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TITLE: Combinatorial Biosignatures of Network Dysfunction to Predict Posttraumatic Epilepsy:
Clinical Translation from a Robust Mouse Model

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14. ABSTRACT Traumatic brain injury (TBI) is a leading cause of mortality and morbidity. Early life-saving treatments have improved survival but optimal development of preventive and therapeutic interventions for late sequelae such as post-traumatic epilepsy (PTE) require additional work. The latent period between the biomechanical insult and epilepsy onset presents a unique window of opportunity for antiepileptogenesis treatments in high-risk patients, and effective early preventive treatments in high PTE risk subgroups could provide substantial clinical benefit. The development of preventive interventions has been hampered however by the limited knowledge of the mechanisms underlying TBI-induced epileptogenesis and by the lack of validated non-invasive biomarkers for identifying patients at-risk for PTE. Using an in vivo mouse model of PTE, we will investigate whether changes in specific pathologic neuronal network leading to epilepsy after TBI, can be detected early post-injury by combining EEG and brain MRI analyses, together with measurements of selected blood neuroinflammatory molecules, autoantibodies and circulating micro(mi)RNAs. The key drivers/ key biosignatures identified in the preclinical model will be subjected to preliminary clinical verification using archived acute, subacute and chronic serum samples from TBI patients with/without PTE.					
15. SUBJECT TERMS Post-traumatic epilepsy; mouse model; combinatorial biomarker(s); validation of biomarkers in human PTE; EEG; magnetic resonance imaging; blood and brain analyses; proteomic analysis; microRNA; system biology					
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1. INTRODUCTION:

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity. Early life-saving treatments have improved survival but optimal development of preventive and therapeutic interventions for late sequelae such as post-traumatic epilepsy (PTE) require additional work. There is a latent period between the insult and epilepsy onset that presents a unique window of opportunity for antiepileptogenesis treatments in high-risk patients. The development of such treatments has been hampered by the lack of non-invasive biomarkers for identifying the patients at-risk of developing PTE, or for early prediction of disease severity, thereby allowing to design affordable clinical studies. In this project, we will collect biosignatures from brain electrophysiological and MRI signals as well as selected blood molecules such as neuroinflammatory and brain-derived proteins and circulating microRNAs (miRNAs). Predictivity of the proposed biosignatures will be tested in our robust PTE mouse model, where epilepsy with frequent seizures occurs in ~50% of mice 3-5 months post-TBI as determined by 24/7 video-EEG recordings. The key drivers/ key biosignatures identified in the preclinical model will be subjected to preliminary clinical verification using archived acute, subacute and chronic serum samples from TBI patients with/without PTE collected within the CENTER-TBI cohorts of patients. Clinically validated PTE biosignatures could ultimately serve as PTE risk and diagnostic biomarkers as well as lead candidates for novel therapeutic targets.

2. KEYWORDS:

Post-traumatic epilepsy; mouse model; combinatorial biomarkers; biomarker validation in human PTE; EEG; brain imaging; blood and brain analyses; proteomic analysis; microRNA; system biology

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1 - To monitor PTE development by ECoG and brain MRI analyses in mice, and sample collection	Duration (mo.)	PI/ Co-PI	Perform. Site	% Completed
Major Task 1 - Project regulatory review and approval to initiate animal studies				
Subtask 1.1: Protocol for regulatory review and approval by local IACUC animal protocol, and by the USAMRMC Animal Care and Use Review Office (ACURO)	1-3	ERZ	IRFMN	
<i>Milestone 1 - Local IACUC approval</i>	2			100%
<i>Milestone 2 - USAMRMC ACURO approval</i>	3			100%
Major Task 2 - To perform a longitudinal analysis of video-ECoG, and to collect blood and brain tissue samples at critical time points of PTE development in mice				
Subtask 2.1: Longitudinal video-ECoG monitoring (24/7) at 1-7 days, and 70 days and 5 months for 3 weeks each monitoring session, blood (7 days, 2 and 5 months) and tissue (5 months) sampling post TBI/sham injury in mice (8 weeks old CD1 mice, Envigo). Number of mice needed: 54 injured mice (randomized to 27 shams)	4-12	ERZ, TR	IRFMN	100%

To take into account to the mortality rate after TBI induction, the sporadic loss of ECoG implants and failure to record high quality ECoG, and problems during longitudinal blood sampling, the number of mice exposed to TBI will be increased by 20% (total of 65 injured mice)				
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Subtask 2.2: Quantitative analysis of ECoG signal during the first 7 days after TBI (acute symptomatic seizures, spikes and nonlinear dynamics)	4-12	ERZ, TR	IRFMN	75%
Subtask 2.3: Longitudinal blood collection and tissue harvesting at sacrifice obtained from video-ECoG-recorded mice for subsequent biochemical analyses (to be done at Aim 2)	4-12	ERZ, TR	IRFMN	100%
Subtask 2.4: Identification of PTE onset time and reckoning of number and duration of spontaneous seizures in chronic phase	4-16	ERZ, TR	IRFMN	100%
Subtask 2.5: Set-up and monitoring of logistics for coordination of mouse sample storage and shipment	4-12	ERZ, TR, KW, WH	IRFMN, UF, GB	80%
Subtask 2.6: Longitudinal behavioral evaluation at acute and chronic stages (sensorimotor function, locomotor activity and cognitive deficits)	4-16	ERZ, TR	IRFMN	100%
Milestone 3 - Transfer and banking of blood and tissue samples to be used for PTE combinatorial biosignatures discovery	12	ERZ, TR	IRFMN	30%
Milestone 4 - Definition of the relationship between early ECoG signal and PTE (primary outcome) or neurological dysfunction (secondary outcome)	16	ERZ, TR	IRFMN	
Major Task 3 – To perform longitudinal MRI analysis, and collect blood and brain tissue samples at critical time points of PTE development.				

Subtask 3.1: Longitudinal <i>in vivo</i> MRI (7 days and 2 months) and blood (7 days, 2 and 5 months) and tissue (5 months) sampling post TBI/sham injury in mice (8 weeks old CD1 mice, Envigo) Number of mice needed: 54 injured mice (randomized to 27 shams) To take into account to the mortality rate after TBI induction, the sporadic loss of ECoG implants and failure to record high quality ECoG, and problems during longitudinal blood sampling, the number of mice exposed to TBI will be increased by 20% (total of 65 injured mice)	13-22	ERZ, TR	IRFMN	80%
Subtask 3.2: Acquisition of MRI-based images using structural T2w and functional connectivity maps using diffusion tensor images (DTI)	13-22	ERZ, TR	IRFMN	80%
Subtask 3.3: Acquisition of molecular MRI-based images to assess endothelial activation/dysfunction by using antibody-based microparticles of iron oxide targeting p-selectin and VCAM-1	13-22	ERZ, TR	IRFMN	
Subtask 3.4: Longitudinal blood collection and tissue harvesting at sacrifice for subsequent biochemical analyses (to be done at Aim 2)	13-22	ERZ, TR	IRFMN	80%
Subtask 3.5: Quantification of MRI images to assess structural and molecular changes	13-28	ERZ, TR	IRFMN	50%
Subtask 3.6: Set-up and monitoring of logistics for coordination of mouse sample storage and shipment	13-22	ERZ, TR, KW, WH	IRFMN, UF, GB	45%
Subtask 3.7: Longitudinal behavioral evaluation at acute and chronic stages (sensorimotor function, locomotor activity and cognitive deficits)	4-16	ERZ, TR	IRFMN	80%
Milestone 5 - Transfer and banking of blood and tissue samples derived from MRI-exposed mice to be used for PTE combinatorial biosignature discovery	22	ERZ, TR	IRFMN	
Milestone 6 - Definition of the relationship between MRI measures and PTE (primary outcome) or neurological dysfunction (secondary outcome)	28	ERZ, TR	IRFMN	
Aim 2 - To monitor PTE development using blood and tissue samples in mice	Duration (mo.)	PI/ Co-PI	Perform. Site	

