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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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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**	Completed
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	Completed
Major Task 2: to investigate if the GBM-eCBome is altered in a murine model of mTBI, using a novel integrative approach, the endocannabinoidomics	**Y2Q1Q2**	
Subtask 1: Gut microbiome and eCBome analyses	Y2Q1-Q4	In progress
Major Task 3: to characterize the mTBI- mediated behavioral and biochemical dysfunctions in a transgenic AD mouse model.	**Y2Q3Q4**	**In progress**
Subtask 1: Inducing mTBI in APP mice and behavioral characterization (N=50)	**Y2Q3**	**Completed**

Subtask 2: Specific markers analysis, brain immunohistochemistry and molecular biology analyses	**Y2Q4**	**In progress**

What was accomplished under these goals?

We have performed in vivo experiments as indicated in the subtask 1 and we have performed statistical analysis to compare the in vivo tests in APP mice to WT animals. Specifically, behavioral tasks have been performed at different time points in both genotypes (WT and APP), as follows:

- Aggressiveness at day 15 (Resident intruder, RIT).
- Exploratory activity at days 30 and 60 (Open field, OF).
- Cognitive performance and memory at 30 and 60 days (Y-maze, Novel object recognition test, NOR)
- Depressive-like behavior at 15, 30, 45 and 60 days (Tail suspension Test, TST)
- Social interaction at 60 days (Three chamber sociability).

As compared with controls, WT-mTBI mice showed an aggressive phenotype. However, sham APP mice showed per sè an aggressive behavior and APP-mTBI mice reported no significant difference in comparison to APP-sham and WT-mTBI (Fig. 1).

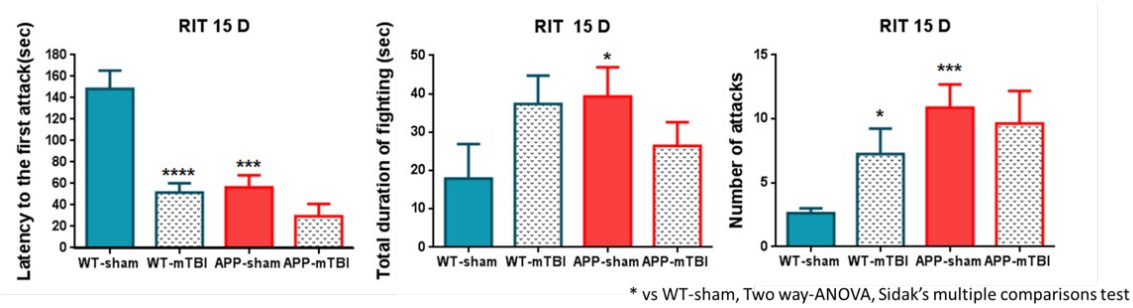


Figure 1. Resident intruder test

The open field test was used to evaluate the general exploratory activity, as well the possible anxiety-like behaviour induced by the trauma and to explore if the trauma worsened this behavior in APP mice. We found that while the travelled distance or the total number of transitions was not changed in WT-mTBI mice, as compared with control, the time spent in the center was reduced (at both 30 and 60 days) suggesting an anxiety-like behavior (Fig. 2). On the other hand, in APP-sham and mTBI mice, data showed an increase of total travelling distance, indicating an hypermotility (Fig. 2).

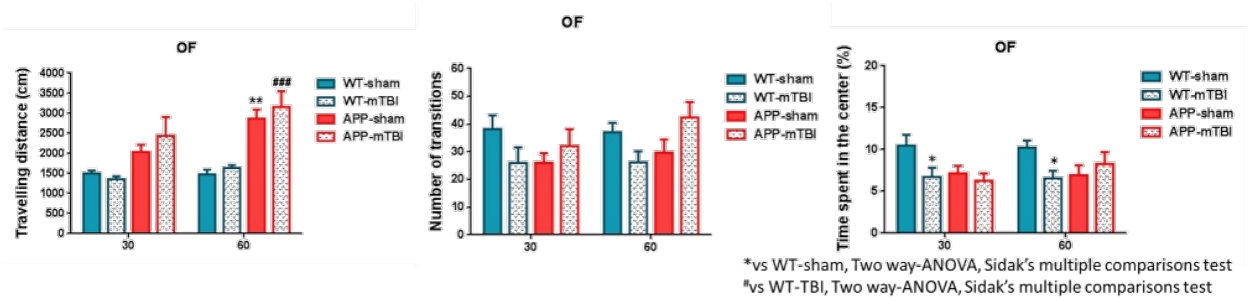


Figure 2. Open field test

Cognitive impairments in term of spatial memory, 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 in WT mice. In APP mice, both sham and mTBI, we found a decrease in the latency to enter, as well as, in the time spent in the novel arm.

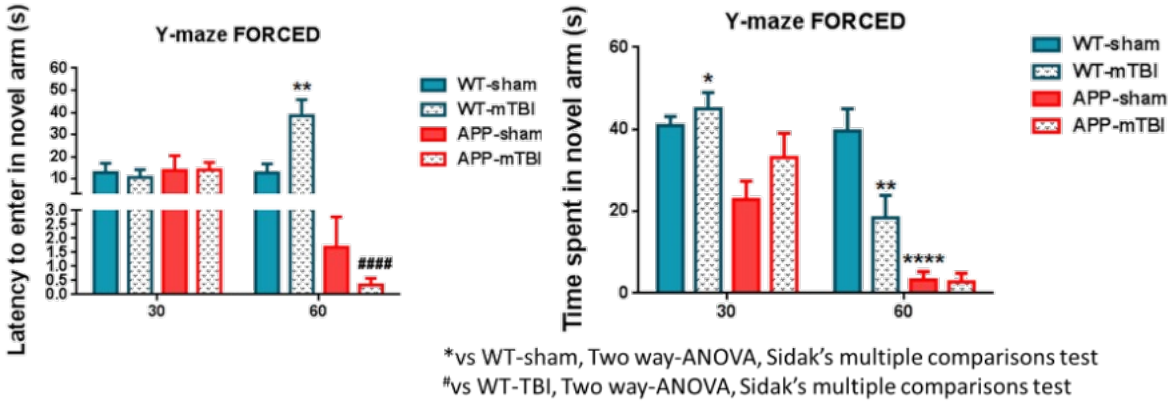


Figure 3. Forced Y maze test.

At 60 days, WT mTBI animals showed a reduced recognition index as well as APP-mTBI mice at 30 days, indicating an impaired discriminative memory performance (fig.4).

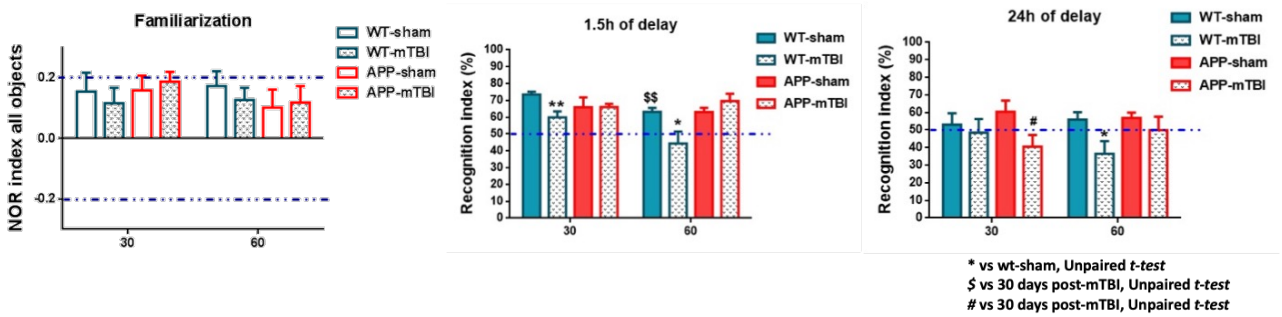


Figure 4. Novel Object Recognition test.

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. However, compared to WT, APP mice presented a strong reduction of immobility time.

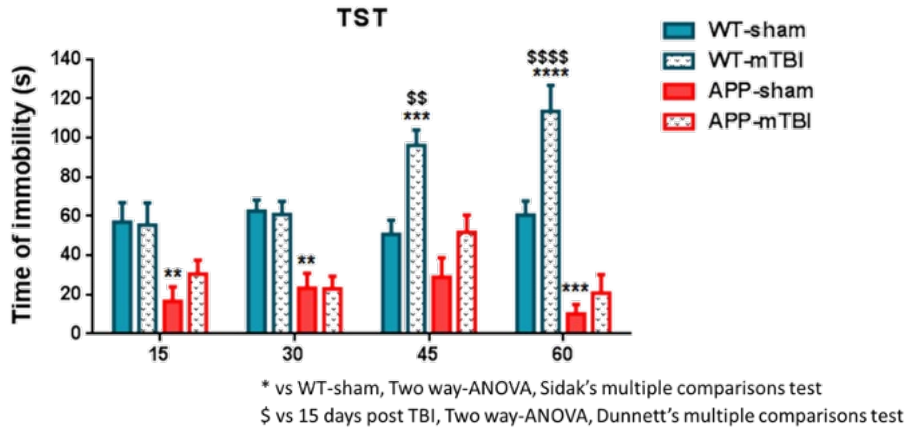


Figure 5. Tail suspension test.

This data was also supported by the reduced social interaction observed in the Three chamber sociability for WT mice (fig. 6). The reduced social interaction was not observed in APP injured animals. APP sham mice showed an alteration in social behavior as compared with WT ones, but no significant change between APP sham and mTBI groups was observed.

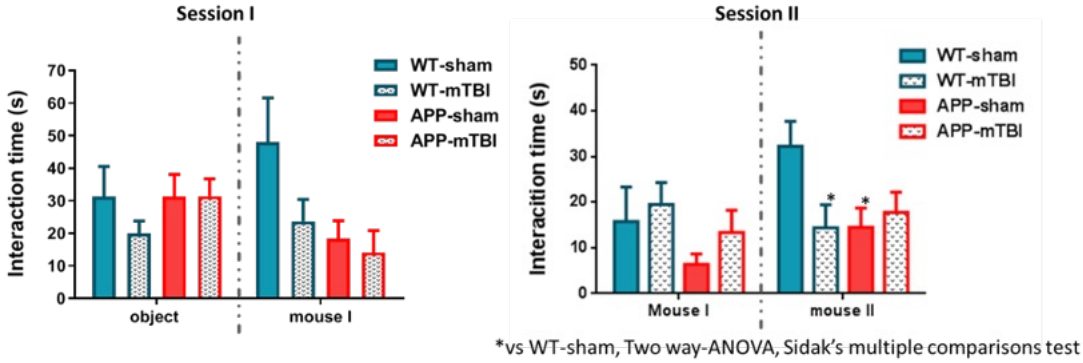
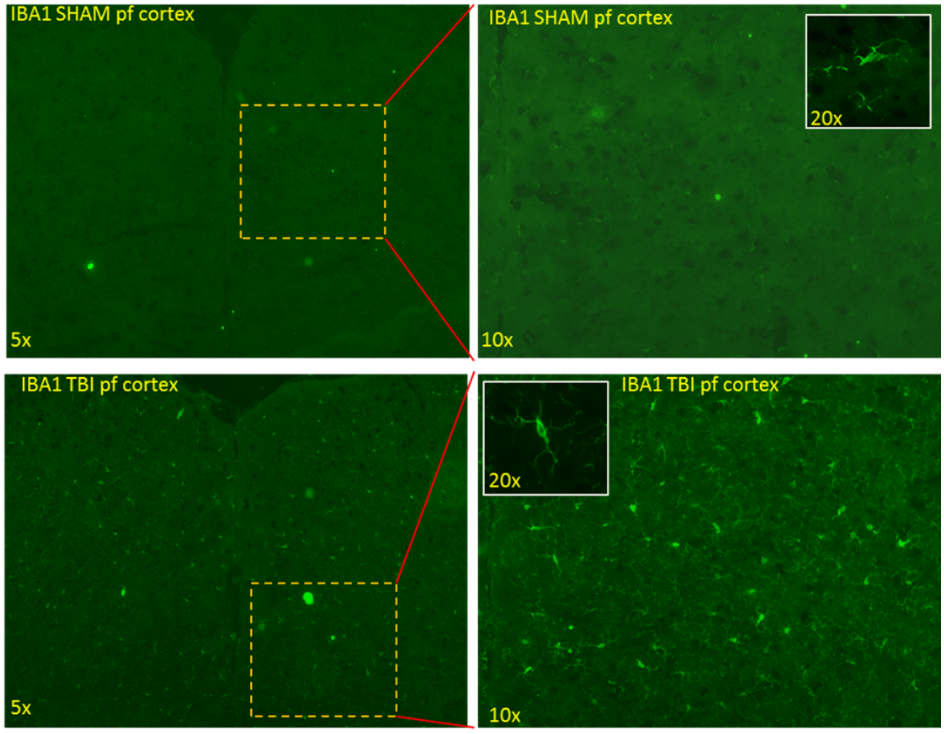


Figure 6. Three chamber sociability test.

