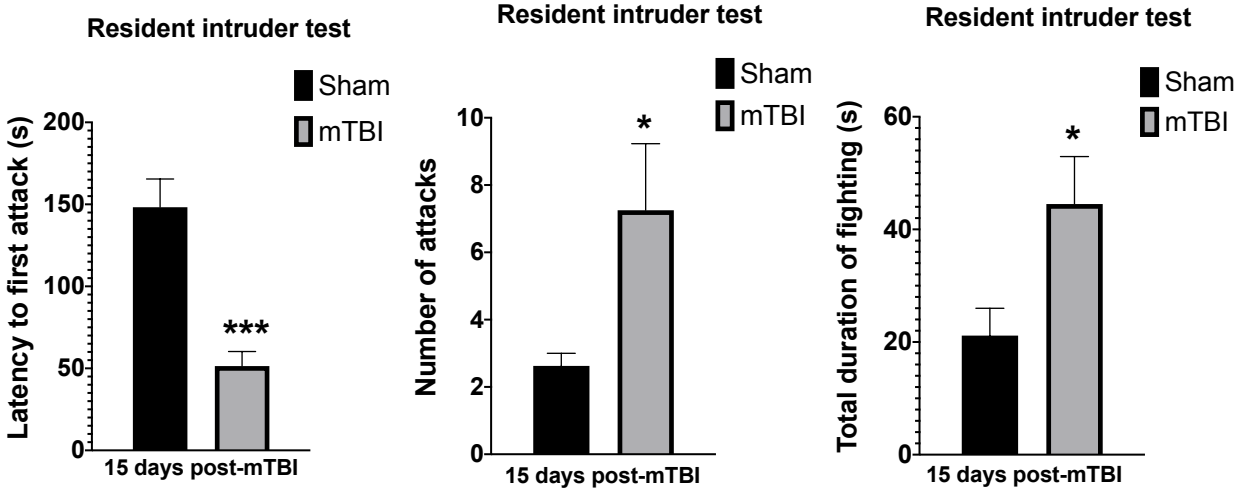

Figure 1. Resident intruder test

The open field test was used to evaluate the general exploratory activity, as well the possible anxiety-like behaviour induced by the trauma. We found that while the travelled distance or the total number of transitions was not changed in traumatized mice, as compared with control, the time spent in the center was reduced suggesting an anxiety-like behavior (fig. 2).