Major Task 4 - Proteomic analysis with unbiased and targeted LC- MS/MS				
Subtask 4.1: Qualitative and quantitative analysis of blood and brain tissue specimens with LC-MS/MS	9-24	WH	GB	40%
Subtask 4.2: Comprehensive examination of LC-MS/MS data with computer algorithms	9-24	WH	GB	30%
<i>Milestone 7 - Volcano plots to prioritize and visualize combinatorial PTE biosignatures (primary outcome) and ROC plots to determine the sensitivity/specificity of each measure and their combinations (secondary outcome)</i>	24	WH	GB	
<i>Milestone 8 - Prioritization of combinatorial PTE biosignatures in brain tissue to inform on combinatorial PTE biosignatures to be examined in serum and in silico in Major Tasks 5-9</i>	24	WH	GB	
Major Task 5 - Targeted immunoassays for neuroinflammatory and brain-derived proteins, and autoantibodies				
Subtask 5.1: Analysis of serum samples obtained from Aim 1 for changes in neuroinflammatory and brain-derived proteins and autoantibody biomarker levels to track the risk for development and progression of PTE	9-25	KW	UF	
Milestone 9 – Definition of the relationship between neuroinflammatory and brain protein biomarker identified molecules and PTE (primary outcome) or neurological dysfunction (secondary outcome).	25	KW	UF	
Milestone 10 - Definition of the emergence of a subacute and/or sustained autoantibody response in relation to PTE (primary outcome) or neurological dysfunction (secondary outcome)	25	KW	UF	5%
Major Task 6 - Analysis of micro(mi)RNA biomarker candidates				
Subtask 6.1: Analysis of blood samples obtained from Aim 1 for changes in serum miRNA biomarker candidate panel to track the risk for development and progression of PTE	9-25	KW	UF	5%
Milestone 11 - Definition of the relationship between key miRNA drivers and PTE (primary outcome) or neurological dysfunction (secondary outcome)	25	KW	UF	5%

Major Task 7 - To identify enriched cellular signaling pathways and pathophysiological mechanisms for PTE by systems biology/pathway analysis				
Subtask 7.1: Analysis of combinatorial PTE biosignatures from Major Tasks 4-6 will be analysed with systems biology/pathway analysis tools to investigate enriched cell signaling pathways and pathophysiological mechanisms of PTE.	26-30	WH	GB	
<i>Milestone 12 - Enriched pathways and mechanisms from statistically significant PTE biosignatures mapped and annotated in IPA (primary outcome).</i>	30	WH	GB	
<i>Milestone 13 - Results from causal analysis of enriched pathways and mechanisms with respect to PTE development (secondary outcome)</i>	30	WH	GB	
Aim 3 - Identification of combinatorial biosignatures in mice				
Major Task 8: Combinatorial analyses using mouse data obtained at Aim 1 (ECoG), Aim 2 (MRI) and Aim 3 (blood and tissue)	31-33			
Subtask 8.1: To combine ECoG, MRI, blood and brain measures to identify a palette of biosignatures for PTE in mice with high sensitivity/specificity	31-33	ERZ, TR, KW, WH	IRFMN, UF, GB	
<i>Milestone 14 – Definition of a combination of biosignatures that predict PTE and monitor disease progression</i>	33	ERZ, TR, KW, WH	IRFMN, UF, GB	
Aim 4 - To perform a preliminary clinical verification study in TBI patients with/without PTE				
Major Task 9 - Project regulatory review and approval to initiate human studies				
Subtask 9.1: Protocol for regulatory review and approval by the IRB human/human tissue study and DOD IRB protocol review	1-6	DM	UC	90%
<i>Milestone 15 – DOD IRB protocol approval</i>	6	DM	UC	
Major Task 10 – Autoantibody response and PTE development in patients				
Subtask 10.1: To examine in the Cambridge cohort the association between PTE and the autoantibody response (in terms of epitope profile and degree of response) in serum at different time point after TBI	7-30	DM	UC	95%

Milestone 16 - Definition of the relationship between autoantibody profile and PTE in patients	30	DM	UC	
Major Task 11 - Preliminary clinical verification of emerging PTE biosignatures				
Subtask 11.1: To examine the archived serum samples from the CENTER-TBI cohort to test the predictivity of PTE by combining key drivers identified in the preclinical model with those emerging from Aim 3	31-34	ERZ, TR, KW, WH, DM	IRFMN, UF, GB, UC	
Milestone 17 - Definition of the predictive value of the identified blood biosignature in the risk of PTE development or progression in patients	34	DM	UC	
Aim 5 – Conclusive administrative issues and data dissemination				
Major Task 12 – Manuscript preparation and final report submission	34-36	ERZ, TR, KW, WH, DM	IRFMN, UF, GB, UC	
Subtask 12.1: Final web or phone conference to review all key data	34	ERZ, TR, KW, WH, DM	IRFMN, UF, GB, UC	
Subtask 12.2: Manuscript preparation	34-36	ERZ, TR, KW, WH, DM	IRFMN, UF, GB, UC	20%
Subtask 12.3: Preparation and submission of the scientific report	34-36	ERZ, TR, KW, WH, DM	IRFMN, UF, GB, UC	
Milestone 18 - Manuscript preparation /submission	36	ERZ, TR, KW, WH, DM	IRFMN, UF, GB, UC	20%
Milestone 19 - DOD CDMRP final report submission	36	ERZ, TR, KW, WH, DM	IRFMN, UF, GB, UC	

What was accomplished under these goals?

Major Task 1 - Project regulatory review and approval to initiate animal studies (IRFMN).

IRFMN accomplished Milestone 1 (local IACUC approval) and Milestone 2 (USAMRMC approval).

Major Task 2 - To perform a longitudinal analysis of video-ECoG, and to collect blood and brain tissue samples at critical time points of PTE development in mice

Subtask 2.1-2.3 (IRFMN)

1) Activities.

CD-1 mice (8 weeks old) were exposed to either traumatic brain injury or sham injury. Severe TBI was induced by cortical controlled impact (CCI; antero-posteriority: -2.5 mm, laterality: - 2.5 mm, velocity: 5 m/s, depth: 2 mm) over the left parieto-temporal cortex. Immediately after injury, mice were implanted bilaterally with cortical electrodes, and continuously (24/7) ECoG recorded during the following 7 days. Then, TBI mice were temporarily disconnected from the ECoG set up and left in their home cage until they were again monitored longitudinally (24/7) at 1.5 (time approaching PTE onset) and 5 months (chronic phase of spontaneous seizures) after TBI for 3 weeks each recording period.

In addition, to characterize the effects of surgical procedures on ECoG signatures, naïve mice (electrode implantation only, n=5), were also prepared and analysed. ECoG monitoring in naïve-operated control mice was done as described for TBI mice.

2) Significant results and outcomes.

We completed the ECoG longitudinal monitoring in the TBI-exposed mice (IRFMN).

Based on the ECoG analysis done at 5 months post-TBI, the total number of PTE+ and non-PTE mice since the beginning of the project is:

- **PTE+ mice: n=18**
 - ✓ **15 out of 18 PTE+ completed the longitudinal ECoG analysis (5 mo post-TBI):**
 - ✓ 12 PTE+ mice have the acute ECoG recording.
 - ✓ 3 PTE+ mice did not have the acute ECoG recording (backup mice)
 - ✓ **3 out of 18 PTE+ mice died at 5 months follow up**, but they were considered as epileptic since they develop spontaneous seizures at 1.5 mo. These 3 PTE+ mice have the acute ECoG recording.
- **non-PTE mice: n=12**
 - ✓ **all 12 non-PTE mice completed the longitudinal ECoG analysis (5 mo post-TBI):**
 - ✓ 8 non-PTE mice have the acute ECoG recording;
 - ✓ 4 non-PTE mice did not have the acute ECoG recording (backup mice).

Among the 15 PTE+ mice that completed the longitudinal ECoG analysis, 13 PTE+ mice develop discrete spontaneous seizures (**Figure 1**), while 2 PTE+ showed ECoG traces compatible with an intermittent status epilepticus. **Figure 1** below depicts the daily seizure frequency and the average seizure duration in those 13 PTE+ mice with discrete spontaneous seizures.