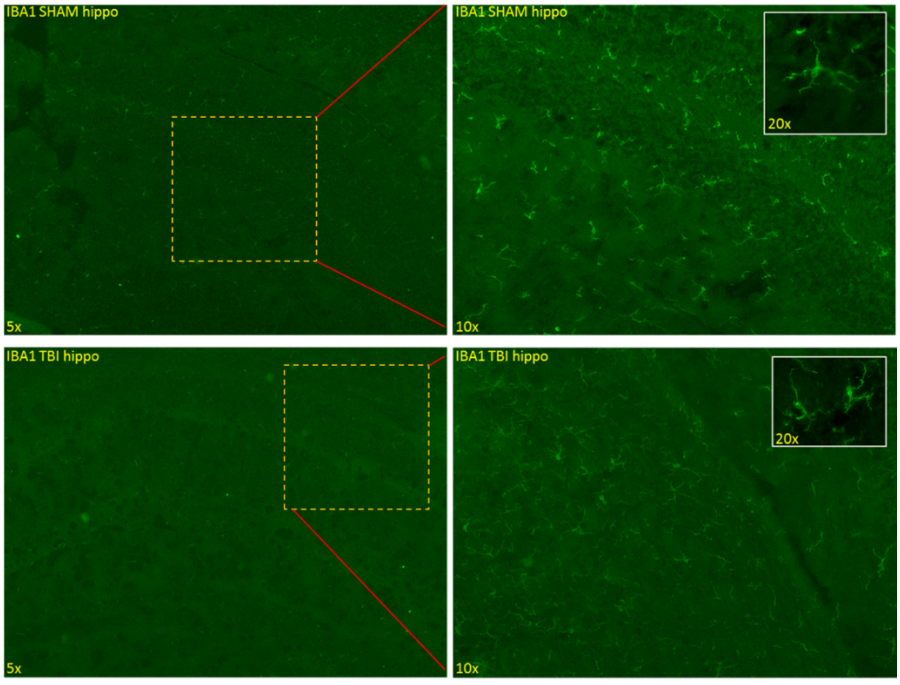
In conclusion our data confirm previous studies showing that the mTBI induces a induces a characteristic dual behavioral phenotype (aggressive/depressive) in WT mice (Guida et al., 2017; Belardo et al., 2019 and Piscitelli et al., 2019). In addition, we demonstrate that mTBI causes significant impairments in the discriminative and spatial memory tasks. However, the experiments performed reveal that mTBI does not induce significant changes in APP animals. This finding may due to the behavioral alterations that we detected in sham APP mice as compared with WT. Indeed, as a modelling of psychological symptoms of dementia, APP transgenic mice present a hyperactive and agitated phenotype (Lalonde et al., 2012). Thus, we cannot exclude that the behavioral impairments induced by the trauma were not evident given the basal behavioral alterations.

In the first quarter of this year we have completed the subtask 3 by performing analysis of brain immunohistochemistry. In particular, according to our previous studies (Rinaldi et al., Neural Plast. 2015), Iba-1-positive cells were identified as resting (with small somata bearing long, thin, and ramified processes), activated microglia (with hypertrophy together with retraction of processes to a length shorter than the diameter of the somata), or dystrophic microglia. Dystrophic microglia were recognized by debris consisting of several cells displaying fragmented processes and an irregularly shaped cell body as previously demonstrated (Cristino et al., Neurobiol. Aging. 2015). Our preliminary data indicate an increase of the total number, activated cell number and dystrophic number, of microglia cells in mTBI as compared to controls in both cortex and hippocampus (Figure 7A and B, respectively).

A


IBA1 SHAM pf cortex
5x
IBA1 SHAM pf cortex
20x
10x
IBA1 TBI pf cortex
5x
IBA1 TBI pf cortex
20x
10x

B


IBA1 SHAM hippo
5x
IBA1 SHAM hippo
20x
10x
IBA1 TBI hippo
5x
IBA1 TBI hippo
20x
10x

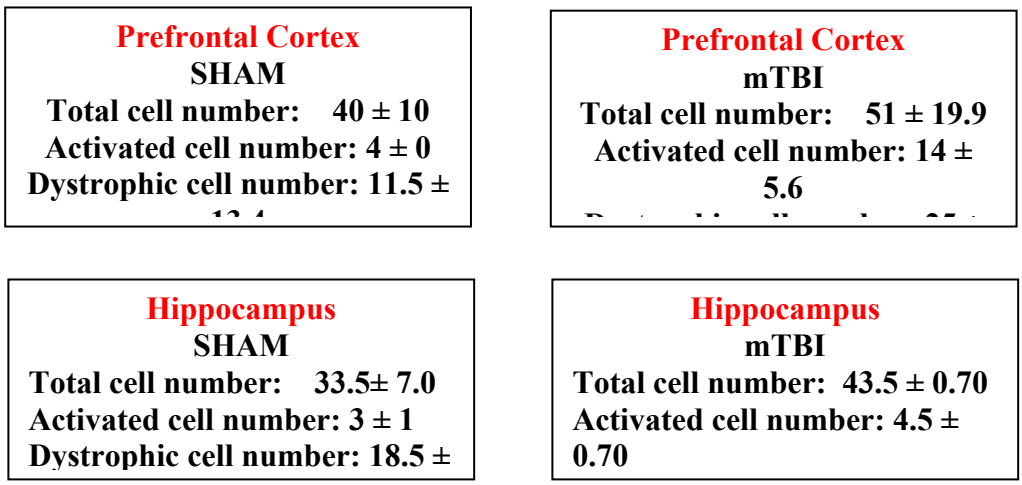


Figure 7. Representative images and quantitative analysis of activated Iba-1 positive cells in prefrontal cortex (A) and hippocampus (B) of sham and mTBI mice 60 days after injury. Data are expressed as the number of the cells/area (n = 2 mice per group), scale bars 5 and 20 µm.

Moreover, we have carried out lipidomic analysis of target tissues to investigate the role of the endocannabinoidome and related lipid mediators. Currently, we have analyzed eCBome mediators in cortex, hippocampus, feces and in the intestine (ileum and cecum) of sham and TBI WT-mice and significant alterations were observed (Figure 8-12).
In particular, as shown in Fig 8, 2-AG levels in cortex of TBI significantly decreased (Figure 8B) and also OEA (Figure 8D), whereas two members of the *N*-acylserotonines family identified (OA5HT and DHA5HT, Figure 8I and L, respectively) increased vey significantly. On the other hand, in the hippocampus, among monoacylglycerols, just 2-DHG levels were altered significantly and decreased in TBI group (Figure 9G). Instead, among *N*-acylserotonines, OA5HT decreased significantly after TBI (Figure 9I). Interestingly, *N*-acylserotonines are known to inhibit eCB inactivation by FAAH and at same time to antagonize TRPV1 channel, another important target for eCBome mediators. This opposite behavior in the two brain areas analysed need further investigations to assess which molecular target is involved and correlate these levels to the in vivo tests.

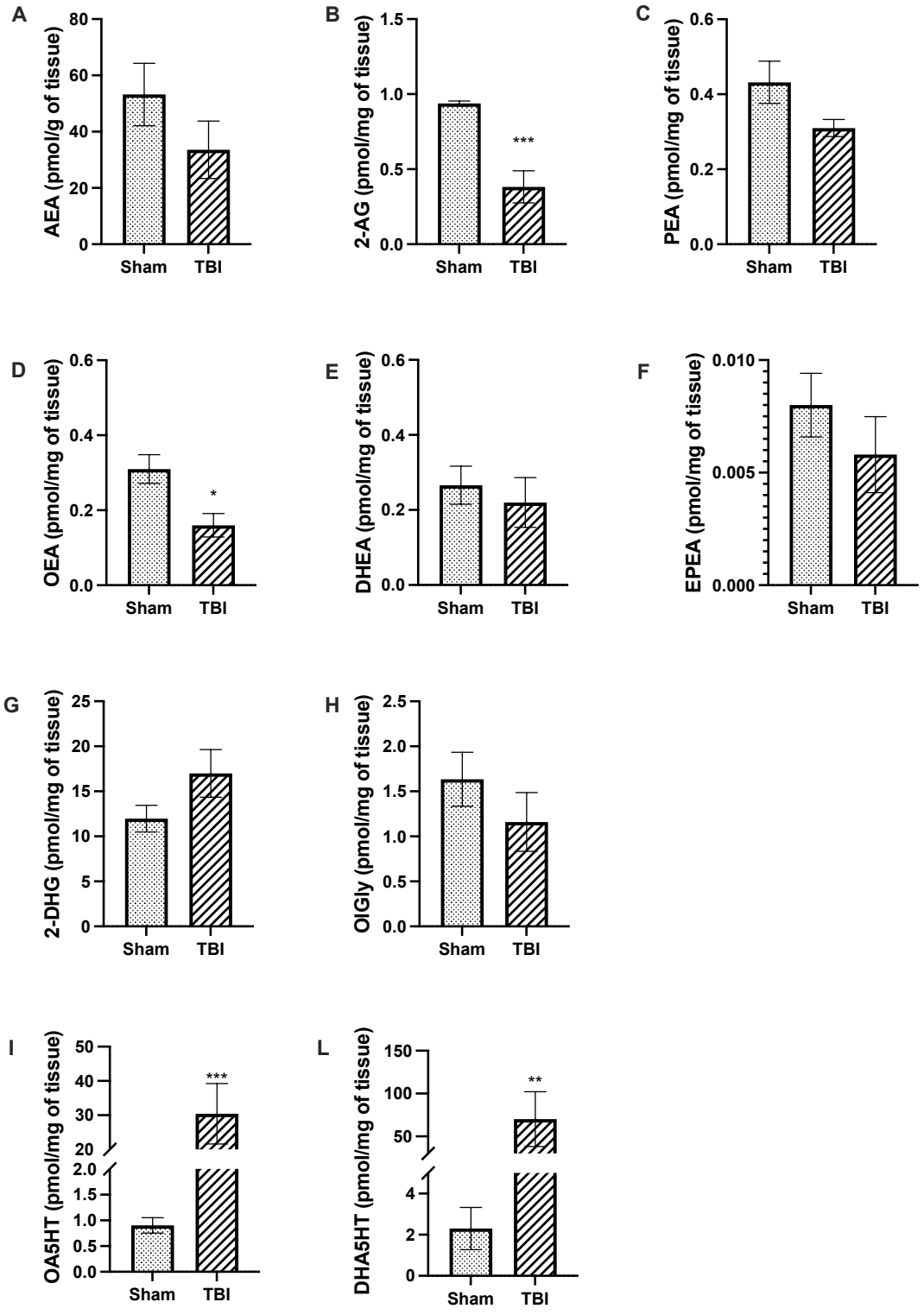


Figure 8. Levels of targeted eCBome mediators in cortex of sham and TBI mice at the end of in vivo tests.

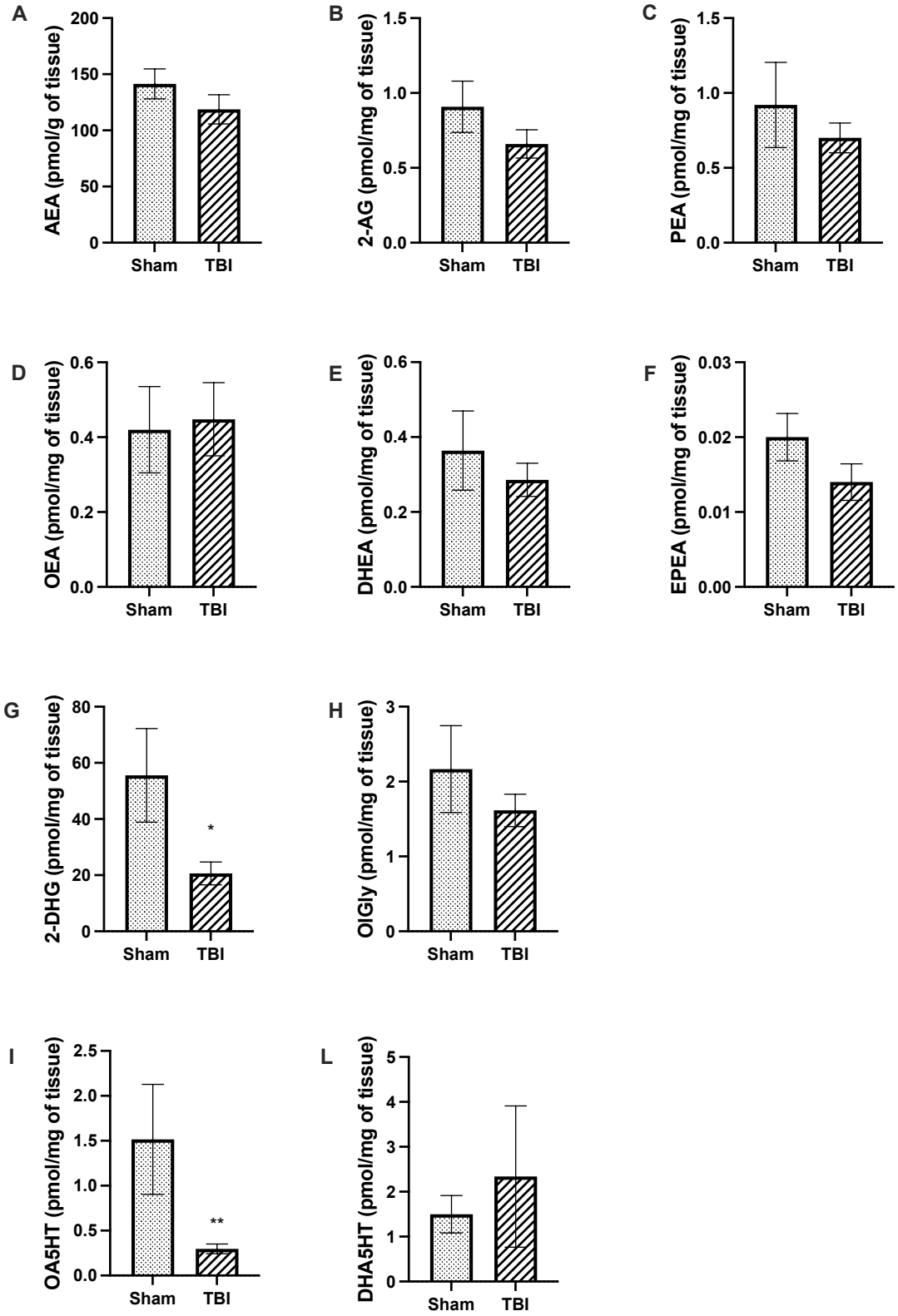


Figure 9. Levels of targeted eCBome mediators in hippocampus of sham and TBI mice at the end of in vivo tests.