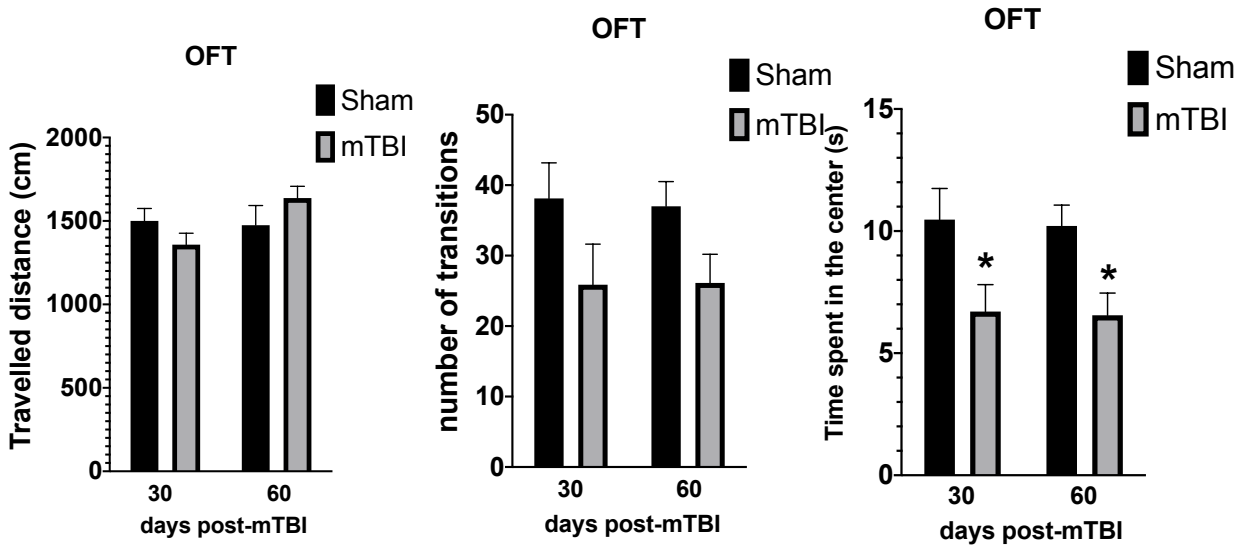

Figure 2. Open field test

Cognitive impairments, measured as time spent in the novel arms, as well as the latency to entry, in the forced Y maze test were detected at 30- and 60-days post trauma (fig. 3).

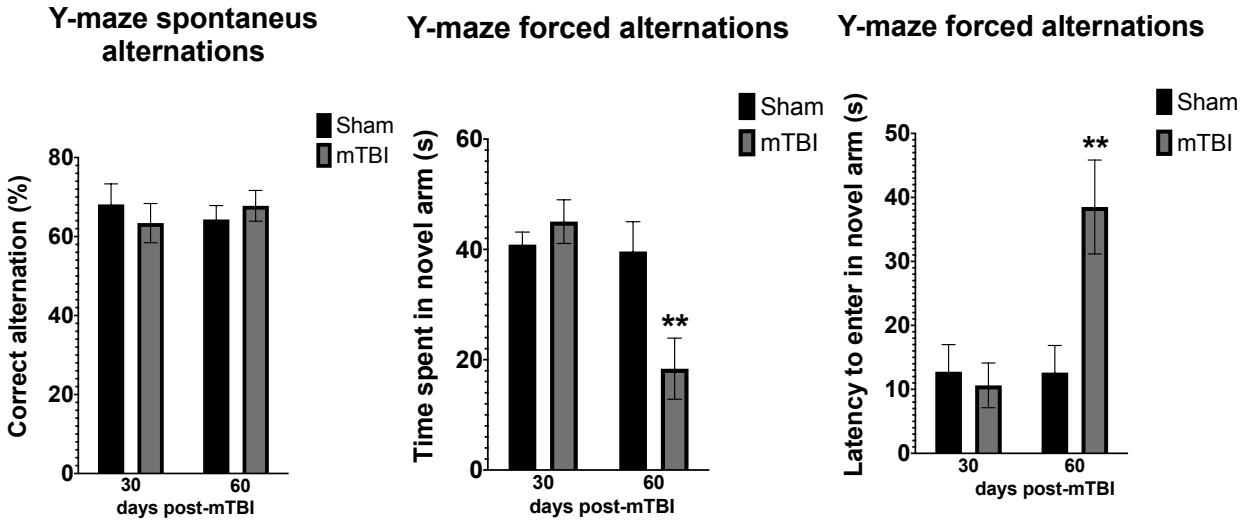

Figure 3. Forced Y maze test.

At 60 days, mTBI animals also showed a reduced recognition index in the novel object recognition, indicating a reduced discriminative memory performance (fig.4).