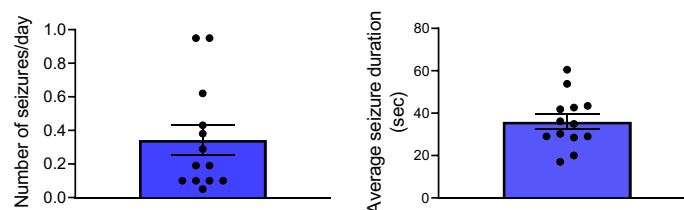


Figure 1. Bargrams depicting the daily seizure frequency (*left*) and the average seizure duration (*right*) in the PTE mice (n=13) that completed the longitudinal ECoG analysis.

We completed the ECoG longitudinal monitoring also in the control groups. In details, we generated:

- **sham mice (electrodes and craniotomy): n=5**
- **naïve mice (electrodes only): n=8**

None of these 13 control mice developed spontaneous seizures during the longitudinal ECoG recording.

Quantitative analysis of ECoG signal (IRFMN)

Acute phase post-TBI (day 1-7 after TBI or surgery)

Overall, the acute ECoG recording is available for 15 PTE+ mice and 8 non-PTE mice, and for 13 controls. We started the analysis of these traces, by quantifying:

1. the number of early seizures (i.e., seizures occurring within 7 days after TBI) and their average duration;
2. the number of spikes and sharp waves recorded in the 4 screw electrodes over time.

Early seizures

The incidence of mice developing early seizures, their total number and their average duration were evaluated in TBI and control animals. After defining mice at 5 months post-TBI as epileptic (PTE+) or non-epileptic (No SRS, PTE-) based on the presence/absence of ECoG spontaneous seizures, we retrospectively analysed and compared early seizure occurrence among experimental groups. Data are summarized in **Table 1** below.

Table 1. Quantification of early seizure in the various experimental groups.

	Early seizures (<7 days after TBI or sham injury)		
	Number of mice	Total number	Average duration (sec)
Sham controls	8/13	6.8±2.0	65.0±7.9
PTE+	4/18	2.0±0.7	44.3±9.3
No SRS (PTE-)	2/12	2.5±1.5	59.7±4.2

Within each experimental group, early seizures were detected only in a percentage of mice.

There was a slight increase in the occurrence of early seizure parameters in sham controls vs TBI mice, but they did not reach statistical significance. We think that this phenomenon may be due to the presence of a cerebral edema in TBI mice that possibly altered the detection of the ECoG signals during the time window closer to the brain injury. Thus, if early seizures occurred, they elapsed within 3 days after TBI or sham injury.

Notably, although early seizures developed in sham control mice, none of them developed spontaneous seizures, thus suggesting that in this model their occurrence is not a biomarker of PTE.

Quantification of the number of spikes and sharp waves.

Preliminary result (PTE+, n=7; non-PTE, n=4) showed that the quantification of the number of spikes/sharp waves in one of the two contralateral screw electrodes (green electrode; see Figure 3), distinguish PTE+ from non-PTE mice.

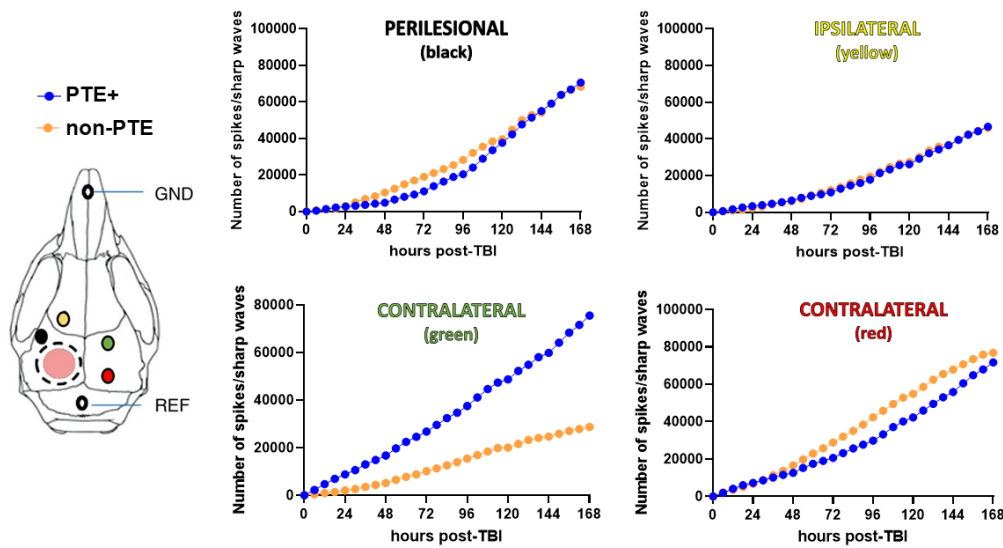


Figure 3. Quantification of the number of spikes and sharp waves during the first 7 days after TBI. *Left panel:* Schematic skull reproduction depicting the placement of cortical electrodes used for the ECoG recording (colored dots), ground (GND) and reference (REF) electrodes, the craniectomy (dotted circle), and the CCI injury (pink circle). *Right panels:* Graphs depicting the cumulative number of spikes during progressive 6 hrs intervals in the 4 screw electrodes used for the ECoG recording

ECoG recording at 1.5 months post-TBI.

We analysed the ECoG traces obtained at 1.5 months in TBI and control mice. Seven out of eighteen TBI mice identified as PTE+ at 5 months, develop spontaneous seizures already during this recording period. **Figure 4** below depicts at which day post-TBI the first spontaneous seizure was recorded, the daily seizure frequency during the recording period, and the average seizure duration. In the remaining 11 PTE+, no spontaneous seizures were detected at 1.5 months. These data are consistent with previous published data (Gao et al, PlosOne, 2013). None of the PTE- and control mice developed spontaneous seizures during the longitudinal ECoG recording.

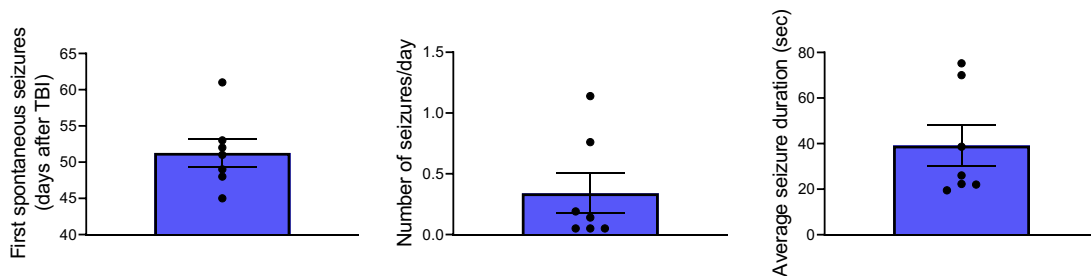


Figure 4. Bargrams depicting the day at which the first spontaneous seizure was detected (*left*), daily seizure frequency (*middle*) and the average seizure duration (*right*) in the PTE+ mice (n=8).

Blood sampling. In all the ECoG-recorded mice, five days before TBI or surgery (baseline), and at 8 days and 1.5 months post-TBI or surgery, blood was collected from the retromandibular plexus in TBI and naïve control mice. At sacrifice in the same animals, blood was withdrawn from the heart, and the selected brain areas were dissected out and stored at -80°C, as described in the experimental plan. Plasma was isolated, aliquoted and stored at -80°C.

Subtask 2.5 (UF and IRFMN)

Set-up and monitoring of logistics for coordination of mouse sample storage and shipment.

UF (Dr. Wang) will coordinate with IRFMN (Dr. Zanier) the shipment of the new available biosamples. The second set of brain tissues and plasma samples will be shipped by beginning of October from IRFMN to UF for subsequent -omic analyses.

Subtask 3.1, 3.2, 3.4 (IRFMN)

1) Activities

A second group (Group 2) of adult (8-week-old) CD-1 mice exposed to TBI (n=22) or sham injury (controls, n=8) underwent MRI analysis at 5 months post TBI/sham injury as follows: (i) RARE, 3D T2-weighted sequence (structural MRI); (ii) DTI sequence. After the imaging session, mice were implanted with screw electrodes, and ECoG-recorded continuously for 3 weeks to reckon the number and duration of spontaneous seizures.

2) Significant results and outcomes

Mortality rate and dropout. Two additional mice died before electrode implantation. Nineteen TBI mice underwent surgery for electrodes implantation using the robot drill device that we purchased. This improvement in the surgery procedure abolished the mortality rate during the recovery and reduced overall mortality by 12% (**Figure 5**).

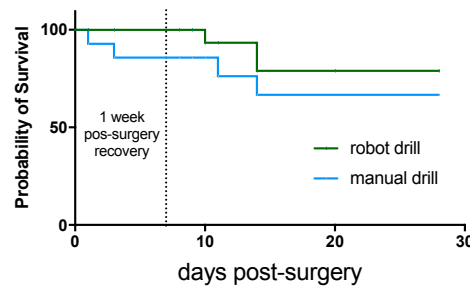


Figure 5. Kaplan-Meier curve comparing mortality associated to TBI and electrode implantation with the robot or the manual drill.

MRI analysis. We analyzed the MRI-DTI images acquired at 5 months post-TBI (or surgery for sham mice), to quantify white matter damage (**Figure 6**). We observed a widespread reduction of fractional anisotropy (FA) in TBI mice vs sham animals (**Figure 6**) with more pronounced reduction in the hemisphere ipsilateral to brain injury. These results were in line with those previously reported in our first group of mice (Group 1, detailed in our previous scientific report). ECoG analysis for the identification of mice with/without PTE is ongoing. As soon as the ECoG analysis at 5 months post-injury will be completed, TBI mice will be split in the two subgroups (PTE+, non-PTE) and the mice compared and analysed.

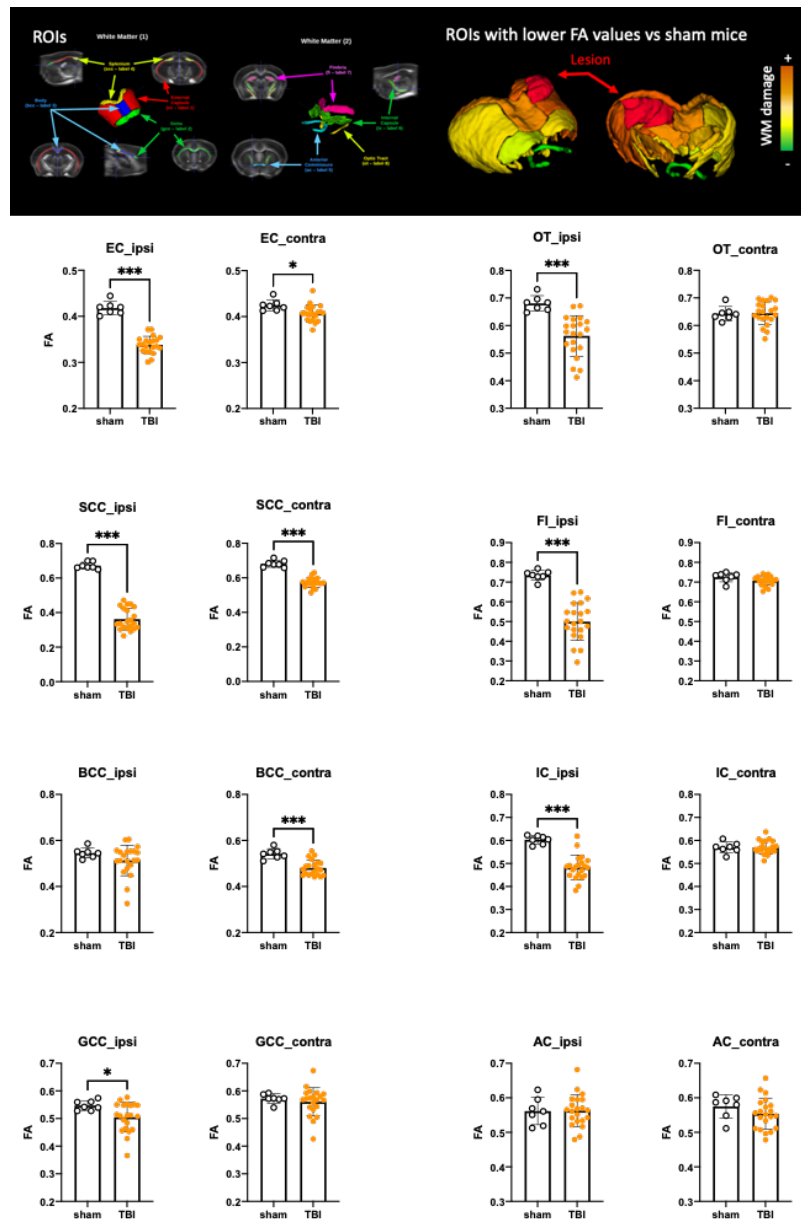


Figure 6. Quantification of FA in TBI and sham mice at 5 months post-surgery (sham) or brain injury (TBI). The first row depicts representative DTI images and 3D reconstructions (colored pictures) of the analysed white matter regions. Bargrams show FA values in these areas. * $p<0.05$, *** $p<0.0001$ by unpaired t-Test. GCC: genu of the corpus callosum; SCC: splenium of the corpus callosum; EC: external capsule; BCC: body of the corpus callosum; FI: fimbria of the hippocampus; OT: optical tract; IC: internal capsule; AC: anterior commissure

DTI analysis done in Group 1 showed that PTE mice had lower FA value in the ipsilateral optic tract (OT) compared to PTE- (No SRS) at 5 months after injury (**Figure 7A**). ROC analysis of Group 1 revealed that setting the cut-off value of FA in the ipsilateral-OT to 0.51 resulted in the diagnostic efficiency for differentiating between PTE and No-SRS mice, with a sensitivity of 83.3% and a specificity of 71.4% ($AUC = 0.81$) (**Figure 7A**). The analysis of the new Group 2 will offer the possibility to verify the predictivity of the identified cut-off. Accordingly, **Figure 7B** shows the mice (in pink) with high risk for PTE based on the ROC analysis. The ECoG analysis in mice of Group 2 at 5 months post-TBI is ongoing to identify PTE+ and PTE- animals.

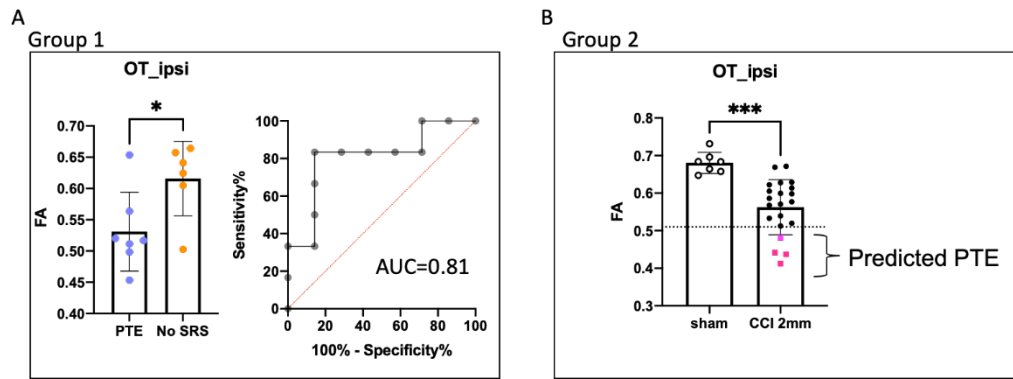


Figure 7. (A) DTI analysis performed in mice of Group 1. FA in the ipsilateral optic tract (OT_ipsi) is reduced in PTE mice and ROC analysis revealed that setting the cut-off value to 0.507 results in the diagnostic efficiency for differentiating between PTE and No SRS, with a sensitivity of 83.3% and a specificity of 71.4% (AUC = 0.81). **(B)** This cut-off is used to classified mice in Group 2 (pink subjects). * $p < 0.05$, *** $p < 0.01$ by unpaired t-Test.