In feces, AEA levels decreased significantly of TBI mice (Figure 10A). The others lipid mediators analyzed did not undergo any significant change.
In the intestinal tissues we were able also to identify and quantify other *N*-acylserotonin species, as PA5HT and EPA5HT.
In ileum 2-AG seems to have the same trend as in the feces, even though in both tissues the decrease in the TBI group is not significant (Fig 11B). However, EPEA levels enhanced significantly in the ileum of TBI mice (Fig 11F).
In the cecum no significant alterations have been observed (Figure 12).

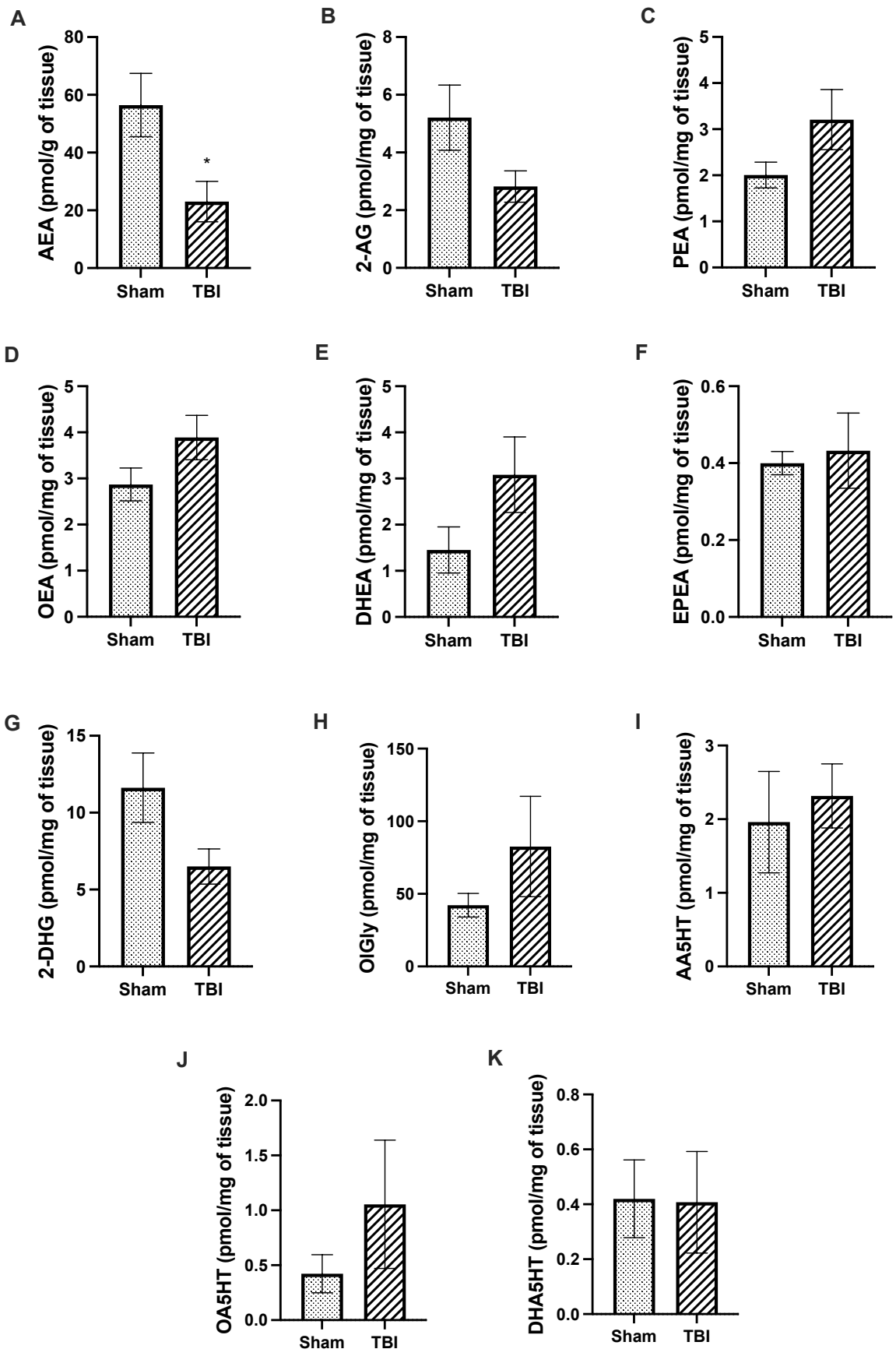

A
AEA (pmol/g of tissue)
80
60
40
20
0
*
Sham
TBI
B
2-AG (pmol/mg of tissue)
8
6
4
2
0
Sham
TBI
C
PEA (pmol/mg of tissue)
5
4
3
2
1
0
Sham
TBI
D
OEA (pmol/mg of tissue)
5
4
3
2
1
0
Sham
TBI
E
DHEA (pmol/mg of tissue)
5
4
3
2
1
0
Sham
TBI
F
EPEA (pmol/mg of tissue)
0.6
0.4
0.2
0.0
Sham
TBI
G
2-DHG (pmol/mg of tissue)
15
10
5
0
Sham
TBI
H
OlGly (pmol/mg of tissue)
150
100
50
0
Sham
TBI
I
AA5HT (pmol/mg of tissue)
3
2
1
0
Sham
TBI
J
OA5HT (pmol/mg of tissue)
2.0
1.5
1.0
0.5
0.0
Sham
TBI
K
DHA5HT (pmol/mg of tissue)
0.8
0.6
0.4
0.2
0.0
Sham
TBI

Figure 10. Levels of targeted eCBome mediators in feces of sham and TBI mice at the end of in vivo tests. (A) Levels of anandamide (AEA) expressed as pmol/g of tissue. (B) Levels of 2-arachidonoyl glycerol (2-AG) expressed as pmol/mg of tissue. (C) Levels of palmitoylethanolamide (PEA) expressed as pmol/mg of tissue. (D) Levels of oleoylethanolamide (OEA) expressed as expressed as pmol/mg of tissue. (E) Levels of *N*-docosahexaenoylethanolamine (DHEA) expressed as expressed as pmol/mg of tissue. (F) Levels of *N*-eicosapentaenoylethanolamine (EPEA) expressed as expressed as pmol/mg of tissue. (G) Levels of 2-docosahexaenoylglycerol (2-DHG) expressed as expressed as pmol/mg of tissue. (H) Levels of *N*-oleoylglycine (OlGly) expressed as expressed as pmol/mg of tissue. (I) Levels of *N*-arachidonoylserotonin (AA5HT) expressed as expressed as pmol/mg of tissue. (J) Levels of *N*-oleoylserotonin (OA5HT) expressed as expressed as pmol/mg of tissue. (K) Levels of *N*-docosahexaenoylserotonin (DHA5HT) expressed as expressed as pmol/mg of tissue. Data are represented as mean ± S.E.M. * indicates values statistically significant versus sham. The Student's t-test was used for statistical analysis. $P<0.05$ was considered statistically significant.

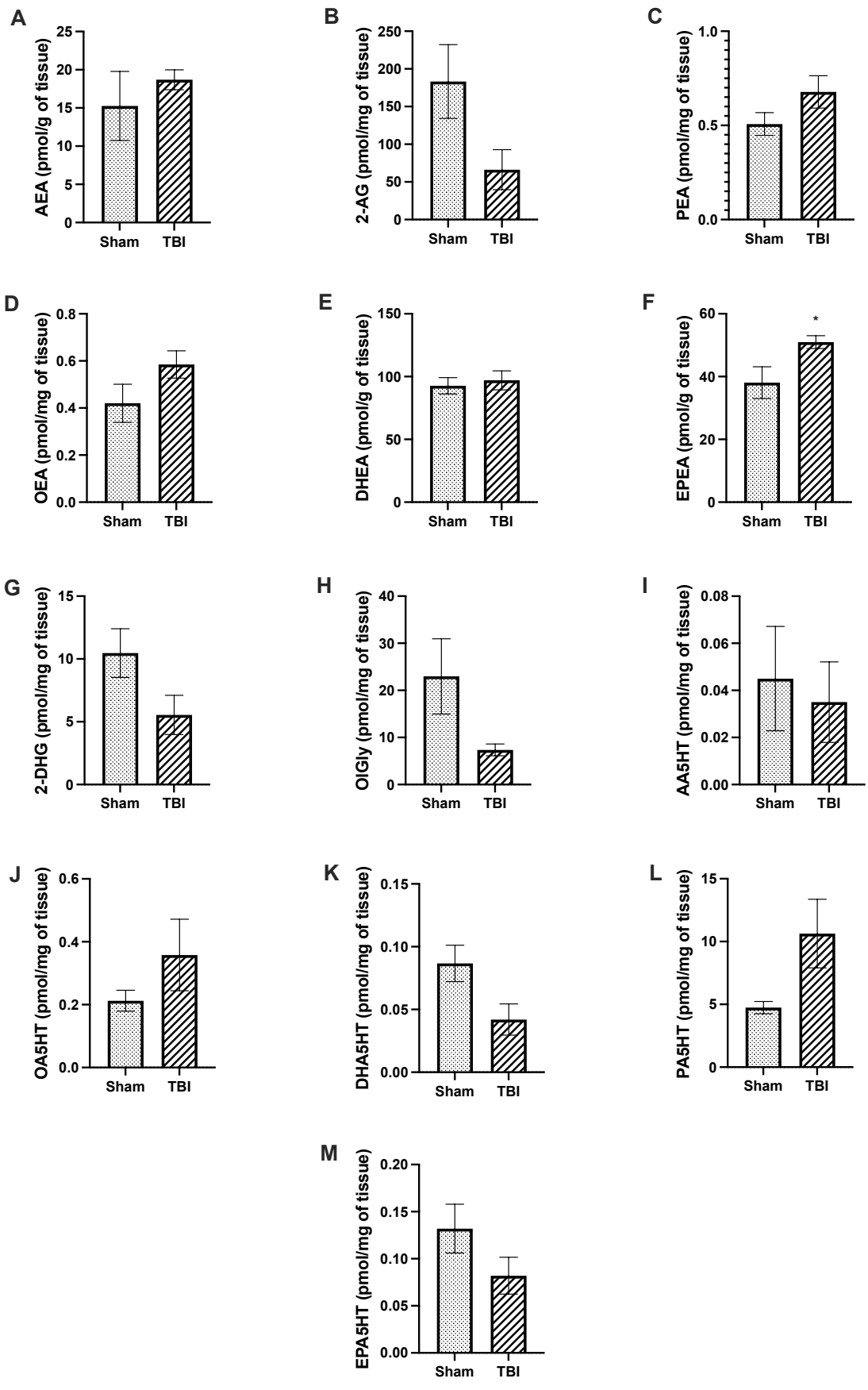
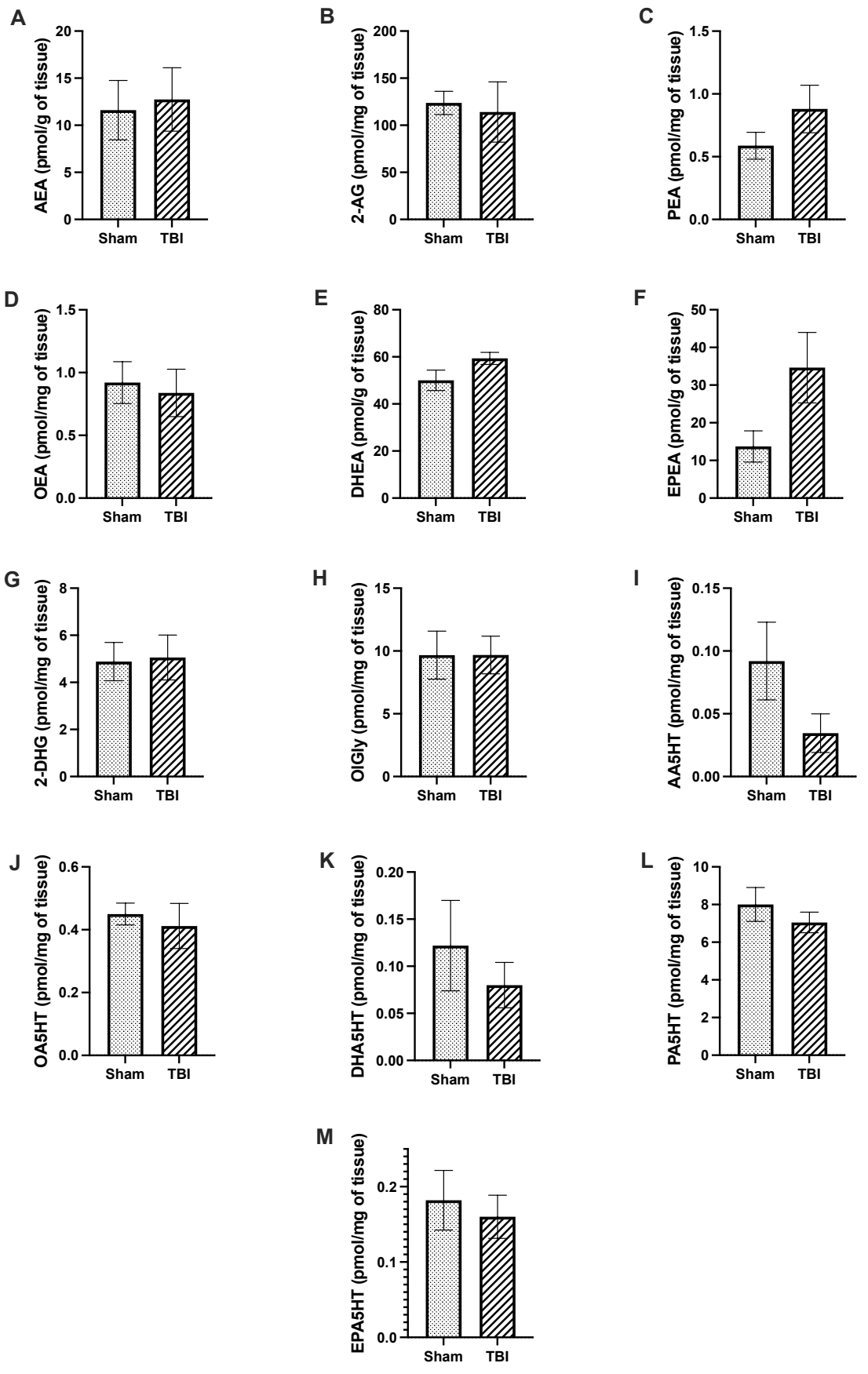