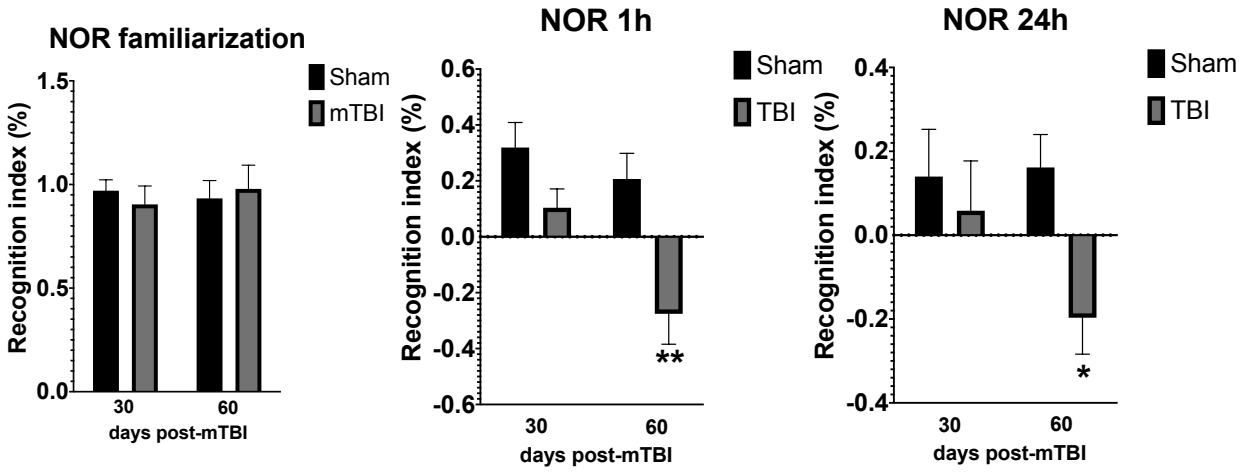

Figure 4. Novel Object Recognition test.

Depressive behavior was monitored across the entire behavioral testing. We observed an increase in the immobility time in tail suspension test at 45 and 60 days after trauma (fig. 5), index of a reluctance to maintain an active escape-oriented behaviour.