Behavioral tests (Subtasks 2.6 and 3.7). Ten days, and 1.5 and 5 months post-TBI mice were longitudinally tested to evaluate their sensorimotor performance using three independent tests, namely composite neuroscore, Simple Neuroassessment of Asymmetric impairment (SNAP) and beam walk. After defining the animals at 5 months post-TBI as epileptic and non-epileptic based on the presence/absence of EcoG spontaneous seizures, we retrospectively analysed and compared their behaviour. We pull together mice obtained from Major Task 1 (Subtask 2.6) and Major Task 2 (Subtask 3.7), since they underwent the same longitudinal behavioral analyses. The SNAP test showed a sensorimotor deficit of a different extent between PTE+ vs No SRS (PTE-) mice at 5 months post-TBI (**Figure 8A**). In the SANP test, PTE mice showed a lower recovery rate compared to No SRS mice that is close to significance between 10 days to 1.5 month ($p = 0.05$), and significantly different between 10 days and 5 months ($p < 0.05$) (Figure 8B). TBI mice, independently or not they developed PTE, showed similar sensorimotor deficits as evaluated with Neuroscore and Beam walk (**Figure 8 C,D**).

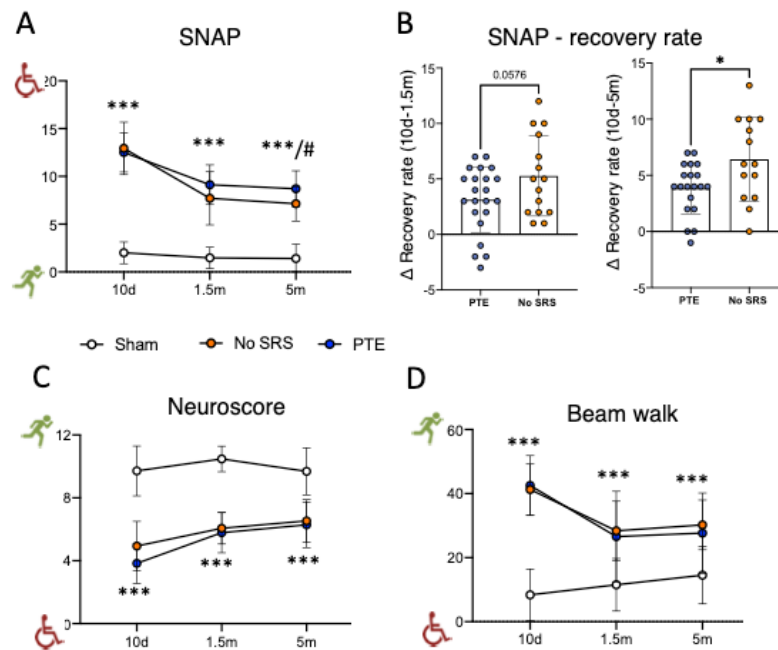


Fig 8. Longitudinal analysis of sensorimotor deficits in TBI and sham mice by SNAP (A,B), composite neuroscore (B), and beam walk (C). *** $p < 0.001$ vs Sham controls, # $p < 0.050$ vs No SRS (PTE-) by mixed model analysis using Tukey's multicomparison test in panel A, C and D. * $p < 0.05$ by unpaired t-test in panel B.

Blood sampling. In all the MRI-exposed mice, five days before TBI or surgery (baseline), and at 8 days and 1.5 months post-TBI or surgery, blood was collected from the retromandibular plexus in TBI and naïve control mice. At sacrifice in the same animals, blood was withdrawn from the heart, and the selected brain areas were dissected out and stored at -80°C, as described in the experimental plan. Plasma was isolated, aliquoted and stored at -80°C.

Subtask 3.6 (UF and IRFMN)

Set-up and monitoring of logistics for coordination of mouse sample storage and shipment.

UF (Dr. Wang) will coordinate with IRFMN (Dr. Zanier) the shipment of the new available biosamples. The second set of brain tissues and plasma samples will be shipped by October from IRFMN to UF for subsequent -omic analyses.

Major Task 4 - Proteomic analysis with unbiased and targeted LC- MS/MS

Subtask 4.1 (GB)

Qualitative and quantitative analysis of blood and brain tissue specimens with LC-MS/MS

Dr. Haskins engaged with Dr. Jin Kho, Proteomics & Mass Spectrometry Scientific Director at the University of Florida (UF) as well as Dr. Olga Vitek and Dr. Sue Abbatiello at Northeastern University (NEU) for future proteomic analysis with unbiased and targeted LC-MS/MS in Gainesville, FL and Boston, MA, respectively. This included discussions on instruments, methods and algorithms required for state-of-the-art data collection and analysis for both unbiased LC-MS/MS-based proteomics (at UF) and targeted LC-MS/MS-based proteomics (at NEU).

An Amazon Web Services (AWS) account has been created by Gryphon Bio to enable shared data and R scripts (e.g., MSStats) for proteomic analysis.

Moreover, Dr. Abbatiello has recently secured funding for a state-of-the-art tandem mass spectrometer with a high sensitivity/high duty cycle (TIMSTOF, Bruker) that Dr. Haskins has already tested with another tandem mass tag (TMT)-based proteomic project and a prototype TIMSTOF instrument (separately funded). Together with Dr. Vitek, Drs. Haskins and Abbatiello plan to implement the TIMSTOF into more traditional unbiased and targeted LC/MS/MS workflows (e.g., unbiased with Orbitrap instruments from Thermo and targeted with QTRAP instruments from Sciex) in future studies. Additional plans under discussion include the incorporation of high-field asymmetric waveform ion mobility spectrometry (FAIMS) for further improved sensitivity of targeted LC-MS/MS-based proteomic assays.

Tissue transferred are from the following 4 experimental groups at 5 mo.

- Naïve (no electrode implantation): n=8
- Naïve-E (Electrode implantation only): n=5
- TBI with PTE (i.e. with spontaneous seizures at 5 months): n=8
- TBI without PTE (i.e. without spontaneous seizure at 5 mo.): n=7

For proteomics studies, we prioritized to analyze mouse ipsilateral and contralateral cortex tissue and completed sample pulverization as followed:

Naïve Ipsi	n=4
Naïve Contra	n=4
Naïve-E Ipsi	n=4
Naïve-E: Contra	n=4
TBI (No SRS) Ipsi	n=4
TBI (No SRS) Contra	n=4
TBI (with PTE) Ipsi	n=4
TBI (with PTE) Contra	n=4
Total: n=40 samples	

Tandem Mass spectrometry-based TMT labeled proteomic study design has been locked down with UF-ICBR proteomic core (subjected to minor modifications). It covers 5 mo. Ipsilateral and contralateral cortex lysate samples (n=4 each group x 8 groups, 32 samples with also four additional pooled samples (naïve, naïve-E, Sham, TBI- no PTE, TBI + PTE) for standardization. Note that for cost consideration, n=4 individual mice / group were used for this stage of proteomic biomarker discovery. Data collection for unbiased LC-MS/MS-based proteomics with TMT labeling has been completed at UF.

Statistical analysis with MSstatsTMT at Northeastern University (NEU), as well as custom R scripts developed on GB's AWS proteomics platform, has been performed for 99% of this dataset, where we (GB and NEU) are verifying the top-scoring differentially abundant proteins (**Figure 9**) by a variety of *in silico* methods prior to verification in the laboratory (e.g., targeted LC-MS/MS and western blotting).