A
AEA (pmol/g of tissue)
25
20
15
10
5
0
Sham
TBI
B
2-AG (pmol/mg of tissue)
250
200
150
100
50
0
Sham
TBI
C
PEA (pmol/mg of tissue)
1.0
0.5
0.0
Sham
TBI
D
OEA (pmol/mg of tissue)
0.8
0.6
0.4
0.2
0.0
Sham
TBI
E
DHEA (pmol/g of tissue)
150
100
50
0
Sham
TBI
F
EPEA (pmol/g of tissue)
60
40
20
0
*
Sham
TBI
G
2-DHG (pmol/mg of tissue)
15
10
5
0
Sham
TBI
H
OlGly (pmol/mg of tissue)
40
30
20
10
0
Sham
TBI
I
AA5HT (pmol/mg of tissue)
0.08
0.06
0.04
0.02
0.00
Sham
TBI
J
OA5HT (pmol/mg of tissue)
0.6
0.4
0.2
0.0
Sham
TBI
K
DHA5HT (pmol/mg of tissue)
0.15
0.10
0.05
0.00
Sham
TBI
L
PA5HT (pmol/mg of tissue)
15
10
5
0
Sham
TBI
M
EPA5HT (pmol/mg of tissue)
0.20
0.15
0.10
0.05
0.00
Sham
TBI

Figure 11. Levels of targeted eCBome mediators in ileum of sham and TBI mice at the end of in vivo tests. (A) Levels of anandamide (AEA) expressed as pmol/g of tissue. (B) Levels of 2-arachidonoyl glycerol (2-AG) expressed as pmol/mg of tissue. (C) Levels of palmitoylethanolamide (PEA) expressed as pmol/mg of tissue. (D) Levels of oleoylethanolamide (OEA) expressed as expressed as pmol/mg of tissue. (E) Levels of *N*-docosahexaenoylethanolamine (DHEA) expressed as expressed as pmol/mg of tissue. (F) Levels of *N*-eicosapentaenoylethanolamine (EPEA) expressed as expressed as pmol/mg of tissue. (G) Levels of 2-docosahexaenoylglycerol (2-DHG) expressed as expressed as pmol/mg of tissue. (H) Levels of *N*-oleoylglycine (OlGly) expressed as expressed as pmol/mg of tissue. (I) Levels of *N*-arachidonoylserotonin (AA5HT) expressed as expressed as pmol/mg of tissue. (J) Levels of *N*-oleoylserotonin (OA5HT) expressed as expressed as pmol/mg of tissue. (K) Levels of *N*-docosahexaenoylserotonin (DHA5HT) expressed as expressed as pmol/mg of tissue. (L) Levels of *N*-palmitoylserotonin (PA5HT) expressed as expressed as pmol/mg of tissue. (M) Levels of *N*-eicosapentaenoylserotonin (EPA5HT) expressed as expressed as pmol/mg of tissue. Data are represented as mean ± S.E.M. * indicates values statistically significant versus sham. The Student's t-test was used for statistical analysis. $P<0.05$ was considered statistically significant.


A
AEA (pmol/g of tissue)
20
15
10
5
0
Sham
TBI
B
2-AG (pmol/mg of tissue)
200
150
100
50
0
Sham
TBI
C
PEA (pmol/mg of tissue)
1.5
1.0
0.5
0.0
Sham
TBI
D
OEA (pmol/mg of tissue)
1.5
1.0
0.5
0.0
Sham
TBI
E
DHEA (pmol/g of tissue)
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60
40
20
0
Sham
TBI
F
EPEA (pmol/g of tissue)
50
40
30
20
10
0
Sham
TBI
G
2-DHG (pmol/mg of tissue)
8
6
4
2
0
Sham
TBI
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OlGly (pmol/mg of tissue)
15
10
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AA5HT (pmol/mg of tissue)
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0.00
Sham
TBI
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OA5HT (pmol/mg of tissue)
0.6
0.4
0.2
0.0
Sham
TBI
K
DHA5HT (pmol/mg of tissue)
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0.15
0.10
0.05
0.00
Sham
TBI
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PA5HT (pmol/mg of tissue)
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8
6
4
2
0
Sham
TBI
M
EPA5HT (pmol/mg of tissue)
0.2
0.1
0.0
Sham
TBI

Figure 12. Levels of targeted eCBome mediators in cecum of sham and TBI mice at the end of in vivo tests. (A) Levels of anandamide (AEA) expressed as pmol/g of tissue. (B) Levels of 2-arachidonoyl glycerol (2-AG) expressed as pmol/mg of tissue. (C) Levels of palmitoylethanolamide (PEA) expressed as pmol/mg of tissue. (D) Levels of oleoylethanolamide (OEA) expressed as expressed as pmol/mg of tissue. (E) Levels of *N*-docosahexaenoylethanolamine (DHEA) expressed as expressed as pmol/mg of tissue. (F) Levels of *N*-eicosapentaenoylethanolamine (EPEA) expressed as expressed as pmol/mg of tissue. (G) Levels of 2-docosahexaenoylglycerol (2-DHG) expressed as expressed as pmol/mg of tissue. (H) Levels of *N*-oleoylglycine (OlGly) expressed as expressed as pmol/mg of tissue. (I) Levels of *N*-arachidonoylserotonin (AA5HT) expressed as expressed as pmol/mg of tissue. (J) Levels of *N*-oleoylserotonin (OA5HT) expressed as expressed as pmol/mg of tissue. (K) Levels of *N*-docosahexaenoylserotonin (DHA5HT) expressed as expressed as pmol/mg of tissue. (L) Levels of *N*-palmitoylserotonin (PA5HT) expressed as expressed as pmol/mg of tissue. (M) Levels of *N*-eicosapentaenoylserotonin (EPA5HT) expressed as expressed as pmol/mg of tissue. Data are represented as mean ± S.E.M. * indicates values statistically significant versus sham. The Student's t-test was used for statistical analysis. P<0.05 was considered statistically significant.

We carried out gene expression analysis by RT-PCR on WT mice. However, the NMR-based metabolomics analysis have provided info on short-chain fatty acid (SCFAs) levels in feces that may predict the trend and the composition in the gut microbiota study.
In particular, gene expression analysis showed a significant reduction of PPARα gene in the hippocampus of TBI group (Fig 13A). The other genes analyzed and that did not undergo to significant alterations in hippocampus (Fig 13A) and cortex (Fig 13B) included *Cb1, Trpv1, Daglα, Daglβ, Napepld, Faah, Gde, Magl* and *Pparγ.*

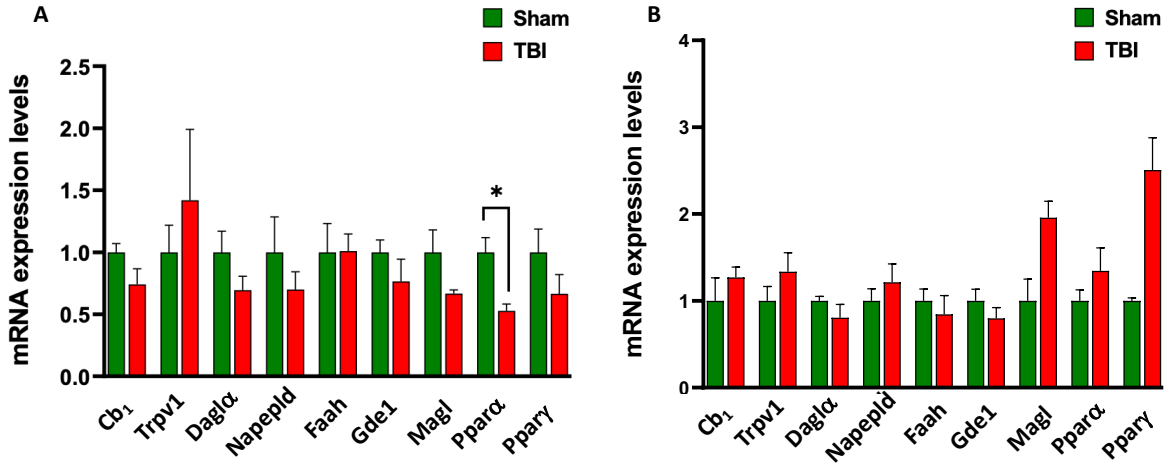


Figure 13. mRNA expression levels of indicated genes analyzed by quantitative PCR analysis. Differences in mRNAs content between groups were expressed as the $2^{\wedge-\Delta\Delta Ct}$ formula as reported in the method section in hippocampus (A) and cortex (B). Data are represented as mean ± S.E.M of at least three independent determinations. The asterisk (*) indicates a p-value <0.05 vs the sham group.

To pinpoint and evaluate the fecal metabolic alterations, through an untargeted approach by NMR-based metabolomics, induced by traumatic brain injury in experimental mice, we first performed OPLS-DA to fecal samples collected from the sham (**CNT, control**) and TBI groups (**TBI**) of WT mice. We obtained a statistical model with 1 predictive and 2 orthogonal components and parameters R2= 80% and Q2=54%. CV-ANOVA test *p* value =0,012 (significant if p<0,05). The parallel or predictive component t[1] accounts for the main differences between the treated (**TBI**) and untreated (**CNT** or **sham**) classes, while the second component to[1] accounts for minor intra-class inhomogeneities (Figure 14).

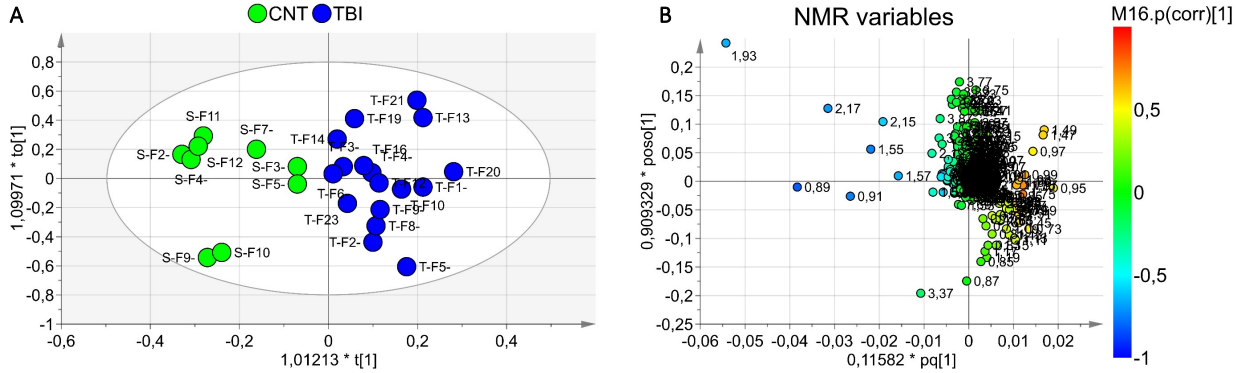


Figure 14. OPLS-DA of NMR metabolic profiles of mice feces. (A) Scores plot. (B) Loadings plot.

The S-plot is used to visualize both the covariance and the correlation structure between the NMR variables and the predictive score t[1]. Thus, the S-plot is a scatter plot of the p[1] vs p(corr)[1] vectors of the predictive component colored according to p(corr) values (Figure 15).

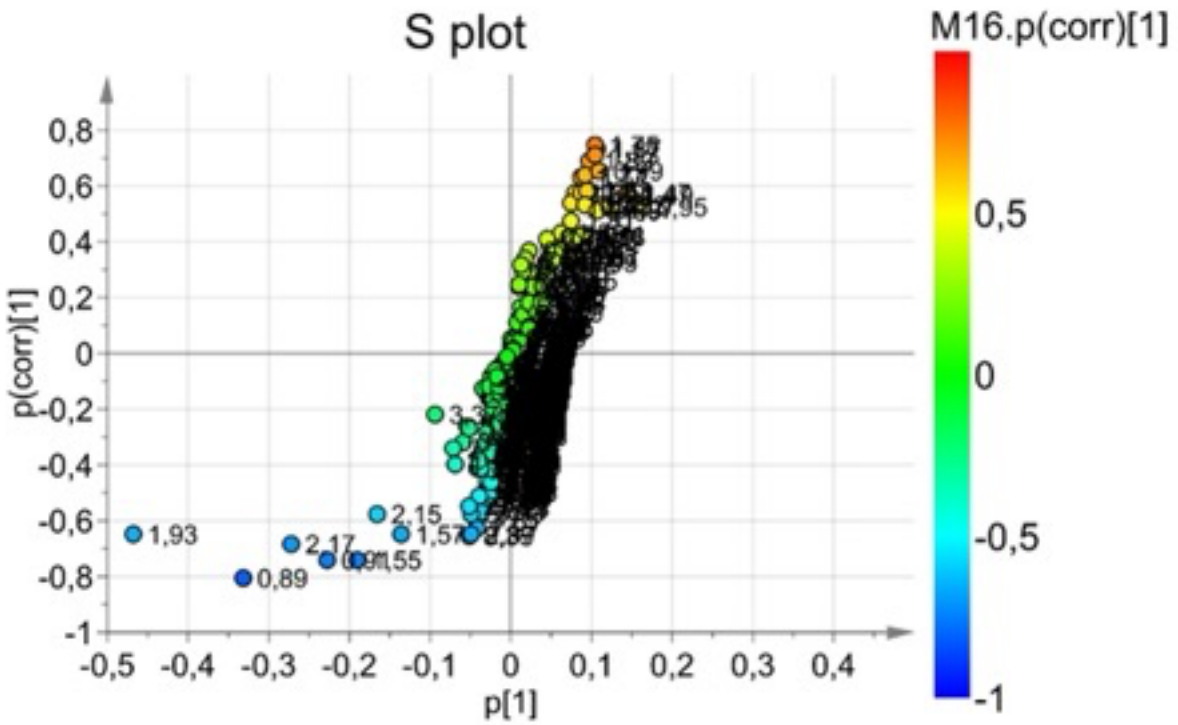


Figure 15. S plot of the p[1] vs p(corr)[1] vectors of the predictive component colored according to p(corr) values.

To identify and highlight the subset of most responsible metabolites in TBI feces metabolic profiles characterizing the discrimination found for sham/TBI classes, NMR variables were selected using a combination of VIP (Variable Influence in Projection) value >1 and correlation loadings values |p(corr)|> 0.5 in the OPLS-DA model classification (Figure 16).

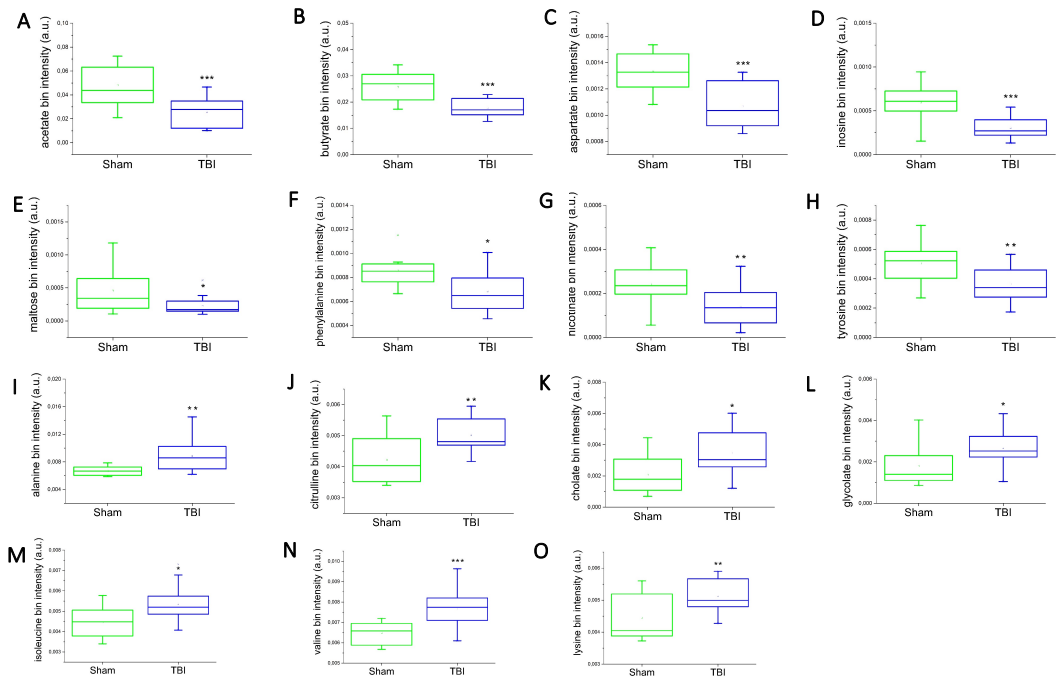


Figure 16. Most relevant metabolites from fecal NMR profiles involved in the biochemical response to traumatic brain injury experiments.

In summary, the following metabolites resulted to be up-regulated in TBI:

- ✓ cholate
- ✓ glycholate
- ✓ alanine
- ✓ isoleucine
- ✓ valine
- ✓ lysine
- ✓ citrulline.

On the other hand the metabolites that were down-regulated in TBI group were:

- ✓ butyrate
- ✓ inosine
- ✓ acetate
- ✓ aspartate
- ✓ phenylalanine
- ✓ maltose
- ✓ tyrosine
- ✓ nicotinate.

Very interestingly, butyrate and acetate, that are two of the main Short chain fatty acids (SCFAs) produced by bacterial fermentation of dietary fiber, are important signaling molecules in the microbiota gut-brain axis. Accordingly, to previous findings, though in a different TBI model, TBI led to a reduction of SCFAs, especially acetate, in stool samples (Opeyemi et al., J Neurotrauma 2021). Moreover, also aspartate and derivatives, as *N*-acetylaspartate (NAA), is a well-known marker of energy metabolism that is found to be decreased in patients suffering from TBI (Arun et al., J Neurotrauma 2010).

MESA or Metabolite Set Enrichment Analysis is a way to identify biologically meaningful patterns that are signicantly enriched in quantitative metabolomic data (Figure 17). In this analysis, the metabolic "pathway-associated metabolite sets" (currently contains 88 entries) from mice library was selected. Over Representation Analysis was implemented using the hypergeometric test to evaluate whether a particular metabolite set is represented more than expected by chance within the given compound list. One-tailed p values are provided after adjusting for multiple testing.

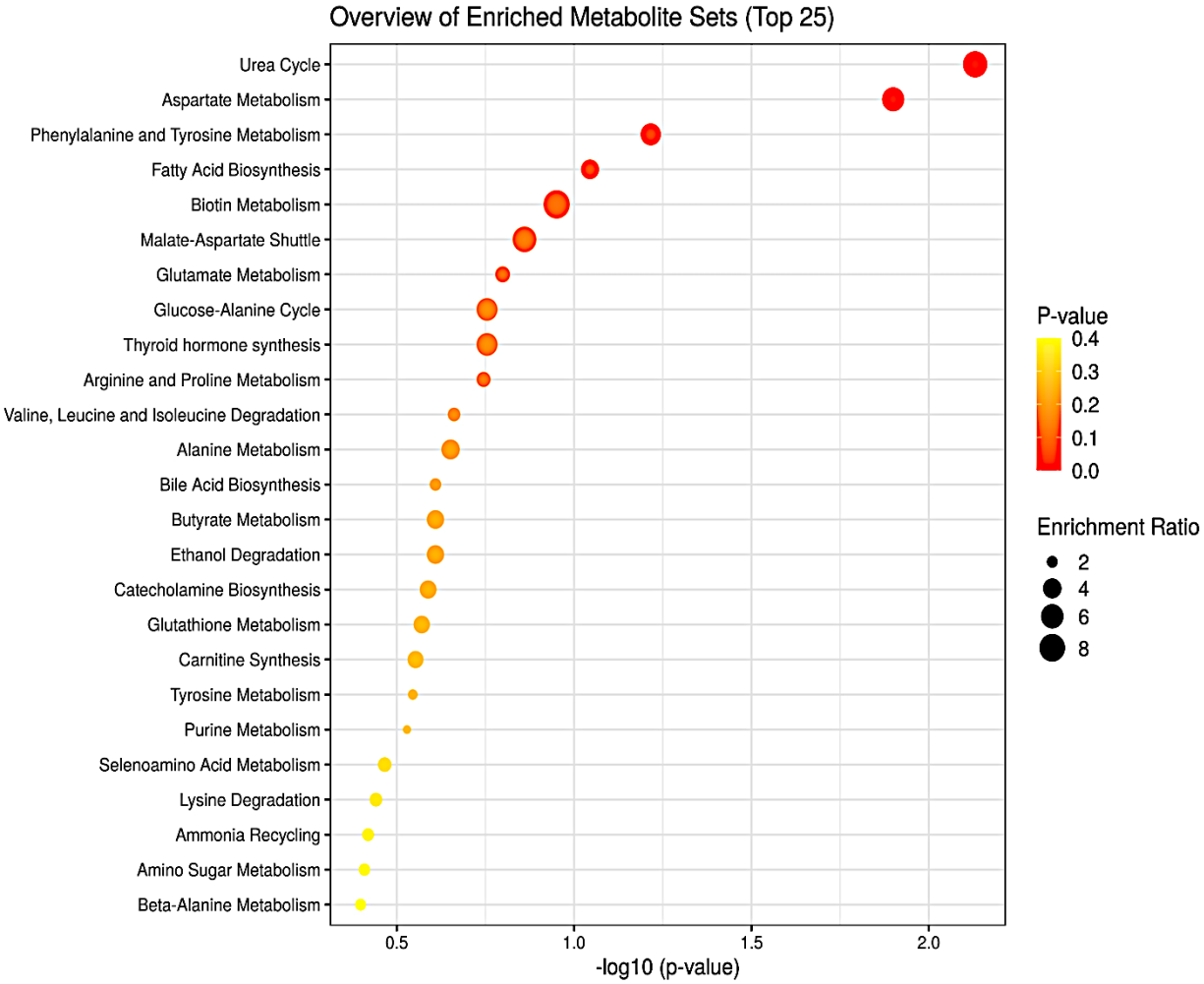


Figure 17. Metabolite Set Enrichment Analysis (MESA) in which are shown the main biologically pathways significantly enriched in quantitative metabolomic data.

We have analyzed also the inflammatory markers in the plasma of WT mice with a multiplexed assay but the kit was not sensitive enough to detect those cytokines for all samples (n=8 for Sham and n=12 for TBI). For this reason, we have decided to repeat the measure using single ELISA kits. However, the results are shown in the figure 18. As reported in the figure, no significant alterations have been observed but only IL-22 (Figure 18C) was detectable in all samples.