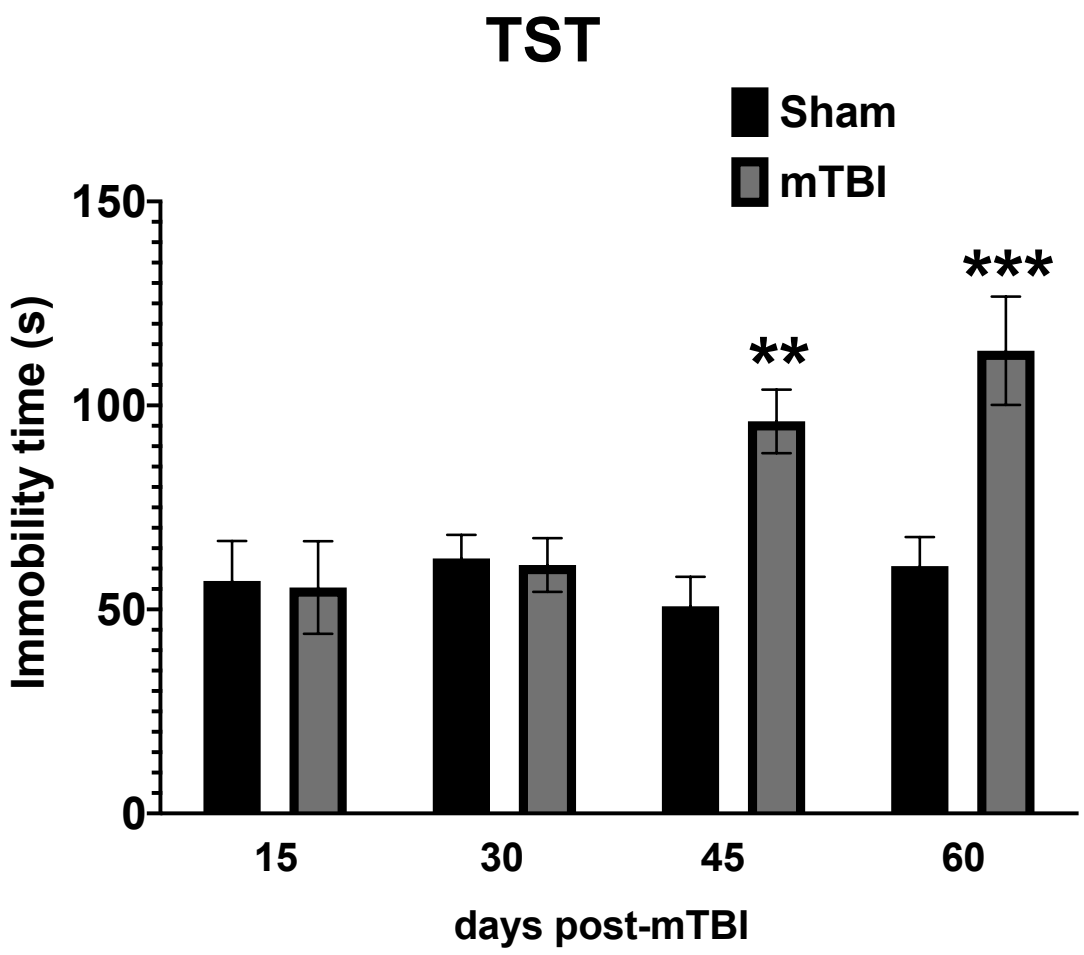

Figure 5. Tail suspension test.

This data was also supported by the reduced social interaction observed in the Three chamber sociability (fig. 6).

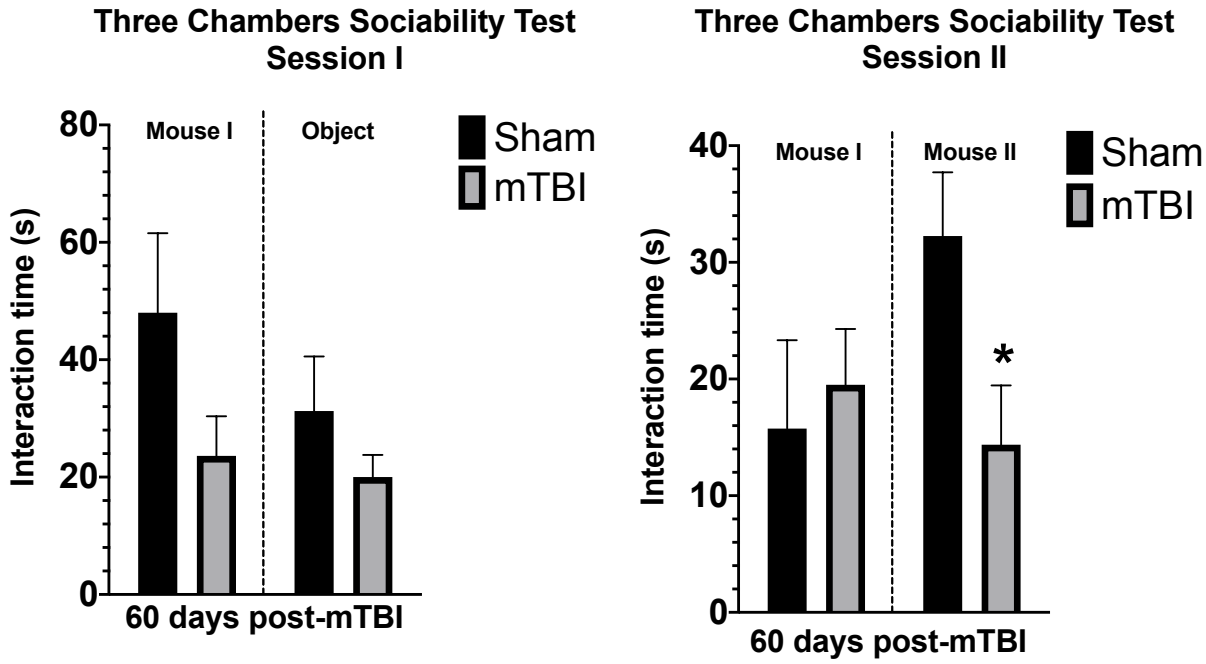

Figure 6. Three chamber sociability test.

In conclusion our data confirm previous studies showing that the mTBI induces a induces a characteristic dual behavioral phenotype (aggressive/depressive) in mice (Guida et al., 2017; Belardo et al., 2019 and Piscitelli et al., 2019). In addition, we demonstrate that mTBI causes significant impairments in the discriminative and spatial memory tasks.