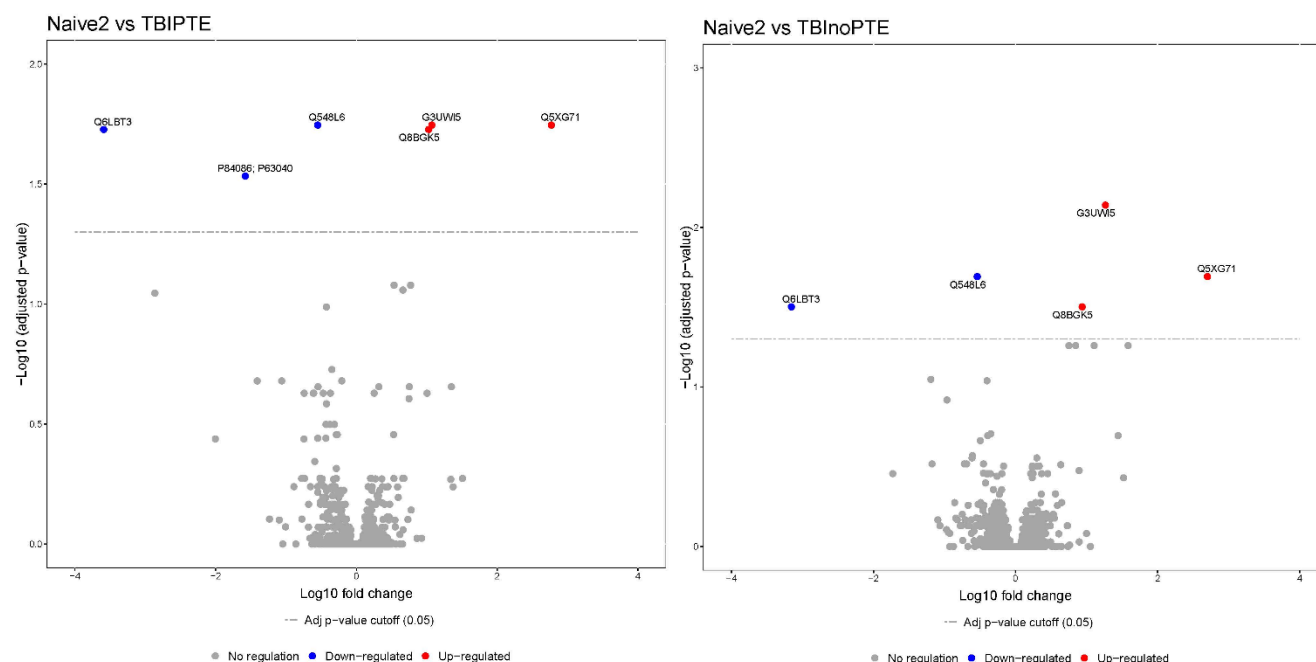


Figure 9 above shows examples of volcano plots for top-scoring differentially abundant proteins revealed by unbiased LC-MS/MS-based proteomics with TMT labeling and MSStatsTMT analysis. The volcano plots show that Uniprot proteins Q6LBT3 (NCAM) and Q543L6 (GAD1) are consistently observed at statistically significant higher levels in both PTE and TBI samples (vs. naïve2, no SRS controls).

Global metabolomics profiling was performed on a Thermo Q-Exactive Orbitrap mass spectrometer with Dionex UHPLC and autosampler. All samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 35,000 at m/z 200 as separate injections. Separation was achieved on an ACE 18-pfp 100 x 2.1 mm, 2 μm column with mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. This is a polar embedded stationary phase that provides comprehensive coverage, but does have some limitation is the coverage of very polar species. The flow rate was 350 $\mu\text{L}/\text{min}$ with a column temperature of 25°C. 4 μL was injected for negative ions and 2 μL for positive ions.

Metabolomic experiments have been completed both in the ipsilateral and contralateral cortices. The analysis of metabolites was started in the ipsilateral cortices.

Data from positive and negative ion modes were separately subjected to statistical analyses. There were a total of 1267 features detected from the positive mode and 1982 features in the negative mode as shown in Figure 6. All subsequent data analyses were normalized to the sum of metabolites for each sample. MZmine (freeware) was used to identify features, deisotope, align features and perform gap filling to fill in any features that may have been missed in the first alignment algorithm. All adducts and complexes were identified and removed from the data set. All settings including the MZmine file can be provided if requested. MZmine is a free metabolomic data processing program. The data tables consist of several columns that are described in the next section. The data

was searched against our internal retention time metabolite library. Subsequent searches against HMDB, METLIN, and KEGG can be performed.

PLS-DA was performed to differentiate between the sham control, PTE+ (TBI A) and TBI B and the score plot shows partial segregation of the studied groups however, low Q2 value was observed even when using 4 principal components.

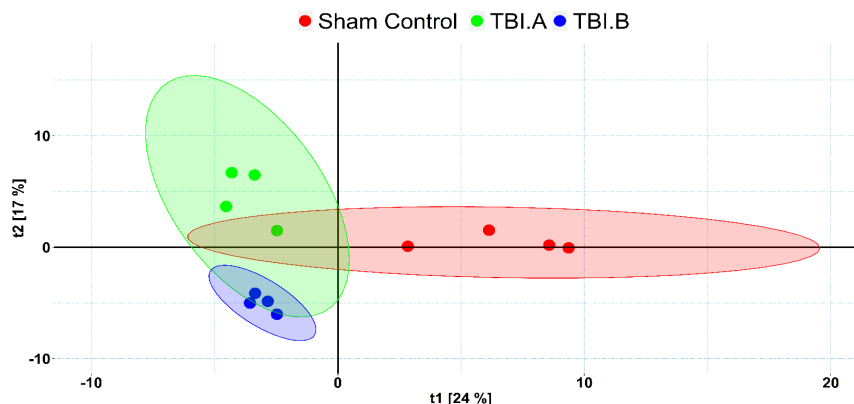
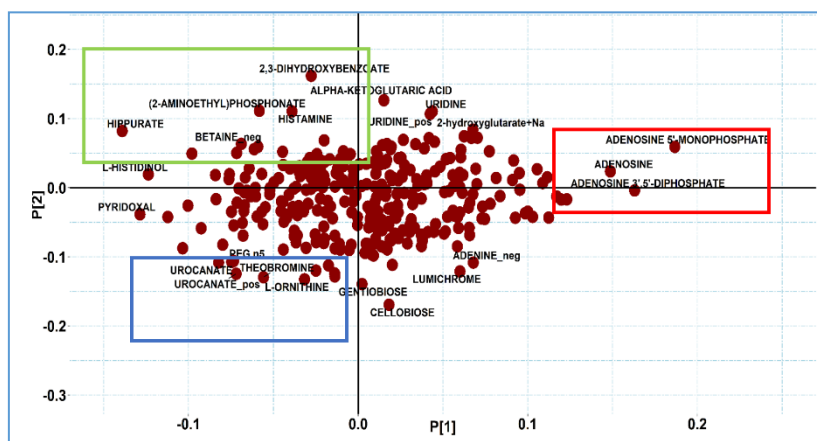


Figure 10 above shows the principal component analysis (PCA) of the positive and negative data set of metabolites including known/unknown metabolites. This can be depicted by looking on the loading plot showing the metabolites associated with such segregation (**Figure 11** below).



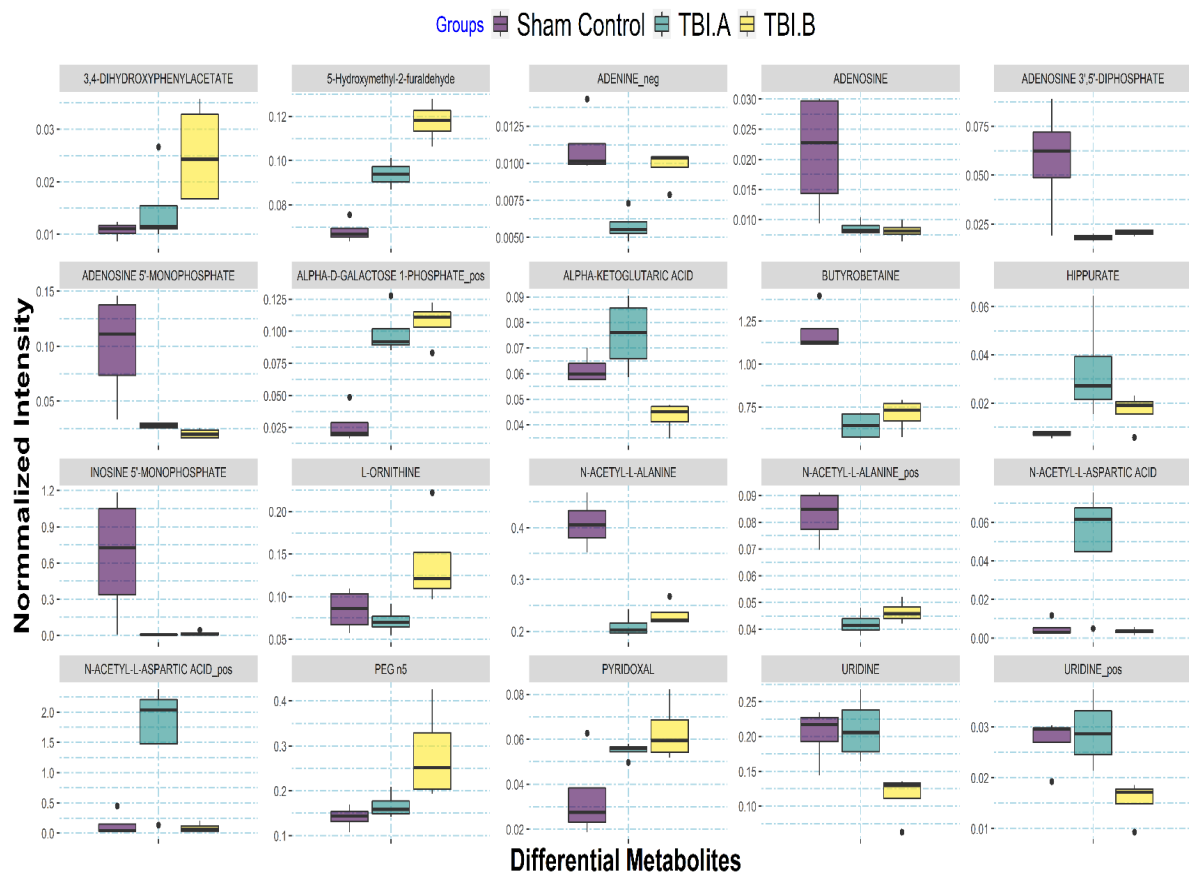
A list of differential metabolomic hits has been generated and plotted to identify those altered metabolites that are differentially expressed between the TBI group (with and without PTE) and Sham group. These metabolites (p values < 0.05, VIP > 1) are listed below in **Table 2**.

Table 2. Top 20 Differential Statistical Metabolites Identified

Differential metabolite	VIP	P value
5-Hydroxymethyl-2-furaldehyde	1.526071655	7.62063E-06
N-ACETYL-L-ALANINE_pos	1.667288276	1.82358E-05
N-ACETYL-L-ALANINE	1.654475091	2.30109E-05
ALPHA-D-GALACTOSE 1-PHOSPHATE_pos	2.493792591	0.000126904
BUTYROBETAINE	1.578809913	0.0001435
ALPHA-KETOGLUTARIC ACID	1.681260662	0.001866486

ADENOSINE 5'-MONOPHOSPHATE	2.350936188	0.001943867
N-ACETYL-L-ASPARTIC ACID	3.39707858	0.009161735
ADENOSINE 3',5'-DIPHOSPHATE	1.902900251	0.009725797
ADENOSINE	1.758919367	0.01145279
N-ACETYL-L-ASPARTIC ACID_pos	3.688203328	0.015198179
PEG n5	1.625487866	0.015424227
URIDINE_pos	1.751541409	0.023069802
ADENINE_neg	1.75272117	0.024439894
URIDINE	1.750814816	0.024439894
PYRIDOXAL	1.517141747	0.027848368
INOSINE 5'-MONOPHOSPHATE	3.415472014	0.0290227
3,4-DIHYDROXYPHENYLACETATE	1.719099504	0.032392694
L-ORNITHINE	1.67059472	0.034210871
HIPPURATE	2.198066583	0.038774208

Figure 12 below shows the changes in the 20 differentially expressed metabolites between the experimental groups [Sham, TBI.A (PTE+), and TBI.B (non-PTE)].



GB/UF teams are scheduled to continue the analysis of metabolites. Lipidomic in the same cortical samples has been just completed, and the analysis is ongoing.

Major Task 6 - Analysis of micro(mi)RNA biomarker candidates

Subtask 6.1 (UF)

UF has worked up Abcam Human Neurology 65 miRNA panel feasibility Analysis (Firefly-bead-based multiplex platform). We identified that they are compatible with mouse samples.

All 65 selected miRNAs were measured in the ipsi- and contralateral cortices at 5 months post-TBI. 54 of them produced measurable signals.

We identified only 5 miRNA with different up- or down-regulation profile in PTE+ (epileptic) vs No SRS (PTE-) vs Sham controls (n=4 each experimental group), namely hsa-mir-342, hsa-mir-338, hsa-mir-125b-5p, hsa-mir-134-5p and hsa-mir-370 (**Figure 13** below).

These results will be confirmed using a larger number of mice, by including additional samples (see Subtasks 2.5 and 3.6).

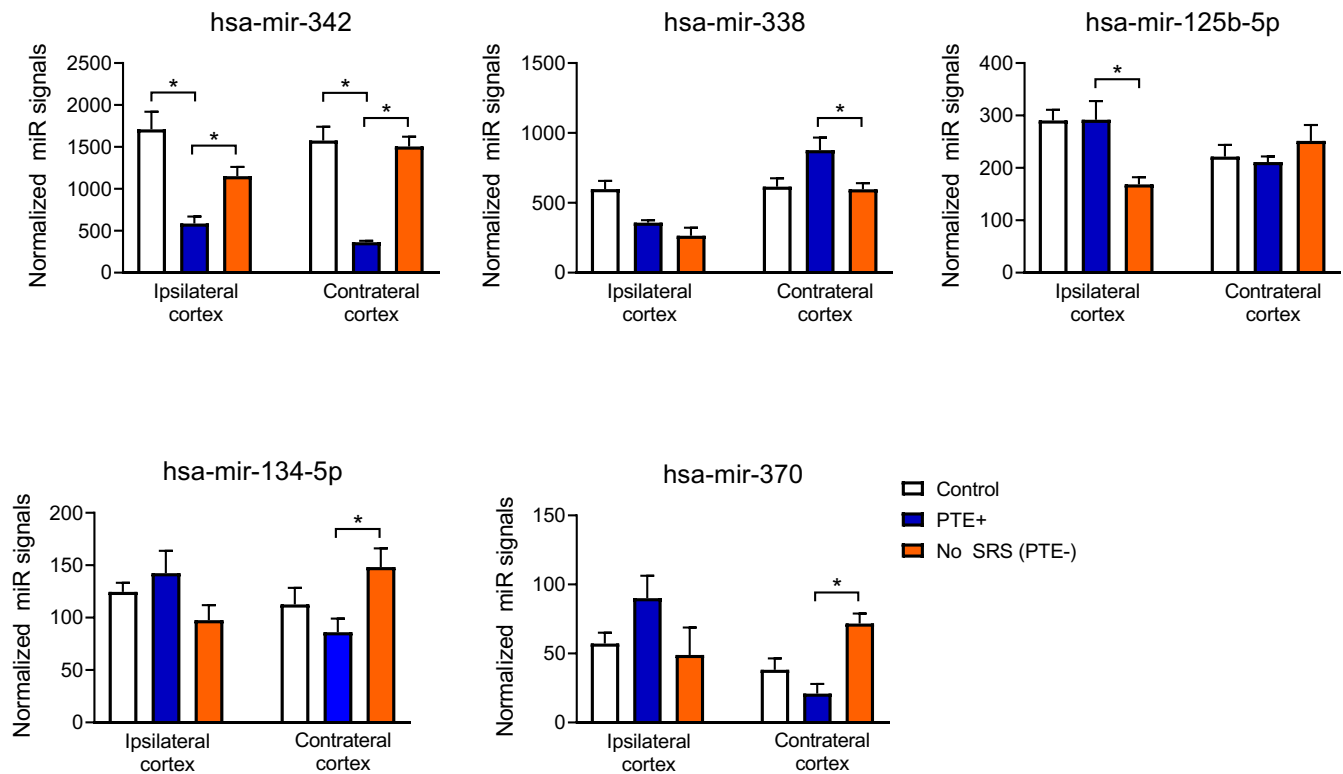


Figure 13. Bargrams depicting the levels of the differentially-expressed miRNAs in the ipsi- and contralateral cortices in the various experimental groups at 5 months post-TBI (n=4 each group). *p<0.05 by Kruskal-Wallis test.

Major Task 9 - Project regulatory review and approval to initiate human studies

Subtask 9.1 (UC, UF)

UC has obtained the approval for the HRPO-IRB protocol.

