CLINICAL STUDY PROTOCOL

Study title	**Long-term recovery and microbiota–gut–brain axis disruption after traumatic brain injury (OVERCOME-TBI)**
Version	2.1
Date	20.6.2024
Principal Investigators	Jussi Posti
Institute	Turku University Hospital, Finland

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SYNOPSIS

Study title:

Long-term recovery and microbiota–gut–brain axis disruption after traumatic brain injury (OVERCOME-TBI)

Study code:

OVERCOME-TBI

Study center and investigators:

- Neurocenter, Turku Brain Injury Center, and Department of Gastroenterology, Turku University Hospital
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Objectives:

1. To study the long-term outcome and symptom development in different severities of traumatic brain injury (TBI) and correlate findings with longitudinal investigations (magnetic resonance imaging, biomarkers) and other clinical variables, and
2. To study the gut microbiota and the intestinal metabolomic and histological differences between different severities of TBI

Potential outcome:

1. To develop objective biochemical and imaging methods to identify patients with progressive post-TBI brain disease (progressive brain ageing, functional and cognitive decline)
2. To identify gut-brain connections that associate with long-term outcome of TBI, creating thus potential intervention targets

Methodology:

Clinical assessment using standardized questionnaires, computerized cognitive testing, MR imaging, biomarker analysis (blood and intestinal mucus), microbiome analysis, colonoscopy

Sample size:

Max. 160 patients with TBI, 30 control patients with orthopaedic injury, and 10 control patients with multiple sclerosis

Main criteria for inclusion:

All alive subjects from the original TBIcare (Evidence-based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries) and PACoS-TBI (Pathophysiological Characterization of Severe Traumatic Brain Injury) study cohorts who give their consent

Assessments:

RPCSQ, GOSE, RAND-36, QOLIBRI, Rome IV Diagnostic Questionnaire, CANTAB testing battery

MR-imaging (3DT1, FLAIR, SWI, DWI), positron emission tomography (PET)

Blood sample for biomarkers (polar metabolites, lipids, NF-L, GFAP, Aβ40, Aβ40, tau, H-FABP, UCH-L1, S100B, cytokines and chemokines, genetics)

Microbiome analysis

Intestinal histology and mucus analyses

INTRODUCTION

Traumatic brain injuries (TBI) are among the most serious health problems worldwide and impose a significant burden on both the injured individuals, their proxies, and the society. It is estimated that >50 million people in the world suffer a TBI each year and that half of the people sustain a TBI in their lifetime. Upon admission, patients are classified as having mild, moderate, or severe TBI (mTBI, moTBI, and sTBI, respectively). However, a more accurate risk stratification and detailed characterisation of the disease phenotype is crucial for improving TBI management and predicting outcome in individual patients. The consequences of long-term disability, provision of care and lost economic activity pose a substantial socioeconomic burden on the individual and the society. Although TBIs in the elderly have increased rapidly, most victims are children or working age adults. Despite the enormous impact on health, many fundamental questions about pathophysiology, diagnostics, and treatment remain poorly understood. The main reason for this is the enormous complexity and individuality of TBI despite its initial severity, where no two injuries are identical. (1)

Until recently, translational and clinical research in TBI has focused on sTBI, but the mortality and morbidity of sTBI have remained unchanged in recent decades. (2) The results of research focused on sTBI have drawn researchers' attention to mTBI. (3,4) New epidemiologic data has underscored the previously unrecognized importance of this patient group and indicated that more than 90% of those seeking medical care after a head injury belong to the mTBI group. (5) This large patient group gained further importance when independent studies showed that a large proportion of these patients cannot return to their normal lives after injury. (6,7) The professional community agrees that mTBI is a misnomer for this type of disease. (2) A major, as yet unexplained, problem is that women in this patient group have a significantly worse outcome profile than men. (8) TBIs are particularly difficult to treat because they are heterogeneous in nature and often involve complex pathogenic pathways. The pathophysiological events following TBI are a collection of highly complex processes. Without tools that can reveal active pathophysiological processes in each injured patient, it is impossible to develop effective targeted interventions and treatments. (9) It has been argued that the classification based on the degree of severity upon admission is hopelessly outdated because it hardly describes the trajectory of recovery, and it is not known which patients develop progressive symptoms as a result of the injury. (10)

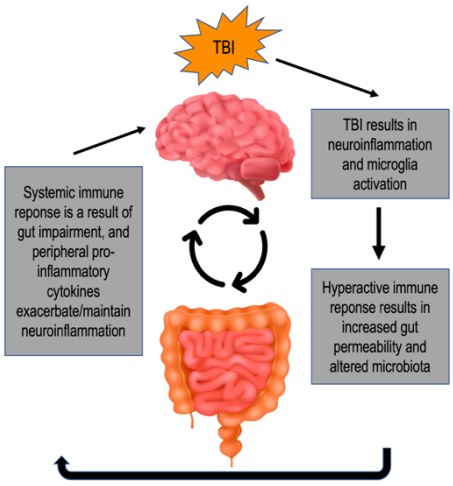


Figure 1. Proposed bidirectional role of microbiota–gut–brain axis in traumatic brain injury, modified from (18, 19)

The key to developing effective therapies for TBI lies in better understanding and identification the precise mechanisms underlying the primary and secondary pathology associated with TBI. Recently, a Commission in *The Lancet Neurology* on TBI highlighted the burden posed by the disease as well as the need for novel approaches to identify and fight the devastating long-term consequences. (2) Primary injury occurs at the time of head impact and causes direct damage to neural tissue, whereas secondary injury develops minutes to months after mechanical trauma and may progressively contribute to neurologic impairment during years after injury. There is a call for more research on the relationship between TBIs of different severities and their long-term effects and likely association with neurodegenerative diseases. (11) In a significant minority (10–30 %) of patients with TBI, TBI has been identified as a trigger of progressive neurological deterioration and a risk factor for chronic neuroinflammation and/or subsequent neurodegenerative disease. (11) There is increasing evidence that the immune system plays a significant role in the TBI pathogenesis. TBIs of varying severity can lead to chronic neuroinflammation, which can potentially be detected on head positron emission tomography (PET) and/or by biomarkers in peripheral blood (12,13), and which can persist years after TBI. (1) A basic premise is that inflammation is associated with almost all brain injuries. (14)

Given the far-reaching influence of the gut microbiome on the central nervous system, there is also growing interest in studying the role of the microbiota–gut–brain axis in patients with TBI. (15) The microbiota–gut–brain axis can be understood as an integrative physiological model encompassing afferent and efferent signals of neural, hormonal, and immunological origin, and dysfunction of this axis has pathophysiological consequences. (15,16) This axis can affect an organism at multiple levels, from modulating behaviour and influencing how and with whom people interact, to regulating mood, cognition, pain, obesity, cancer, and inflammatory and autoimmune diseases. As a result, there is growing interest in the local and systemic impact this communication pathway has on host health and disease burden. (17) Given the increasing recognition that TBI is a chronic disease and a risk factor for the subsequent development of neurodegenerative diseases, and that the underlying mechanisms related to gut microbiome disruption and chronic neuroinflammation are unknown, gastrointestinal dysfunction after TBI

and its impact on patient outcomes should be further investigated. A bidirectional microbiota–gut–brain axis has been proposed to be involved in the consequences of TBI. The revolving mechanism may work as shown in Figure 1. (18,19)

In addition to neuroinflammation, there are several other potential mechanisms that could contribute to the late effects of TBI, but there is no convincing scientific evidence for many of them. One reason for this is the scarcity of high-quality longitudinal studies in which subjects undergo comprehensive examinations, including modern MRI, PET, and biomarker profiling, both acutely and after several years of follow-up. Three studies that have previously addressed this issue show that blood levels of the biomarker neurofilament light (NF-L) correlate with brain atrophy, microstructural brain injury, and functional outcome in the chronic phase TBI. (20–22) One of the above seminal studies is from the PI's research group in which we showed that glial fibrillary acidic protein (GFAP) and NF-L can remain elevated months to years after TBI and exhibit different temporal profiles. These elevations correlate with microstructural injury in both grey and white matter and with accelerated brain ageing on contemporaneous quantitative diffusion tensor MRI and functional decline (Figure 2). (22) Identifying potential therapeutic pathways to prevent secondary brain injury and later neurodegeneration after TBI is of great importance in reducing patient morbidity. Adding the microbiota–gut–brain axis to this entity, which may influence the chronicity of neuroinflammation, may also lead to development of therapies that influence the microbiome.

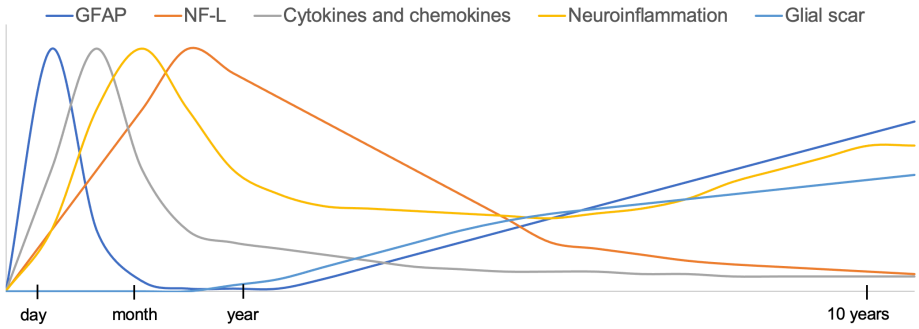


Figure 2. Overview of the proposed biochemical processes and blood biomarker levels in traumatic brain injury in patients who develop progressive disease. GFAP peaks at 24 hours in blood but increases again after one year in response to glial scarring and possibly neuroinflammation. NF-L peaks between months-year in blood and decreases thereafter but remains elevated for up to several years. Pro- and anti-inflammatory cytokines remain elevated due to chronic immune activation. Neuroinflammation involves both a protective and a damaging response, the latter of which dominates in pathology that progresses after months. GFAP, glial fibrillary acidic protein; NF-L, neurofilament light

The starting point for the project is the fact that TBI is a dynamic disease in a large proportion of patients and current diagnostic methods do not take this into account. The new OVERCOME-TBI project continues the line of investigation of PI's earlier projects on acute and subacute assessment of TBI with novel multimodal approaches (biochemical and imaging biomarkers). This time, the project focuses on the chronic (months–years) phases of TBI with the goal of identifying biochemical (proteomic, lipidomic, and metabolomic biomarkers), imaging biomarkers (features on MRI), faecal and gut microbiota and intestinal histological findings that correlate with functional outcome and the development of progressive neurological disease (Figure 2). This will facilitate a shift from guideline-based patient care to patient-specific diagnostics and treatment and may reveal new treatment targets to halt the progression of progression of sequela after TBI.

STUDY HYPOTHESES AND EXPECTED RESULTS

The OVERCOME-TBI project includes two hypotheses:

1. Blood-based protein, metabolomic, and inflammatory biomarkers can identify patients with TBI who exhibit signs of progressive neurodegeneration (progressive brain ageing, functional, and/or cognitive decline) in the chronic phase of TBIs of all severities.
2. Patients with progressive brain ageing, functional and cognitive decline exhibit signs of both neuro- and intestinal inflammation, as well as altered gut microbiota and intestinal permeability in the chronic phase of TBI compared with i) recovered patients and ii) control patients

More specifically, it is hypothesised that TBI-related pathophysiologic processes reflected in biochemical biomarkers in blood have individual patterns (biomarker profiles) in associated with different kinds of disease progression trajectories (hypothesis 1), each biochemical and imaging biomarker has different individual diagnostic time windows in terms of clinically acceptable sensitivity and specificity for outcome prediction (hypothesis 1 and 2), biochemical and imaging biomarkers can identify patients with TBI who exhibit signs of active inflammatory processes in the brain and in the gut as well as altered gut and faecal microbiome and intestinal

permeability in the chronic phase of TBI (hypothesis 2), the above mentioned patients with progressive brain disease (progressive brain ageing, functional, and cognitive decline) showing active inflammation processes can also be identified already in the subacute phase (during hospital stay/at the initial follow-up visit) (hypothesis 1 and 2).

TBI diagnostics has lagged significantly behind that of many other diseases. The current methods for assessing severity and predicting outcomes are based only on initial stage variables, and there are no objective tools for monitoring the progression of the disease.

The current acute severity indices have shown only a modest association with outcome, especially in patients with mTBI. However, even in patients with moderate-severe TBI, clinical predictors and imaging together explain only 35 % of the outcome variance. (23) In patients with mTBI, the most important outcomes relate to the risk of long-term or permanent sequelae or the risk of not fully recovering, whereas in more severe cases, the risk of death or permanent dependence on others is most important.

In the OVERCOME-TBI project, the overall goal is to collect multimodal data approximately 1–4 and 11–14 years after an earlier well-documented TBI and examine factors that influence disease progression to provide new scientific insight into the long-term pathophysiology of TBI and identify new therapeutic targets.

We will examine long-term outcome and symptom development in different severities of TBI and correlate the results with longitudinal biochemical and imaging biomarkers, and other clinical variables. We will also examine the overall neuroinflammation burden (head PET imaging, blood biomarkers), gut and faecal microbiome, and metabolomic and histologic and permeability differences in the intestine between different severities of TBI and controls. Understanding the influence of the microbiota–gut–brain axis in the context of TBI may open new avenues for therapeutic approaches for TBI survivors. One of the purposes of the OVERCOME-TBI project is also to investigate whether women's body's response to TBI is different and whether this explains women's worse recovery from the disease. The research project therefore also aims to promote the elimination of health differences between the sexes.

The expected research results are i) development of objective biochemical, microbiological, and imaging methods to identify patients with progressive post-TBI brain disease and decrease the variance in outcome prediction using novel, rich, and multimodal data, and ii) to identify microbiome–gut–brain connections that associate with long-term outcome of TBI, identifying thus potential intervention targets. These findings could lead to the development of tools that promote an anti-inflammatory and regenerative immune phenotype and prevent the development of chronic neuroinflammation.

Primary Outcome Measures

1. Overall neuroinflammation burden
    - Overall neuroinflammation burden as measured by blood-based biomarkers, brain diffusion-weighted imaging and brain positron emission tomography
2. Functional outcome
    - Functional outcome measured with Glasgow Outcome Scale Extended
3. Changes in microbiota and gut permeability

Secondary Outcome Measures

1. Changes in brain white and grey matter microstructure
    - Changes in brain white and grey matter microstructure as measured with diffusion tensor metrics
2. Accelerated brain ageing
    - Accelerated brain ageing calculated at a whole brain level using T1-weighted images and at a voxel-based level as the annualised Jacobian determinants in white matter and grey matter, referenced to a population of healthy control subjects

STUDY DESIGN

Work plan and schedule

The project includes two existing high-quality datasets, which are described in Figure 3. Additionally, 10 patients with multiple sclerosis (MS) will be recruited from the Neurocenter's neurological outpatient department at the Turku University Hospital, where neurologists aware of the research meet these patients as part of their routine follow-up, for TSPO-PET imaging and MRI imaging. The implementation schedule is as follows:

- **First year (2024)**
    - The last year of follow-up visits for PACoS-TBI (Pathophysiological Characterization of Severe Traumatic Brain Injury), see Figure 3. Blood samples will be sent to metabolomic and lipidomic (Turku, Finland/Örebro, Sweden), proteomic (Gothenburg, Sweden), and cytokine and chemokine analyses (Geneva, Switzerland). Processing of the MRI data starts.

- Screening of living patients and control patients from the TBIcare Evidence-based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries) and PACoS-TBI studies from the patient records for inclusion in the OVERCOME-TBI study
- Contacting patients and control patients from the TBIcare and PACoS-TBI studies for inclusion in the OVERCOME-TBI study by invitation letters
- Contacting patients with multiple sclerosis by invitation letters from the patients of the Neurocenter's neurological outpatient department at the Turku University Hospital

- **Second year (2025)**
  - The first neurological, neuropsychological, imaging, and gastroenterological investigations of the OVERCOME-TBI study.
- **Third year (2026)**
  - The second year of neurological, neuropsychological, imaging, and gastroenterological investigations of the OVERCOME-TBI study.
  - Biochemical biomarker data analysis of the PACoS-TBI is complete. Harmonisation of the MRI data of TBIcare and PACoS-TBI in Turku and in Cambridge.
- **Fourth year (2027)**
  - The third and last year of neurological, neuropsychological, imaging, and gastroenterological investigations of the OVERCOME-TBI study.
- **Fifth year (2028)**
  - The OVERCOME-TBI blood samples will be analysed by collaborators. Intestinal and faecal microbiome (Turku, Finland) and intestine metabolomics and lipidomics analyses (Turku, Finland/Örebro, Sweden) are conducted. Processing of MRI data in Turku and in Cambridge. Preparation of manuscripts starts. New results are presented in conferences.

RESEARCH MATERIALS AND METHODS

The OVERCOME-TBI project is a research project that builds on an extensive body of previous research on two patient cohorts: i) TBIcare (Evidence-based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries) project in which patients were recruited between Dec 7, 2011, and Nov 11, 2013, and ii) PACoS-TBI (Pathophysiological Characterization of Severe Traumatic Brain Injury) in which patients were recruited between Nov 1, 2020, and Dec 31, 2023, The PI has authored tens of publications, supervised one doctoral thesis (Iftakher Hossain, MD, PhD) and is currently supervising multiple doctoral theses based on these data.

The new dataset comes from the OVERCOME-TBI recruitment, which will be a new comprehensive analysis of the TBIcare and PACoS-TBI cohorts at chronic timepoint after TBI. The organisation and content of the datasets included in the OVERCOME-TBI are shown in Figure 3. Regression models and machine learning methods will be fundamental tools for comprehensive data processing.

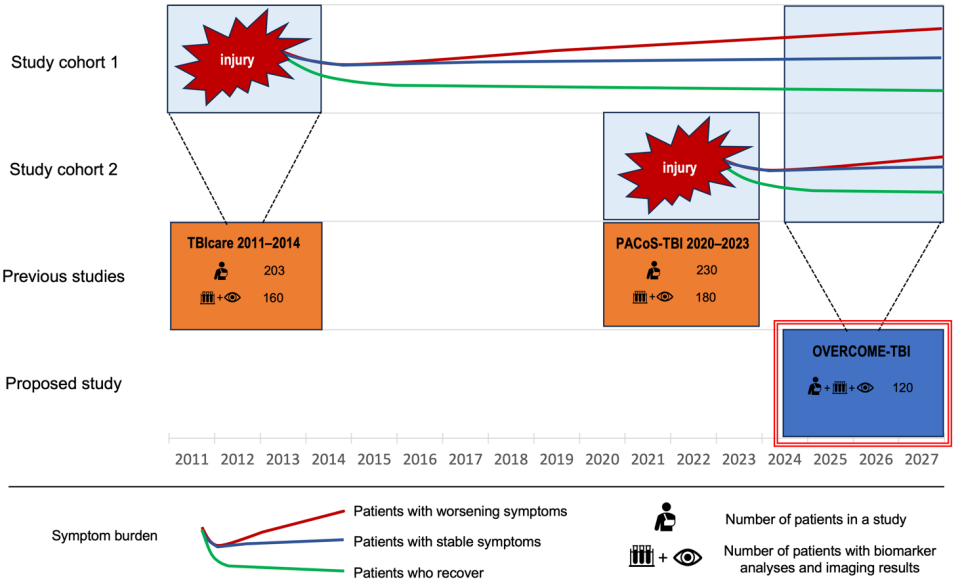


Figure 3. Description of the study materials. The prospective study cohorts recruited earlier (TBIcare and PACoS-TBI) provide the pool of patients with a history of TBI sustained 1–4 and 11–14 years earlier to be recruited. The colored lines show different trajectories of recovery/symptom burden over time. The light blue boxes represent the time points studied in the studies. In the TBIcare study, biomarker levels were examined at admission, the following seven days, and at a follow-up visit approximately nine months after injury. The TBIcare study also includes MRI scans done after injury and at the follow-up visit. The PACoS-TBI data include two daily biomarker assessments during the first 10 days after injury and at follow-up approximately six months after injury. The proposed study box shows an estimate of the number of patients that can be recruited. The red frame in the figure above represents the same study phase, which has the corresponding red frame in Figure 5.

The clinical and scientific facilities are located at the Turku Health Campus (https://www.healthcampusturku.fi). The clinical environment is internationally unique and advanced, as all necessary specialties are united in one department (The Turku Brain Injury Center) to treat patients with TBI, from acute care to follow-up. The PI is the head of The Turku Brain Injury Center, and he and several other members of the research group participate daily in the clinical treatment of patients with TBI in our hospital, ensuring a close relationship between clinical practise and clinical research. Our research facilities have centrifuges and freezers for processing and storing samples, as well as high performance computers for processing imaging data.

Materials

This is an analysis of a cohort of patients and control subjects who were prospectively assessed during the acute stage in the TBIcare and PACoS-TBI studies conducted by our research group in 2011–2013 and 2020–2023, respectively. Thus, this is mainly a prospective study, with some cross-sectional data including new diagnostic assessments. The overall aim is to collect multimodal data about approximately 1–4 and 11–14 years after an earlier well-documented TBI and to investigate factors influencing the course of the disease to gain new scientific knowledge about the long-term pathophysiology of TBI and the role of neuroinflammation, and to identify new potential therapeutic targets.

Patients also undergo a comprehensive clinical examination. Information about the diseases they have been diagnosed with, the medications they have been taking (and how long they have been taking them), and possible new head injuries after the injury are carefully recorded. These data are supplemented with the patients' original health data collected in the TBIcare and PACoS-TBI studies. We will examine i) the long-term outcome and symptom development in different severities of TBI and correlate findings with longitudinal investigations (magnetic resonance imaging, PET imaging, biomarkers) and other clinical variables, and ii) the gut and faecal microbiome and the intestinal metabolomic and histological differences between different severities of TBI and controls. In particular, the study has the following objectives: i) to examine longitudinal functional and neurocognitive outcomes and outcome trajectories (ca. 1–4 and 11–14 years), and quality of life after TBI, ii) to examine if biochemical biomarkers during the acute and subacute stage (1–7 days, and 6–9 months after injury) are predictive of long-term neurocognitive and functional outcome and outcome trajectories, iii) to investigate whether surrogate markers of neuroinflammation (PET imaging, biochemical biomarkers) correlate with the long-term outcome and can distinguish patients with TBI from orthopaedic controls at 1–4 and 11–14 years, iv) to examine longitudinal and long-term changes in brain microstructure, networks and brain ageing on advanced MR imaging, v) to examine association between irritable bowel syndrome (IBS) symptoms, gut and faecal microbiome, and calprotectin levels in patients with TBI and orthopaedic trauma controls, vi) to examine stool samples and endoscopically collected intestinal samples (histology and metabolomics) in IBS-symptomatic and asymptomatic patients with TBI and orthopaedic trauma controls, vii) to examine metabolomic and lipidomic profiles in blood, stool samples and colonoscopy pinch biopsies in IBS-symptomatic and asymptomatic patients with TBI and orthopaedic trauma controls to study overall metabolomic changes in post-TBI health, and viii) to examine associations between brain microstructure, networks, and brain neuroinflammation (biochemical biomarkers, PET) and gut findings (microbiome, histology).

Methods

The TBIcare and PACoS-TBI patients will be contacted by letter explaining why study patients are being contacted again and providing information about the purpose of the study, the methods, and the importance of study results in developing new diagnostic methods and finding treatment targets in patients with TBI.

From those 181 + 25 (patients with TBI + orthopaedic controls) patients who participated fully (= attended the outcome visit) in the TBIcare study, and of those 230 + 20 (patients with TBI + orthopaedic controls) patients who participated fully in the PACoS-TBI study, the estimated number of those patients who will give their consent to participate in this study is 120. We will expect to be able to recruit 20 controls. It is anticipated that the entire recovery period (and the various courses of recovery) of TBI can be covered quite comprehensively, so 70 patients from the TBIcare study (11–14 years) and 50 patients from the PACoS-TBI study (1–4 years) will participate. It is expected that 10 control subjects from both studies will participate. For the TSPO-PET-imaging, 10 patients with multiple sclerosis will be additionally recruited as positive controls. Based on the PI's experience from multiple clinical TBI studies, it must be highlighted that the expected figure of recruited patients is very conservative. The ethical and institution permission will be sought for 160 patients with TBI, 30 control patients with orthopaedic injury, and 10 patients with multiple sclerosis.

The inclusion process will be as follows (Figure 5):

- Primary inclusion criteria
    - All those who are alive and give their consent will be included.
- Secondary inclusion criteria (gastroenterology)

  - All those who are alive and give their consent will be included.

- Inclusion criteria for control patients with multiple sclerosis
  - MS diagnosis in accordance with McDonald 2017 criteria
  - Significant white matter lesion load (a minimum of 10 white matter lesions)
  - Secondary progressive disease (SPMS)

- Primary exclusion criteria
  - For patients with TBI there are no exclusion criteria, and all eligible patients who give consent will be recruited. Those patients and controls with contraindication for head MRI (MRI-incompatible heart pacemaker, weight over 200 kg, mechanical heart valve prosthesis, first trimester of pregnancy, orbital area tattoo) will not undergo head MRI. Instead, they we will undergo the other assessments. For controls, those who have suffered a TBI or any other brain disorder after the TBIcare or PACoS-TBI study will be excluded.

- Secondary exclusion criteria (gastroenterology)
  - Diagnosis of IBD or other diagnosis besides IBS causing severe GI symptoms, such as microscopic colitis or bile acid diarrhea
  - Poorly controlled celiac disease
  - Colorectal cancer diagnosed within five years
  - Antibiotic or probiotic treatment, on-going or previous month.
  - Unwilling or unable to undergo colonoscopy and/or standard bowel preparation.
  - Significantly increased risk of heart or kidney failure or electrolyte imbalances due to bowel preparation (frailty, serious pre-existing heart or kidney insufficiency).
  - Altered bowel anatomy after significant operation. Appendicectomy or cholecystectomy are considered minor operations.

- Exclusion criteria for patients and controls undergoing TSPO-PET imaging
  - Patients with other neurodegenerative disease than MS
  - Patients with history of traumatic brain injury necessitating hospital admission
  - Patients with other autoimmune disease than MS
  - Patients with other significant or malignant underlying disease of any other organ system
  - Patients that are pregnant or breast-feeding
  - Corticosteroid treatment within 4 weeks of imaging
  - Patients with claustrophobia, or a history of moderate to severe anxiety disorder or panic attacks (which could potentially lead to preterm termination of the imaging)
  - Contraindication to PET scan investigations
  - Exposure to experimental radiation in the past 12 months such that radiodosimetry limits would be exceeded by participating in this study.
  - Intolerance to previous PET scans, i.e. previous hypersensitivity reactions to any PET ligand or imaging agent or failure to participate in and comply with previous PET scans.

As for neurological and cognitive examination, withdrawals are unlikely. Not all patients are necessarily willing to undergo colonoscopy and histological sampling. However, they may be included in neurological and faecal microbiome examinations. There will be no replacement for any dropouts. Data collected prior to dropout will be used. The consenting subjects will receive compensation of 50 euros for the first phase of the study.

The patients will undergo neurological and cognitive testing, blood sampling for blood-based biomarker analyses, head magnetic resonance imaging, PET imaging (radioligand [^{11}C]PK11195) using PET-MRI, and colonoscopy. Patients with orthopaedic injuries will serve as controls and will undergo similar assessments (Figure 4 and Figure 5). For PET-MRI-imaging, control patients include patients with orthopaedic injuries from the TBIcare and PACoS-TBI cohorts and also patients with MS. Patients (from all groups) who will participate in the PET imaging will receive a compensation of 50 euros.

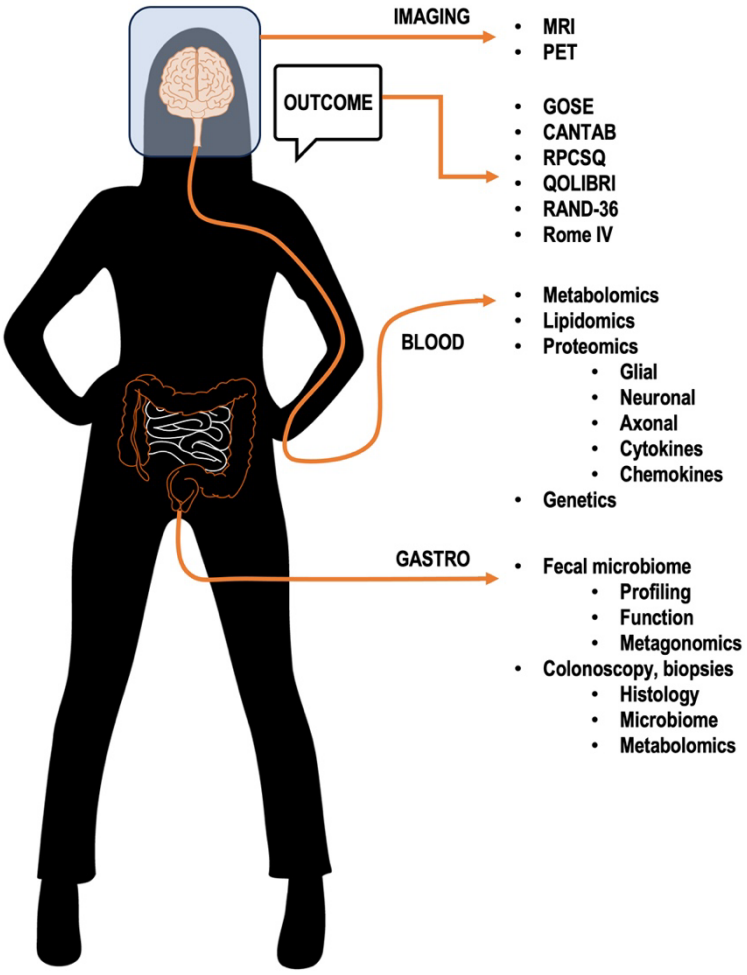


Figure 4. Description of the study assessments included in the clinical (neuro and gastro) and gastroenterological visits. MRI, magnetic resonance imaging (conventional and advanced); PET, positron emission tomography. See body text for the description of the assessments.

The previous study cohorts offer an exceptional possibility to study factors which contribute to the long-term outcome. Because these patients have both imaging and comprehensive biomarker data from the acute and subacute phase, and from a 9-month follow-up visit, new data 1–4 and 11–14 years after a TBI can be used to assess factors associated with long-term outcome. Up to 60 patients with TBI and 10 patients with orthopaedic injury and 10 patients with MS will be undergoing PET-MRI imaging.

The clinical assessments of the OVERCOME-TBI will include the same evaluation tools that were used in the original TBIcare and PACoS-TBI studies: i) Glasgow Outcome Scale Extended (GOSE), ii) Rivermead Postconcussion Symptom Questionnaire (RPCSQ), iii) QOLIBRI (a quality-of-life assessment tool), iv) RAND-36 (general health questionnaire), v) gastroenterological questionnaire panel including the Rome IV Diagnostic Questionnaire (functional gastrointestinal disorders) (Figure 4).

Cognition of the participating subjects will be tested using the CANTAB computerized testing battery (same eight subtests as in TBIcare and PACoS-TBI). MRI will be performed using identical sequences with the original TBIcare study. We will also supplement the previous DTI sequences with 45–60 directions acquired with a higher b-value, e.g., 2500 s/mm^2, which will allow for neurite orientation dispersion and density imaging (NODDI) and multi-shell tractography. Accelerated brain ageing will be calculated at a whole brain level as the predicted age difference defined using T1-weighted images, and at a voxel-based level as the annualized Jacobian determinants in white matter and grey matter, referenced to a population of 600 healthy control subjects. (22) A 10 ml blood sample from the antecubital vein will be drawn into one plasma and one serum tube (= 20 ml altogether) and will be divided in aliquots.

Neuroinflammation will be measured with PET imaging. PET imaging is done using the [^{11}C]PK11195 radioligand and performed with a GE Signa PET-MRI scanner. The target radioactivity dose of the administered [^{11}C]PK11195 radioligand is 500 MBq. 60-min dynamic imaging is started simultaneously with the intravenous, smooth bolus injection of the radioligand. Head movements during the scan are minimized using support around the head. Image reconstruction is performed using 17 timeframes (2 x 15, 3 x 30, 3 x 60, 7 x 300, and 2 x 600 s; total, 3,600 s) as described previously.(24) The dynamic data is then smoothed using a Gaussian 2.5 mm postreconstruction filter. (25) The possible displacements between frames are corrected using mutual information realignment in SPM8. Finally, all the PET images are resliced to match 1 mm MR voxel size. For the estimation of [^{11}C]PK11195 distribution volume ratio (DVR), the time–activity curve (TAC) corresponding to a reference region devoid of specific translocator protein (TSPO) binding is acquired for each PET session using a supervised cluster algorithm (SCA) approach with 4 predefined kinetic tissue classes (SuperPK software, SCA4 classification) (26,27) as described previously. (28) As an additional step, the intersection of the clustered reference region maps of each session is used as a common clustered reference region. Regional DVRs are estimated by using reference tissue input Logan method (29), with time interval from 20 to 60 minutes.

The faecal microbiome assessments will be done on the stool samples collected at the first visit and undertaken primarily by the laboratory of Turku Microbiome Biobank. For microbiome (both taxonomical profiling and

function) analysis, DNA will be extracted from the one aliquot of the collected stool samples by utilizing Powerbeads and TissueLyser II (Qiagen) & Chemagic™ DNA Stool 200 Kit (PerkinElmer) on a Chemagic 360 machine in 96-well format. (19). To analyse the DNA from faecal microbiome, we aim at deep shot gun metagonomics approach, (>6 Gb) which guarantees a high coverage and depth for strain level resolution, critical for studying transmission patterns from donor to patient. This step is purchased from a local core sequencing provider Finnish Functional Genomics Centre, Turku Bioscience. As basis for the development of these methods for standard analyses of gut microbiome composition, diversity and function are available. (31)

Faecal and intestine omics study is performed in collaboration with the System Medicine Group at Örebro University and University of Turku. Both targeted and non-targeted metabolomics with mass spectrometric techniques coupled with chromatography will be utilised.

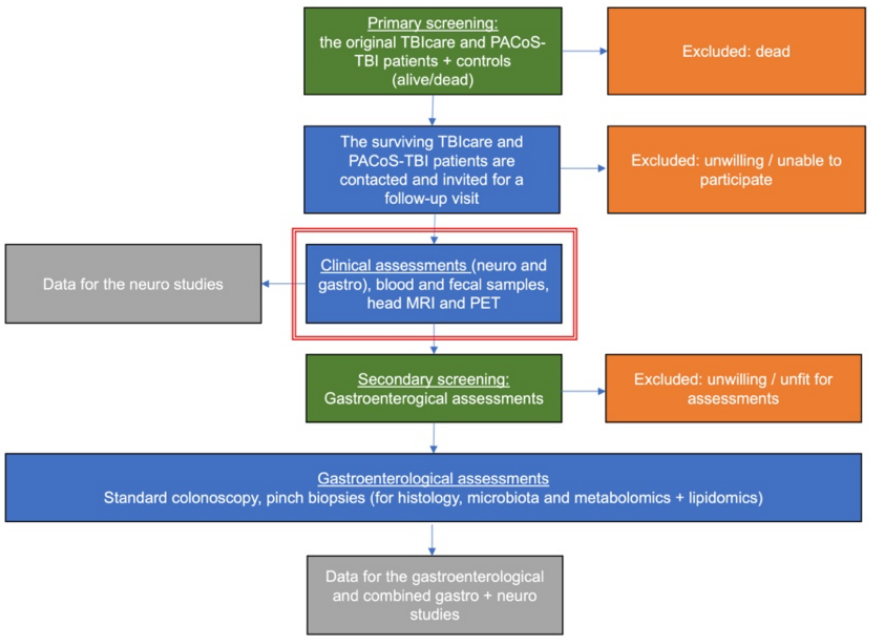
It is not expected that all 120 potential patients will consent to colonoscopy. The consenting subjects will receive compensation of 50 euros for this step. We expect 90 % of the patients to be included in the gastroenterological investigations. However, it is assumed that a stool sample will be obtained from all participants. All consenting patients and orthopaedic controls included in the second screening (Figure 5) will undergo standard colonoscopy including pinch biopsies from the bowel wall at several levels (terminal ileum, ascending colon, transverse colon, descending colon, and rectosigmoideum) for histology, microbiome analysis and metabolomics. The patients and controls are also asked to bring another stool sample from which calprotectin level will be analysed. The aim is to investigate the inflammatory state and permeability changes in the intestine to assess the overall inflammatory burden together with faecal microbiome profile, blood biomarkers and head imaging (PET) and to correlate the findings with the neurological disease progression.

Interventions

All consenting patients and orthopedic controls included in the second screening will undergo standard colonoscopy including pinch biopsies from the bowel wall at several levels (terminal ileum, ascending colon, transverse colon, descending colon, and rectosigmoideum) for histology, microbiota analysis and metabolomics.

Bleeding risks involved with pinch biopsies are considered minimal. Therefore discontinuation of anticoagulant medication is not recommended in the current clinical practice.

Figure 5. OVERCOME-TBI study flow chart. The red frame in the figure above represents the same study phase, which has the red frame in Figure 3.



STUDY RISK MANAGEMENT

The OVERCOME-TBI project concerns a highly important research topic with a major impact on public health. The project involves a highly experienced research team including several renowned national and international collaborators. There are many critical issues for success. The extensive multidisciplinary experience gained in the project ensures conceptualisation of causal mechanisms behind the study results, as well as scientific breakthroughs based on a wealth of data and novel analytical approaches (including systems biology, machine

learning, and artificial intelligence). Furthermore, the patient cohort of the INFLUX-TBI already exists and these patients have healthcare connections to the main study site. The sample size of the project has been calculated very conservatively, and the TBI experts behind the study application believe it is very likely that the planned 120 patients will be recruited for the study, and it is possible that even more patients than the target will be included. The calculation of this sample size has been based on experience from previous international and national studies, so the risk of recruitment failure is considered minimal making the OVERCOME-TBI a very trustworthy funding target.

Because the project includes material from two clinical studies, the following risks exist (in descending order of likelihood): (i) The number of patients in TBIcare and PACoS-TBI remains too small to answer all hypotheses– the risk is because reliable performance calculations cannot be made in this kind of setting; the solution to this problem is that we are part of the global InTBIr consortium (https://intbir.incf.org), so it is possible to initiate a larger follow-up study, (ii) inefficient use and/or pooling of a wealth of data, (iii) failure to synchronise work input from different collaborators, (iv) failure to change study designs efficiently when needed, and (v) due to the highly complex brain pathophysiology of TBI, it may be that the causalities behind obtained results remain uncertain and will require further studies. The overall likelihood of risks is very low. The PI has personal relationships with all collaborators. If some of the risks materialise, alternatives will be quickly sought (brainstorming, online meetings, face-to-face meeting).

STATISTICS

Statistical plan and analysis

The associations between early (= original TBIcare and PACoS-TBI studies) and late (this study) variables will be analysed using logistic regression analysis in multivariate models. Differences between groups will be assessed using parametric and non-parametric tests. A biostatistician and system biologists will be consulted when deciding the appropriate machine learning tools for analysing the data.

DATA HANDLING AND RECORD KEEPING

Case report forms

An electronic case report form (CRF) will be filled for each patient, including all questionnaires used as well as thorough medical history and information about imaging, blood sampling, and cognitive testing.

Blood, faecal and tissue samples

Blood samples will be stored in a –70 C freezer with pseudonymized identifiers. Faecal and intestinal tissue samples will be stored in a deep freeze –70 C in department of microbiology at the University of Turku.

Data management

The case report forms will be stored at the research office in a locked cupboard. The electronic records will be stored at the hospital's server, secured by a username and password. To give access for the data also for researchers outside the research group, the data will be transferred to an anonymized database, which can be given based on data sharing agreement.

Study subject register

The study register will be built according to current EU data protection legislation.

ETHICS

Ethical review

The Ethical Committee of the wellbeing services county of Southwest Finland will review the study.

Ethical conduct of the study

The study will consist mainly of non-interventional assessments that do not bear special ethical concerns. The risk of serious complications, including bowel wall perforation, is less than 1/1000 for diagnostic colonoscopy. (32) General anaesthesia is not required, but intravenous sedation and pain relief will be provided if requested by the patient. We expect these colonoscopies to reveal several important incidental findings (mostly advanced colonic polyps) that can be treated immediately and are likely to benefit patients. Bleeding risks involved with pinch biopsies are considered minimal. Therefore discontinuation of anticoagulant medication is not recommended in the current clinical practice.

For the PET-imaging part, the participants will receive one [^{11}C]PK11195 injection. The total effective dose is 2,4 mSv, which is equivalent to the average radiation dose a Finn receives in 5 months. On the basis of this, those patients involved in the research will not be exposed to a significantly harmful radiation burden, and therefore

from the point of view of radiation biology the study can also be considered ethically acceptable. Furthermore, the treatment of the patients will not be affected by the study. The research methods are PET and MRI scans and blood sampling, which cause patients some degree of discomfort (venous cannulation, sacrifice of their time), but are not dangerous. Participation in the research is completely voluntary and subjects may at any time suspend the study.

All subjects will be able to give informed consent and the examinations performed will not pose any health risks to the subjects. The principles of the Declaration of Helsinki will be followed.

Subject information and informed consent

A written information sheet about the study will be sent to all potentially eligible subjects, together with an informed consent sheet. The subjects can ask additional questions about the study before giving their consent. In case there is any doubt about the subject's ability to understand the study and give consent, the same information will be given to the proxy.

FINANCING AND INSURANCE

The study subjects will be covered by the usual hospital insurance. Financing of the study will be covered by major funding acquired from the Research Council of Finland (to the PI), Sigrid Jusélius Foundation (to the PI) and State Research Funding Tied to Academic Research in Health Sciences (to the PI) for 2024–2028.

STUDY REPORT AND PUBLICATIONS

This study will produce numerous publications because of the nature of the data and the possibility of comparison with results obtained from the same individuals at different timepoints after TBI. The main focus will be on i) the long-term outcome of TBI and its prognostic indicators and ii) the altered gut microbiota and intestinal permeability after TBI.

The results of this study may reveal potential targets for reducing the inflammatory disturbance of gut-brain axis and thus to reduce its deleterious effects on the brain.

ARCHIVING

All research data will be archived in electronic form in the Turku University Hospital servers and stored for eventual later needs for 30 yrs. The blood samples will be stored in the PI's research group's freezers.

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