Methods

Mild TBI Induction. Experimental mTBI was performed using a weight-drop device developed in our laboratory. Mice were anesthetized with intraperitoneal injection of Tribromoethanol (250 mg/kg) and placed in a prone position on a spongy support. The head was not fixed. After a midline longitudinal incision, the skull was exposed to locate the area of impact and placed under a metal tube device where the opening was positioned directly over the animal's head. The injury was induced by dropping a cylindrical metal weight (50 g), through a vertical metal guide tube from a height of 20 cm. The point of impact was between the anterior coronal suture (Bregma) and posterior coronal suture (Lambda). Immediately following injury, the skin was closed with surgical wound clips and mice were placed back in their cages to allow for recovery from the anesthesia and mTBI. Sham mice were submitted to the same procedure as described for mTBI, but without release of the weight (Guida et al., 2017, Frontiers).

Resident-Intruder. At 15 days after mTBI or sham surgery, mice were tested for aggressive behavior using a resident intruder test. Mice were individually housed for 1 week in Plexiglas cages to establish a home territory and to increase the aggression of the resident experimental mice. To begin, food containers were removed, and an intruder mouse of the same gender was placed in a resident home cage and resident–intruder interactions were analyzed for 10 min. The aggressive

behavior of resident socially-isolated mice was characterized by an initial pattern of exploratory activity around the intruder, which was followed by rearing and tail rattle, accompanied in a few seconds by wrestling and/or a violent biting attack. The number of these attacks and latency to the first attack during the 10 min observation period was recorded

Open Field. At 30 and 60 days after mTBI or sham surgery, mice were tested for motor activity and anxiety-like behavior. Test Motor activity was also evaluated by open field test in sham and mTBI mice. Briefly, each mouse was individually monitored for 5 min in an open arena (l × w × h: 25 cm × 25 cm divided into 16 square grids). Parameters evaluated included: (1) number of transitions; and (2) number of rearings; and (3) time spent in the periphery or center (s).

Novel Object Recognition (NOR). To assess learning and long-term memory the Novel Object Recognition (NOR) task was used at 30 and 60 days after mTBI . Two identical objects were placed into the arena during a 6 min sample phase. One of the objects was exchanged by a new object and memory was assessed by comparing the time spent exploring the novel object as compared with the time spent exploring the familiar object during a 5 min test phase. One week before the NOR experiments, the animals experienced handling by the experimenter and habituation to the arena for 5 consecutive days and before the habituation, respectively. For habituation, mice were placed into the empty arena (40 × 30 × 30 cm width × length × height, PVC) for 60 min. For NOR experiments custom-built plastic pieces (Polyoxymethylen, POM), were used with different shapes (bell: 5 cm diameter, 6 cm height; diamond: 7 x 7 x 7 cm; cube 5 x 5 x 5 cm) and same colour (black) or different colour and size (glass: 8.3 cm diameter, 8.5 cm height; cup: 6 cm diameter, 6 cm height). The objects were cleaned thoroughly with 70% ethanol followed by distilled water between trials to remove olfactory cues. During the sample phase on the first day of the NOR test, the mice were allowed to explore the two identical black objects (two bells) for 6 min. For the short-delay test phase (1.5 h) one of the sample objects was replaced by a new one (bell by diamond) and exploration was measured for 5 min. For the long-delay test phase (24 h) the new object was again exchanged by another new object. The location of the novel object at 24 h was always different from that at 1.5 h, either first left then right, or vice versa. Consequently, the location of the familiar object also switched between the two test phases. Objects with the same colour but different shapes were considered to be similar to acquisition object. Active exploration was defined as direct sniffing or whisking towards the objects or direct nose contact. Climbing over the objects was not counted as exploration. The relative exploration was quantified by normalizing the difference between the exploration time of the novel (Tn) and familiar object (Tf) by the total time of exploration (Ttot) to calculate the NOR discrimination index: NOR index=(Tn–Tf)/Ttot. With identical acquisition objects the NOR index was always less than 0.2 indicating that there was no side preference in the mice used for the study.