We are addressing some questions from UF IRB, thus the approval is still pending. We anticipate approval of this IRB protocol imminently

Dr. Zanier (IRFMN), Dr. Wang (UF) and Dr. Menon (UC) are working together to obtain non-human DOD HARPO approval.

Major Task 10 - Autoantibody response and PTE development in patients

Subtask 10.1 (UC)

The new custom protein microarray has been developed, printed, delivered and passed quality control checks. Profiling of the CENTER-TBI serum samples is currently undergoing. 600 samples have already been processed,

and preliminary analysis reveals comparable results to our pilot data, with subacute polyantigenic responses occurring over the first two weeks, with dominant responses to particular antigens being apparent. Once all the analysis has been undertaken, blinding to which patients have PTE will be broken, and the association between autoantibody production and PTE ascertained.

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

Longitudinal studies in the PTE mouse model (IRFMN) are conducted involving a PhD student and a Post-Doc thus providing training opportunities for staff with mentoring by Drs Elisa R Zanier and Teresa Ravizza.

Proteomic analysis with unbiased and targeted LC- MS/MS (GB): This project's subaward to Gryphon Bio will provide training opportunities for staff at Northeastern University's Mass Spectrometry Core/Barnett Institute with mentoring by Drs. Haskins, Vitek and Abbatiello.

How were the results disseminated to communities of interest?

IRFMN. Data related to the project have been presented during a Department meeting. The title of the presentation was: "In-depth characterization of a mouse model of post-traumatic epilepsy for biomarker and drug discovery" (May 11th, 2021).

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

Subtask 2.1-2.4 (IRFMN): To continue the analysis of acute ECoG traces.

The activities planned are:

- i) to complete the quantification of the number of spikes/sharp waves;
- ii) to start the spectral analysis;
- iii) to start the analysis of non-linear dynamics.

Subtask 3.1, 3.2, 3.4 (IRFMN): To continue the ongoing experiments in MRI-exposed mice.

The activities planned are:

- i) to identify PTE+ and non-PTE in the second group of mice exposed to longitudinal MRI analyses, based on the presence/absence of ECoG spontaneous seizures at 5 months post-TBI;
- ii) to finalize structural MRI analysis (Group 2).
- iii) to design the MRI-based molecular studies.

To ship the second set of brain region tissue and serum samples from IRFMN to USA analytical sites for biomarker discovery studies (Major Task 4, 5 and 6; IRFMN, UF, GB)

- i) the second shipment to UF site is scheduled by the beginning of October 2021 by World Courier cold chain transport.

To complete and verify Proteomic analysis with unbiased LC- MS/MS (Subtask 4.1; GB)

The activities planned are:

- i) to verify the top-scoring differentially abundant proteins by a variety of *in silico* methods prior to verification in the laboratory (e.g., targeted LC-MS/MS and western blotting).
- ii) to process more mouse samples (controls, and TBI with and without spontaneous seizures) ifor further verification.

To continue the analysis for targeted immunoassays and miRNA (Major Task 5 and 6; UF, GB)

The activities planned are:

- i) to process mouse sample (controls, and TBI with and without spontaneous seizures).

To continue autoantibody analysis in PTE patients (Subtask 10.1; UC)

The activity planned is:

- i) to extract clinical data in order to identify those patients with PTE.

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?

This project is likely to make an impact on Gryphon Bio's trajectory for development and commercialization of novel CNS therapeutics. As one of the two first grants awarded to Gryphon Bio (as a Subaward), this project is expected to reveal signatures for novel biomarkers and/or therapeutic targets for PTE that might be of future interest to Gryphon Bio and/or its partners. Therefore, technology transfer is likely to play an important role in the long-term.

What was the impact on society beyond science and technology?

This project is likely to generate results for PTE that will lead to long-term changes in public knowledge of PTE biomarker signatures and change the behavior of PTE patients with worsening PTE biomarker signatures, including decision making with respect to treatment, sleep, diet, exercise, etc.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

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

IRFMN site. At the beginning of March 2020 the unexpected spread of the new coronavirus in Lombardy, where we are located, highly challenged our lives and work arrangements. Italy was in total lockdown as per authority orders until May. Our Institute and labs were closed and the PI of the project was involved in the organization of a new COVID-19 ICU in a dedicated hospital in Milan. We could get slowly back to bench work from June, but only in September 2020 the Mario Negri Institute officially reopened the administrative and scientific activities, although still with reduced personnel to comply with the physical distancing obligations, since in the meantime a second wave of coronavirus spread took place. Only in May 2021, we were back to normal. Therefore, we were not able to start the MRI-based molecular studies.

University of Cambridge: COVID-19 has impacted significantly on this project. The core team at UC are Intensive Care doctors, and had to prioritise clinical care during the peaks of the pandemic; recruitment to non-COVID-19 trials was unilaterally halted by the University. Furthermore, given the impact of COVID-19 on workplaces, our industrial partners were unable to manage processing the high numbers of samples required for this project. Therefore, we had to undertake a substantial portion of the laboratory processing ourselves. The combination of all of the above have slowed our expected progress; however, we are making good headway, and the results are encouraging.

Nothing to Report (GB, UF)

Changes that had a significant impact on expenditures

Nothing to report.

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

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Not applicable

6. PRODUCTS:**Publications, conference papers, and presentations**

The following original article has been published:

Rossella Di Sapia, Federico Moro, Marica Montanarella, Valentina Iori, Edoardo Micotti, Daniele Tolomeo, Kevin K.W. Wang, Annamaria Vezzani, Teresa Ravizza and Elisa R. Zanier. *In-depth characterization of a mouse model of post-traumatic epilepsy for biomarker and drug discovery*. Acta Neuropathologica Communication, 2021; 26;9(1):76.

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)

News item on this award are highlighted by IRFMN and GB:

IRFMN

<https://www.marionegri.it/laboratories/lab-of-acute-brain-injury-and-therapeutic-strategies>

<https://www.marionegri.it/dipartimenti/neuroscienze>

GB

www.gryphonbio.com

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	Project Role	ORCID ID	Person month worked	Contribution to the project	Funding Support
Elisa R Zanier	PI (IRFMN)	0000-0002-3011-8718	2.5	Project coordination, experimental planning, data analysis	Institutional support
Teresa Ravizza	Co-PI (IRFMN)	0000-0002-9578-018X	2.4	Experimental planning, data analysis	Institutional support
Federico Moro	Post-Doc (IRFMN)	0000-0002-2682-5583	8.5	TBI induction, behavioral analysis	Institutional support
Martina Di Nunzio	PhD student (IRFMN)	0000-0003-3903-5705	12	Electrode implantation, ECoG analysis, blood sampling	This award
William Haskins	Subaward site PI (GB)	0000-0001-7972-2142	0.73	Proteomic analysis with unbiased and targeted LC- MS/MS (planning)	This award + Gryphon Bio, Inc contribution of 16% of the 0.73 person months effort
Kevin Wang	Subaward site PI (UF)	0000-0002-9343-6473	0.3	Site coordination, discussion with Dr. Zanier, collaboration and discussion with Dr. Haskins at Gryphon	Institutional support
Zhihui (Sunny) Yang	Co-I (UF)	0000-0002-5075-5108	0.24	Logistic preparation for receiving biosamples and assaying, miRNA assay optimization, reporting to Dr. Wang	Institutional support
Fan Lin	Research Assistant	(none)	0.84	Logistic preparation for receiving biosamples from IRFMN	Institutional support

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?

Proteomic analysis with unbiased and targeted LC- MS/MS:

Organization Name: Northeastern University/Barnett Institute

Location of Organization: Boston, MA

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

- Financial support; None, other than subaward funding from Gryphon Bio, Inc.

- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff); Instrumentation that is part of the Mass Spectrometry Core/Barnett Institute Facilities (e.g., project staff use the partner's facilities for project activities); Mass Spectrometry Core/Barnett Institute
- Collaboration (e.g., partner's staff work with project staff on the project); Drs. Olga Vitek and Sue Abbatiello
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); None yet.
- Other. N/A

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHART is submitted as separate attachment.

9. APPENDICES.

Nothing to report.