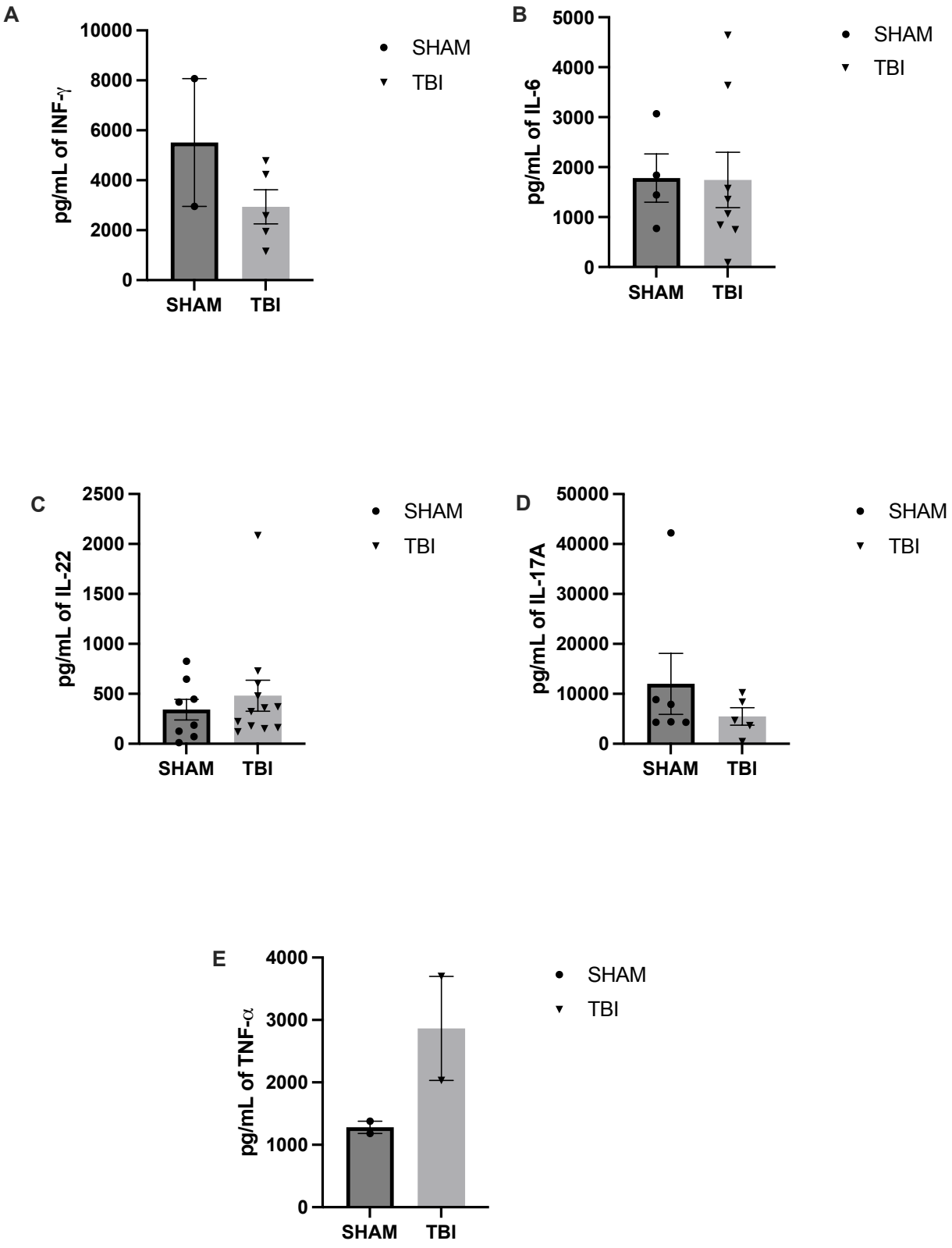

A
10000
8000
6000
4000
2000
0
pg/mL of INF-γ
SHAM
TBI
SHAM
TBI
B
5000
4000
3000
2000
1000
0
pg/mL of IL-6
SHAM
TBI
SHAM
TBI
C
2500
2000
1500
1000
500
0
pg/mL of IL-22
SHAM
TBI
SHAM
TBI
D
50000
40000
30000
20000
10000
0
pg/mL of IL-17A
SHAM
TBI
SHAM
TBI
E
4000
3000
2000
1000
0
pg/mL of TNF-α
SHAM
TBI
SHAM
TBI

Figure 18. Inflammatory markers measured in plasma samples from WT mice (sham and TBI). The asterisk (*) indicates a p-value <0.05.

As reported in Figure 19, the new experiments using the single ELISA kit for each cytokine allowed us to measure the plasmatic levels of these molecules. In particular, IFN-γ increased significantly in APP sham mice in comparison to WT-sham ($p<0.01$) and in APP-mTBI as compared to WT-mTBI ($p<0.01$). IL-17A, showed in Fig 19D, increased significantly in APP-mTBI compared to WT-mTBI ($p<0.01$) and APP-sham ($p<0.001$). Instead, TNF-α (Fig. 19E) increased significantly only in WT-mTBI compared to WT-sham ($p<0.05$), but no significant alteration was observed in APP mice. IL-1β (Fig. 19F) increased significantly in WT-mTBI ($p<0.01$ vs. WT-sham) and in APP-mTBI compared to APP-sham and WT-mTBI ($p<0.01$ for both).

The proinflammatory cytokines IFN-γ and IL-1β are thought to be the major mediators of neuro-inflammation, however, a recent study aimed to examine the effect of IFN-γ and IL-1β on survival of cortical neurons in stab wound injury in mice showed that IFN-γ and IL-1β induced astrogliosis, microgliosis, enhanced the secretion of BDNF, one of the many neurotrophic factors after brain injury, and promoted the survival of cortical neurons in stab wound brain injury (Abd-El-Basset et al., Neurosci Insights. 2020). Moreover, very recently it was shown that IL-17A, that has been demonstrated to be involved in neuroinflammatory diseases and microglial activation (Anyong Yu, Haizhen et al., Molecular Immunology. 2016), accumulate in the brain and in the meninges of 3xTg-AD mice and the increase of IL-17 producers associates with short-term memory deficits (Brigas et al. Cell Reports. 2021). TNF-α is a pro-inflammatory cytokine, involved in Alzheimer's disease pathogenesis (Wyss-Coray et al., Cold Spring Harb Perspect Med. 2012). Anti-TNF-α therapeutic approaches currently used in autoimmune diseases have been proposed as a therapeutic strategy in AD. Very interestingly, TNF blocking agents are associated with lower risk for Alzheimer's disease in human patients (Zhou et al., Plos One. 2020). Therefore, our results indicated that mTBI, at 60 days post-injury, is accompanied to a persistent circulating inflammation that worsened AD pathology.

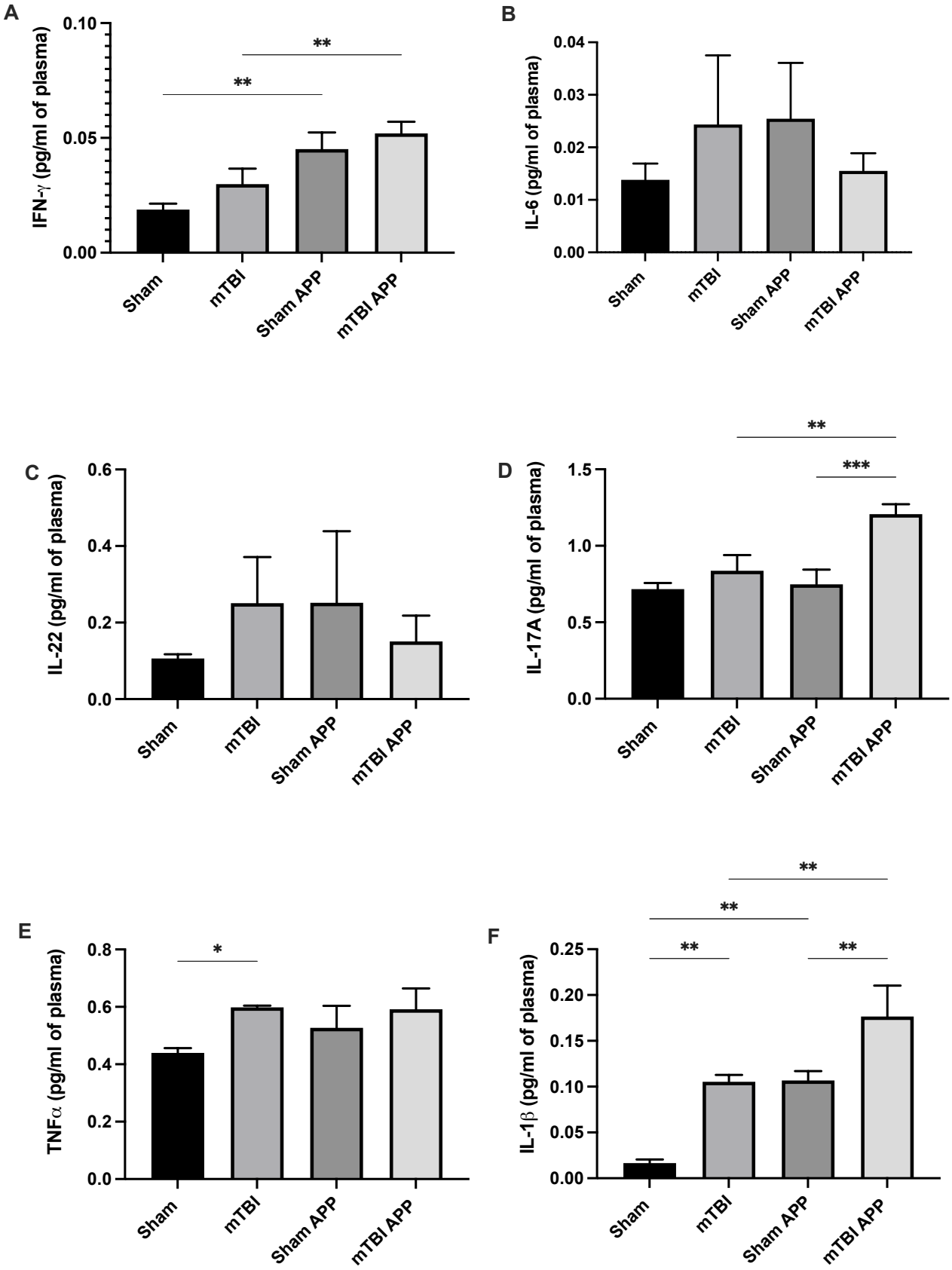

A
IFN-γ (pg/ml of plasma)
0.10
0.05
0.00
**
**
Sham
mTBI
Sham APP
mTBI APP
B
IL-6 (pg/ml of plasma)
0.04
0.03
0.02
0.01
0.00
Sham
mTBI
Sham APP
mTBI APP
C
IL-22 (pg/ml of plasma)
0.6
0.4
0.2
0.0
Sham
mTBI
Sham APP
mTBI APP
D
IL-17A (pg/ml of plasma)
1.5
1.0
0.5
0.0
**
***
Sham
mTBI
Sham APP
mTBI APP
E
TNFα (pg/ml of plasma)
0.8
0.6
0.4
0.2
0.0
*
Sham
mTBI
Sham APP
mTBI APP
F
IL-1β (pg/ml of plasma)
0.25
0.20
0.15
0.10
0.05
0.00
**
**
**
Sham
mTBI
Sham APP
mTBI APP

Figure 19. Inflammatory markers measured in plasma samples from WT and APP mice (sham and TBI). The asterisk (*) indicates a p-value <0.05.

These data are in line with the measurement of Aβ(1-40) and 1-42. In fact, our data show an increase of Aβ(1-42) in the cortex of APP mice subjected to mTBI in comparison to APP-sham and WT-mTBI, while the Aβ (1-40) is reduced in WT-mTBI and APP-sham in comparison to WT-sham. No significant effect in hippocampus was observed.

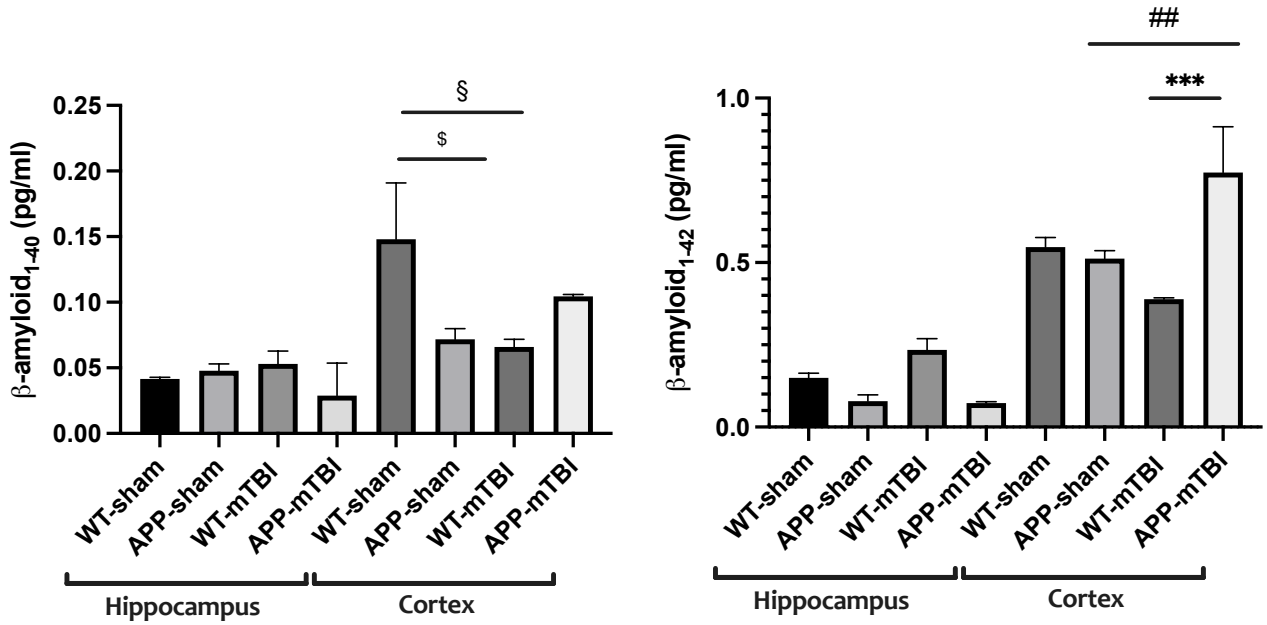


Figure 19. Aβ(1-40) and Aβ(1-42) measured in cerebral samples (hippocampus and cortex) from WT and APP mice (sham and TBI).

Therefore, our data showed that mTBI induced in APP mice (9-18 weeks) led to increased levels of Aβ(1-42) in the cortex of these mice at the end of the in vivo tests (17-25 weeks) suggesting that AD is a long-term consequence of mTBI.
Moreover, given their proposed neuroprotective role, both the endocannabinoidome and gut microbiome mediators whose concentrations were shown here to be modified following trauma, might be partly responsible for the pathological, behavioural and biochemical, alterations observed in this condition

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.

Immunoistochemistry.

Under pentobarbital anesthesia (50 mg/kg, i.p.), animals were transcardially perfused with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were excised, post fixed for 3 h in the perfusion fixative, cryoprotected for 72 h in 30% sucrose in 0.1 M phosphate buffer, and frozen in Optimal cutting temperature-embedding compound. Prefrontal cortex and hippocampus were analyzed. Transverse sections (20 μm) were cut using a cryostat and thaw-mounted onto glass slides. Slides were incubated overnight with primary antibody solutions for the microglial cell marker Iba-1 (rabbit antiionized calcium binding adapter molecule-1; 1:1000; Wako Chemicals, Germany). Possible non-specific labeling of rabbit secondary antibody was detected by using secondary antibody alone. Following incubation, sections were washed and incubated for 2 h with secondary antibody solution (donkey anti-rabbit Alexa FluorTM 488; 1:1000; Molecular Probes, USA). Slides were washed, coverslipped with Vectashield mounting medium (Vector Laboratories, USA), and visualized under a Leica fluorescence microscope. Quantitative analysis was performed by counting in areas measuring 1.7×10^4 μm2.

RNA Extraction and quantitative PCR (qPCR)

Total RNA was isolated from hippocampus and cortex by use of the TRIzol Reagent (Cat# 15596026; ThermoFisher, Italy), reacted with DNase-I (Cat# 180680151U/µl; ThermoFisher, Italy) for 15 min at room temperature, followed by spectrophotometric quantification. The final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥ 1.7. Isolated mRNA was reverse-transcribed by the use of iScript™ Reverse Transcription Supermix (Cat# 1708840; Biorad, Italy). Quantitative PCR (qPCR) was carried out in a real-time PCR system CFX384 (Bio-Rad) using the SYBR Green PCR Kit (Cat# 1725274, Biorad; Italy) Each sample was amplified simultaneously in quadruplicate in a one-assay run with a nontemplate control blank for each primer pair to control for contamination or primer-dimer formation, and the cycle threshold (Ct) value for each experimental group was determined. The housekeeping gene ribosomal protein S16 was used to normalize the Ct values, using the $2^{\wedge-\Delta Ct}$ formula. Differences in mRNAs content between groups were expressed as $2^{\wedge-\Delta\Delta Ct}$, as previously described (Iannotti et al. 2018).

Lipid Extraction and eCBome analysis.

Feces and intestinal were frozen in liquid nitrogen immediately after dissection, which took place within 5 min from sacrifice. Frozen tissues were then homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal deuterated standards for AEA, 2-AG, PEA, OEA, DHEA, EPEA, OlGly and *N*-acylserotonines quantification by isotope dilution (5 pmol for $[^2H]_8$AEA; 50 pmol for $[^2H]_5$2-AG, $[^2H]_4$PEA, and $[^2H]_2$OEA; 10 pmol for $[^2H]_4$DHEA, $[^2H]_4$EPEA, $[^2H]_2$OlGly, $[^2H]_{17}$OA5HT). Then the lipid extract was purified using open bed chromatography with silica gel. Fractions enriched in eCBs, *N*-acylethanolamines, *N*-acylglycines and *N*-acylserotonines (9:1, CHCl3/CH3OH, v/v) were analyzed by liquid chromatography-atmospheric pressure chemical ionization-single quadrupole mass spectrometry, as previously described (Guida et al, BBI 2018; Piscitelli et al., ACS Chem Neurosci 2020). Endogenous levels of eCBome mediators were calculated on the basis of their area ratio with the internal deuterated standard signal areas, all *N*- acylserotonines were calculated on the basis of their area ratio with the OA5HT deuterated standard signal areas.

NMR-metabolomics analysis

To extract the metabolites of interest (e.g., lipids, carbohydrates, amino acids and other small metabolites), leaving other compounds (e.g. DNA, RNA, proteins) in the pellet, the feces were mechanically broken down. The extraction of polar metabolites was performed using the methanol / chloroform protocol suggested by the Standard Metabolic Reporting Structures working group (Lindon et al., 2005). Homogenization of 30 mg of frozen stool samples was performed in 8 ml / g of wet tissue of methanol and 1.70 ml / g of wet tissue of water (all solvents were cold) with UltraTurrax for 2 minutes on ice . At this point, 4 ml / g of wet chloroform tissue was added and the homogenate was gently shaken and mixed, on ice (the solution must be monophasic). Subsequently, an additional 4 ml / g of chloroform wet tissue and 4 ml / g of water wet tissue were added and the final mixture was shaken well and centrifuged at 12,000 g for 15 minutes at 4 ° C. Through this procedure, three phases are separated: water / methanol in the upper part (aqueous phase, with the polar metabolites), denatured proteins and cellular debris in the middle and chloroform in the lower part (lipid phase, with lipophilic compounds). The upper and lower layers were transferred into glass vials and the solvents were removed under a stream of dry nitrogen and stored at -80 ° C. For each extract, two samples are obtained for NMR measurements. The first sample was resuspended in 700 µl of phosphate buffer saline (PBS, pH 7.4) and transferred to an NMR tube. The second

sample was used for two-dimensional (2D) analyzes and resuspended in 700 µl of D2O. This procedure avoided possible differences in chemical displacement due to separate extractions. The mono (1D-NMR) and two-dimensional (2D-NMR) spectra were obtained at a temperature of 27 ° C (300K) on a high resolution Bruker Avance spectrometer operating at the frequency of 600.13 MHz and equipped with TCI CryoProbe ™ technology. For one-dimensional proton spectra (1H), the water peak signal was suppressed by using the excitationsculpting sequence. To improve the resolution of the peaks in the one-dimensional spectra and to facilitate their assignment, homonuclear 1H-1H (TOCSY = clean Total Correlation SpectroscopY) and hetero nuclear 1H-13C (HSQC = Heteronuclear Single Quantum Coherence) NMR experiments were acquired. The TOCSY spectra were recorded using the standard pulse sequence that incorporates the suppression of the water signal (excitation-sculpting). After transformation, each spectrum was calibrated on the peak of the TSP standard set at $\delta = 0.00$ ppm. The HSQC spectra were recorded on the same Avance-600 spectrometer operating at 150.90 MHz for the 13C core. After the appropriate transformation, each spectrum was calibrated on the lactate signal (βCH3) consisting of a resonant doublet at $\delta = 1.33$ ppm for 1H and $\delta = 20.76$ ppm for 13C. The values of the chemical shifts (positions) of the peaks identified in the spectra were then compared with the data present in the dedicated on-line databases.

Aβ and pro-inflammatory markers measurement. The levels of Aβ (1-40 and 1-42) and pro-inflammatory cytokines (IFN-γ, IL-6, IL-22, IL-17A, TNF-α and IL-1β) were measured in the saliva samples, in the different experimental conditions, by SinglePlex ELISA kit (Elabscience and Diaclone) with a GENios-Pro Reader (Tecan) following the manufacturer's instructions

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 or Tukey for all other tests.

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

Conferences
International Symposium on Pathomechanisms of Amyloid diseases

Catania, Italy - August 25-27, 2022
Poster presentation: Potential role of the microbiome-endocannabinoidome connection in the gut-brain axis after traumatic brain injury and its association with Alzheimer's disease

Seminar
On-line seminar September 27, 2022
Presentation title: Potential role of the microbiome-endocannabinoidome connection in the gut-brain axis after traumatic brain injury and its association with Alzheimer's disease

How were the results disseminated to communities of interest?

Dissemination activities
Video for Global Conversation on Sustainability, IUPAC activity
September 25, 2022
https://www.youtube.com/watch?v=sRclYbfhyKE&t=3s

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

In next quarter, we will continue brain immunohistochemistry, molecular biology, targeted lipidomics and gut microbiome analysis.

4. **IMPACT:***or*

What was the impact on the development of the principal discipline(s) of the project?

The results provided so far confirmed that also a mild brain trauma could lead to Alzheimer and an increase of inflammatory profile that could worsen the pathology. However, our research in progress is working to find new predictive biomarkers that may help in early diagnosis and in developing a therapeutic strategy.

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

Delay in microbiome results for technical problems.

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.
In order to provide a timely financial report for the last year, we need to request anticipated funds (at least one half of the remaining funds) to proceed with the other expenses for the experimentation of the third period.

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.

International Symposium on Pathomechanisms of Amyloid diseases
Catania, Italy - August 25-27, 2022
Poster presentation: Potential role of the microbiome-endocannabinoidome connection in the gut-brain axis after traumatic brain injury and its association with Alzheimer's disease

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

In the occasion of the Global Conversation on Sustainability, the group of Italian IUPAC Young Observers wanted to express its commitment for sustainability. In this short video we present, in "pills", some of the main challenges related to sustainability that we are facing in our work, and possible solutions that chemists are developing.

Video for Global Conversation on Sustainability, IUPAC activity
September 25, 2022
https://www.youtube.com/watch?v=sRclYbfhyKE&t=3s

On-line seminar September 27, 2022
Presentation title: Potential role of the microbiome-endocannabinoidome connection in the gut-brain axis after traumatic brain injury and its association with Alzheimer's disease

https://www.icb.cnr.it/en/potential-role-of-the-microbiome-endocannabinoidome-connection-in-the-gut-brain-axis-after-traumatic-brain-injury-and-its-association-with-alzheimers-disease-3/

- **Technologies or techniques**

Nothing to Report

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

  Nothing to Report

- **Other Products**

Video for Global Conversation on Sustainability, IUPAC activity
September 25, 2022
https://www.youtube.com/watch?v=sRclYbfhyKE&t=3s

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: *2*
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: *2*
Contribution to Project: *Prof. Luongo is conducting experiments for the avaluation of neuroinflammation*

Name: *Roberta Verde*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Dr. Verde has performed targeted lipidomics analysis.*

Name: *Adele Cutignano*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Dr. Cutignano has performed targeted lipidomics analysis.*

Name: *Debora Paris*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Dr. Cutignano has performed untargeted metabolomics analysis.*

Name: *Monica Iannotta*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *full-time*
Contribution to Project: *Dr. Iannotta started her post-doc position to work full-time in the project.*

Name: *Fabio Arturo Iannotti*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Dr. Iannotti has performed gene expression analysis.*

Name: *Vincenzo Di Marzo*
Project Role: *Co-PI*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: Dr. Di Marzo is working on gut microbiome analysis.

Name: *Pal Pacher*
Project Role: *Co-PI*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: Prof Pacher is working on inflammatory markers analysis.

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

During this year we have recruited a PD and a second PD has started for the last period.

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
Partner's contribution to the project (identify one or more)
- *Facilities;*
- *Collaboration;*
- *Personnel exchanges*

Organization Name: ***Université Laval***
Location of Organization: (if foreign location list country) **1050, avenue de la Médecine Quebec City, Quebec Canada**

Partner's contribution to the project (identify one or more)
- *Facilities;*
- *Collaboration;*

Organization Name: ***NIH***

Location of Organization: (if foreign location list country) **5625 Fishers Lane Rockville, MD 20852 USA**

Partner's contribution to the project (identify one or more)

- *Facilities;*
- *Collaboration;*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Abstract International Symposium on Pathomechanisms of Amyloid diseases
Catania, Italy - August 25-27, 2022

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

F. Guida[1], R. Verde[2], R. Infantino[1], S. Boccella[1], M. Iannotta[1,2], L. Luongo[1], F.A. Iannotti[2], A. Lauritano[2], I. Cipollone[2], D. Paris[2], C. Moriello[2], P. Pacher[3], C. Silvestri[4], S. Maione[1], V. Di Marzo[2,4] and F.Piscitelli[2]

[1]Università della Campania "L. Vanvitelli", Dipartimento di Medicina Sperimentale, Italy, 80138 –Napoli; [2] Istituto di Chimica Biomolecolare-Consiglio Nazionale delle Ricerche (ICB-CNR), Italy, 80078 – Pozzuoli (NA);National Institute of Health (NIH), USA, MD 20852 – Rockville; [4]Université Laval, Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health, Canada, QC G1V 0A6 – Québec;

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). Moreover, TBIs represent a major health issue, especially for football players and soldiers who have frequent experiences of multiple brain injuries. The main challenge in this area is the development of new diagnostic and therapeutic approaches. Recent evidence suggests that both TBI and AD are characterised by an alteration in the microbiota-gut-brain axis that may significantly contribute to their pathogenesis and represent the missing link to understand their association. Furthermore, accumulating evidence in literature shows that the endocannabinoid (eCB) system with the accompanying "endocannabinoidome" (eCBome) play a key role in numerous physiological and pathological conditions. In particular, eCB signaling is increasingly emerging as a system of lipid mediators of the health-disease continuum. Its strong connection with the gut microbiome has been so far suggested only in the context of inflammatory, metabolic and intestinal disorders and never been investigated in other disorders. Therefore, the objective of the project funded by the "US Army Medical Research and Development Command" (USAMRDC) for the Peer Reviewed Alzheimer's Research Program (PRARP) 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 microbiota composition and how the latter may determine the severity of these disorders by regulating the activity of endocannabinoids and related mediators using a multidisciplinary approach. To date, our data in control mice confirm previous studies showing that mTBI induces a characteristic dual behavioural phenotype (aggressive/depressive) in mice, and significant impairments in the discriminative and spatial memory tasks. Brain immunohistochemistry showed an increase of the total number, activated cell number and dystrophic number of microglia in mTBI as compared to controls in both the cortex and hippocampus. Moreover, we analysed the eCBome in the hippocampus and cortex of these mice through gene expression analysis by RT-PCR and targeted lipidomic analysis. Gene expression analysis showed a significant reduction of PPARα in the hippocampus of the TBI group. The targeted lipidomics analysis of the eCBome in the hippocampus, cortex, feces and in the intestine of sham and TBI mice reported significant alterations. Very interestingly we have identified for the first time *N*-acylserotonins in the brain of mice that underwent to significative changes after the injury. Finally, TBI led to a reduction of butyrate and acetate in feces, as assessed by NMR-based metabolomics.

Poster

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

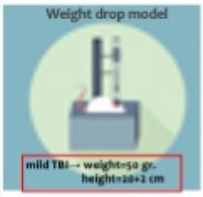
F. Guida[1], R. Verde[2], R. Infantino[1], S. Boccella[1], M. Iannotta[1,2], L. Luongo[1], F.A. Iannotti[2], A. Lauritano[2], I. Cipollone[2], D. Paris[2], L. Palomba[2], C. Moriello[2], P. Pacher[3], C. Silvestri[4], S. Maione[1], V. Di Marzo[2,4] and F.Piscitelli[2]

[1]Università della Campania "L. Vanvitelli", Dipartimento di Medicina Sperimentale, Italy, 80138 – Napoli; [2]Institute of Biomolecular Chemistry, Unit of Pozzuoli, 80078 – Pozzuoli (NA); [3]National Institute of Health (NIH), USA, MD 20892 – Rockville; [4]Université Laval, Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health, Canada, QC G1V 0A6 – Québec

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). Moreover, TBIs represent a major health issue, especially for football players and soldiers who have frequent experiences of multiple brain injuries. The main challenge in this area is the development of new diagnostic and therapeutic approaches. Recent evidence suggests that both TBI and AD are characterised by an alteration in the microbiota-gut-brain axis that may significantly contribute to their pathogenesis and represent the missing link to understand their association. Furthermore, accumulating evidence in literature shows that the endocannabinoid (eCB) system with the accompanying "endocannabinoidome" (eCBome) play a key role in numerous physiological and pathological conditions. In particular, eCB signaling is increasingly emerging as a system of lipid mediators of the health-disease continuum. Its strong connection with the gut microbiome has been so far suggested only in the context of inflammatory, metabolic and intestinal disorders and never been investigated in other disorders. Therefore, the **objective** of the project funded by the "US Army Medical Research and Development Command" (USAMRDC) for the Peer Reviewed Alzheimer's Research Program (PRARP) is to investigate the effects of a mild TBI on the subsequent development of AD-related neuropathology and cognitive impairments in an APPSWE mice, the role of inflammation, the potential perturbation of the gut microbiota composition and how the latter may determine the severity of these disorders by regulating the activity of endocannabinoids and related mediators using a multidisciplinary approach.

mTBI model



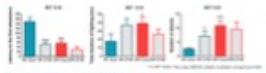
The injury severity is directly related to the mass and the height from which the brass weight is released.
The brass weight is released between bregma and lamba.
This model of cerebral trauma is widely utilized to replicate "diffuse traumatic brain injury" (DTBI) , without focal lesion to characterize changes characteristic of human DTBI cause by motor vehicle accidents or falls.

Pre-symptomatic stage

APPSWE-Model 1349-RDI-M 9-18 weeks of age (Taconic)
This model carries a transgene coding for the 695-amino acid isoform of human Alzheimer β-amyloid (Aβ) precursor protein carrying the Swedish mutation. It expresses high concentrations of the mutant Aβ, develops significant amyloid plaques and displays memory deficits

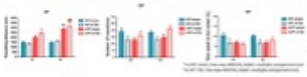
Behavioral timeline

Behavioral and cognitive tests



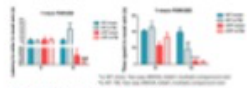
mTBI mice showed an aggressive phenotype. Sham APP mice showed per sé an aggressive behavior

Resident intruder (RI) for the aggressiveness at day 15



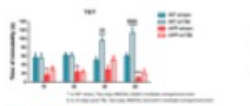
In WT-mTBI group the time spent in the center was reduced (at 30 and 60 days), suggesting an anxiety-like behavior; while in APP-sham and mTBI mice, data showed an increase of total traveling distance, indicating an hyperactivity

Open field (OF) for the exploratory activity at days 30 and 60



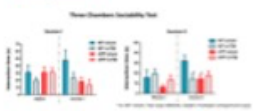
Cognitive impairments in term of spatial memory, measured as time spent in the novel arms, as well as the latency to entry, in the Forced Y maze test were detected at 30- and 60-days post trauma in WT mice. In APP mice, both sham and mTBI, we found a decrease in the latency to enter, as well as, in the time spent in the novel arm.

Forced Y maze test at days 30 and 60.



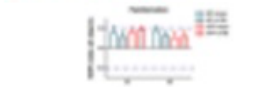
Increased immobility time at 45 and 60 days after trauma in WT animals. Compared to WT, APP mice presented a strong reduction of immobility time.

Tail suspension test



Reduced social interaction in WT, but not APP-injured animals. APP sham mice showed an alteration in social behavior as compared with WT ones, but no significant change between APP sham and mTBI groups was observed.

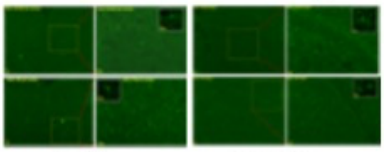
Three Chambers Sociability Test



At 60 days, WT mTBI animals showed a reduced recognition index and also APP-mTBI at 30 days, indicating an impaired discriminative memory performance.

Novel Object Recognition test at days 30 and 60

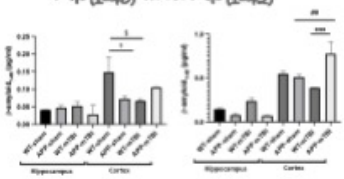
Brain immunoistochemistry



Cortex | Hippocampus

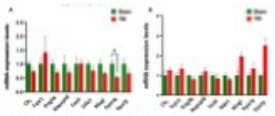
Our preliminary data indicate an increase of the total number, activated cell number and dystrophic number, of microglia cells in WT-mTBI as compared to controls in both cortex and hippocampus.

$A\beta_{(1-40)}$ and $A\beta_{(1-42)}$

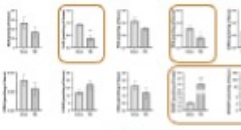


Our data show an **increase of Aβ(1-42) in the cortex of APP mice subjected to mTBI** in comparison to APP-sham and WT-mTBI, while the **Aβ(1-40) is reduced in WT-mTBI and APP-sham** in comparison to WT-sham. No significant effect in hippocampus was observed.

Endocannabinoidome analysis

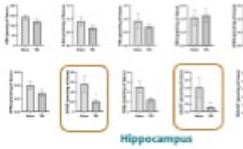


Gene expression analysis showed a significant reduction of PPARa gene in the hippocampus of TBI group (Fig 1A). The other genes analyzed and that did not undergo to significant alterations in hippocampus (Fig 1A) and cortex (Fig 1B) included Cb1, Trpv1, Dagla, Daglb, Napepld, Faah, Gde, Magl and Pparg.



2-AG levels in cortex of WT-mTBI significantly decreased and also OEA, whereas two members of the N-acylserotonines family identified (OAsHT and DHAsHT) increased vey significantly.

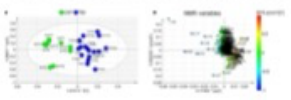
Cortex



In the hippocampus, among monoacylglycerols, just 2-DHG levels were altered significantly and decreased in WT-mTBI group. Instead, among N-acylserotonines, OAsHT decreased significantly after TBI.

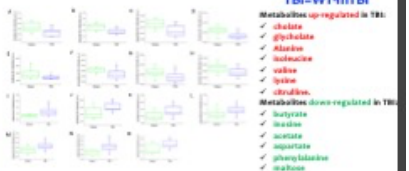
Hippocampus

Untargeted metabolomics



OPLS-DA of NMR metabolic profiles of mice feces.
(A) Scores plot. (B) Loadings plot.

CNT=WT-sham
TBI=WT-mTBI



Metabolites up-regulated in TBI:
- cholate
- glycholate
- Alanine
- Isoleucine
- valine
- lysine
- citrulline.

Metabolites down-regulated in TBI:
- butyrate
- inosine
- acetate
- aspartate
- phenylalanine
- maltose
- tyrosine
- nicotinate.

Discussion and conclusions

- ✓ In conclusion our data confirm previous studies showing that mTBI induces a characteristic dual behavioral phenotype (aggressive/depressive) in mice (Guida et al., 2017; Antonio et al., 2019 and Piscitelli et al., 2013). In addition, we demonstrated that mTBI causes significant impairments in the discriminative and spatial memory tasks. However, the experiments performed reveal that mTBI does not induce significant changes in APP animals. This finding may due to the behavioral alterations that we detected in sham APP mice as compared with WT. Indeed, as a modelling of psychological symptoms of dementia, APP transgenic mice present a hyperactive and agitated phenotype (Lalonde et al., 2002). Thus, we cannot exclude that the behavioral impairments induced by the trauma were not evident given the basal behavioral alterations.
- ✓ Our data showed that mTBI induced in APP mice (9-18 weeks) led to increased levels of $A\beta_{(1-42)}$ in the cortex of these mice at the end of the in vivo tests (17-25 weeks) suggesting that AD is a long-term consequence of mTBI.
- ✓ Investigations of the eCBome in WT mice revealed that a significant alteration of the endogenous tone after mTBI occur. We identified N-acylserotonin for the first time in the brain of these animals that are known to inhibit eCB inactivation by FAAH and at same time to antagonize TRPV1 channel, another important target for eCBome mediators.
- ✓ Metabolomics analysis showed that TBI reduced both butyrate and acetate, that are two of the main Short chain fatty acids (SCFAs) produced by bacterial fermentation of dietary fiber and are important signaling molecules in the microbiota gut-brain axis. Accordingly, to previous findings, though in a different TBI model, TBI led to a reduction of SCFAs, especially acetate, in stool samples (Opeyemi et al., 2021). Moreover, also aspartate and derivatives, as N-acetylaspartate (NAA), is a well-known marker of energy metabolism that is found to be decreased in patients suffering from TBI (Amin et al., 2015).
- ✓ Given their proposed neuroprotective role, both the endocannabinoidome and gut microbiome mediators whose concentrations were shown here to be modified following trauma, might be partly responsible for the pathological, behavioural and biochemical, alterations observed in this condition

Acknowledgements: Funded by USAMRDC Peer Reviewed Alzheimer's Research Program Convergence Science Research Award Program Announcement (Funding Opportunity Announcement Number W81XWH-19-PRARP-CSRA)