Y maze. To assess spatial memory the Y maze test was used at 30 and 60 days post injury. The apparatus consisted of three enclosed arms (30 × 5 × 15 cm; length x width x height) converging on an equilateral triangular center (5 × 5 × 5 cm). At the beginning of each experimental session, each mouse was placed in the center platform and the number of spontaneous alternations (defined as number of successive triplet entry into each of the three arms without any repeated entries) was monitored in a 5 min test session. The percentage of alternation was calculated as the percentage of the ratio of the number of alternations/ (total number of arm entries − 2). The forced alternation was performed according to Wolf et al., 2014, PlosOne.

Tail Suspension Test (TST). The Depression like behavior was evaluated at 15, 30, 45 and 60 days after mTBI or sham surgery, mice were individually suspended by the tail on a horizontal bar (55 cm from floor) using adhesive tape placed approximately 4 cm from the tip of the tail. The duration

of immobility, recorded in seconds, was monitored during the last 4 min of the 6-minute test by a time recorder. Immobility time was defined as the absence of escape-oriented behavior. Mice were considered to be immobile when they did not show any body movement, hung passively and completely motionless.

Three Chambers Sociability. Test at 60 days after mTBI or sham surgery, mice were tested for social interaction using a three-chambered social interaction apparatus. A plexi-glass three-chambered box was custom-built as follows: doorways in the two dividing walls had sliding covers to control access to the outer-side chambers. The test consisted of two consecutive stages of 5 and 10 min each. During the 5-minute first stage of habituation the mouse was allowed to freely explore the three chambers of the apparatus, detecting at this stage any innate side preference. After that the mouse was gently encouraged into the central chamber and confined there briefly by closing the side chamber doors. During the following 10-minute stage sessions, a custom made stainless-steel barred cup (6.5 cm × 15 cm) was placed upside down in one of the side chambers. A never before-met intruder, previously habituated, was placed into an upside-down identical cup in the other chamber. The time spent sniffing each upside-down cup, the time spent in each chamber and the number of entries into each chamber were recorded.

Statistical analysis

Data analysis was performed by Prism Software 9.0. Data are represented as mean ± SEM of 8-10 mice per group, (∗) P< 0.05, (∗∗) P 0.01, and (∗∗∗) P <0.001 versus Sham group. Unpaired T test in the Resident intruder and Sociability. Two way ANOVA followed by post hoc Sidak for all other tests.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

In next quarter, we will start the specific markers analysis, brain immunohistochemistry and molecular biology analyses as stated in the SOW.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Actual or anticipated problems or delays and actions or plans to resolve them

As stated in all quarter reports, we have encountered many delays due to failure in requesting anticipating funding (we have asked for 4 GV but requested have failed). While the project is proceeding quite well from the scientific point of views, lack or delay in receiving feedback from the Financial Officer risk to create some troubles or delay in the correct submission of the financial report.
One mouse died at the arrival day but since the target required for statistical significance was 24 for each group, this issue was not considered as a problem.

Changes that had a significant impact on expenditures

Increased cost for mice and delays in recruiting PD in the second period due to delay in proceed the GV requested.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

No use of human subjects

Significant changes in use or care of vertebrate animals

One mouse died at the arrival day but since the target required for statistical significance was 24 for each group, this issue was not considered as a problem.

Significant changes in use of biohazards and/or select agents
Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications.

  Nothing to Report

  Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

  Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Fabiana Piscitelli*
Project Role: *PD*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Dr Piscitelli has prepared all the documentation needed to start the project, order mice and has supervised all the experimentation.*

Name: *Francesca guida*
Project Role: *co-PI*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Dr. Guida has prepared the documentation for the Italian Ministry of Health approval. Dr. Guida has participated in the in vivo experimentation and supervised the behavioral tests.*

Name: *Serena Boccella*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Dr. Boccella has done in vivo experimentation.*

Name: *Sabatino Maione*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Prof. Maione conducted animal surgery and behavioral analysis*

Name: *Livio Luongo*
Project Role: *Other professional*

Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Prof. Luongo is conducting experiments for the avaluation of neuroinflammation

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Provide the following information for each partnership:
Organization Name: ***Università della Campania "L. Vanvitelli"***
Location of Organization: (if foreign location list country) ***Via Costantinopoli 16, 80138 Napoli, Italy***

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: