ALLEN INSTITUTE for BRAIN SCIENCE

Aging, Dementia and TBI Study

TECHNICAL WHITE PAPER: OVERVIEW

OVERVIEW

The Aging, Dementia and Traumatic Brain Injury (TBI) Project is a detailed neuropathologic, molecular and transcriptomic characterization of brains of control and TBI exposure cases from a unique aged population-based cohort from the Adult Changes in Thought (ACT) study. This project was developed by a consortium consisting of the University of Washington, Kaiser Permanente Washington Health Research Institute and the Allen Institute for Brain Science and was supported by the Paul G. Allen Family Foundation (PIs: Richard Ellenbogen and C. Dirk Keene, University of Washington; PI: Eric Larson, Kaiser Permanente Washington Health Research Institute; and PI: Ed Lein, Allen Institute for Brain Science). The original aim of the project was to search for a molecular and neuropathologic signature of mild to moderate TBI by aggregating a large-scale, internally consistent dataset consisting of detailed patient clinical metadata, quantitative neuropathology and cutting edge transcriptomics. This document is centered on this original intention to study the consequences of TBI. However, the use of the Adult Change in Thought (ACT) cohort (see ACT Cohort whitepaper in Documentation), with its extensive aged patient population, detailed medical histories and brain tissue availability, created a unique "data cube" that can be used to study molecular hallmarks of aging, brain pathology, dementia and many other variables in addition to TBI (see Weighted Analysis whitepaper in Documentation).

The World Health Organization (WHO) has predicted that by 2020 TBI will be the third leading cause of death and disability for all ages worldwide, and TBI is currently the leading cause of death and disability in adolescents[1,2]. Public awareness regarding the possible progressive effects of TBI have increased following recent high profile cases of suicides and neurological sequelae in soldiers and athletes who have suffered TBI. These events have raised concern among soldiers and athletes and their families, coaches, trainers, and caregivers, as well as the federal government.

The severity of a TBI ranges from "mild" (transient changes in baseline cerebral function) to "severe" (prolonged neurological deficits) following injury. Concussions and other mild head trauma represent the majority of TBIs reported annually. Severe acute TBI results in upregulation of amyloid precursor protein (APP)[3] and results in innate immune activation and free radical injury[4]. Autopsies of professional boxers, American football players, and military combat veterans with numerous mild TBIs have revealed pathological deposition of phospho-tau species within multiple brain regions[5-9]. However, until more is known about the late biological and structural changes that result from TBI, particularly mild to moderate TBI, the prognostic implications of TBI remain unknown and diagnostic and therapeutic approaches cannot be developed. The aim of the project is to allow the coordinated analysis of neuropathological markers with molecular measurements including the transcriptome and targeted proteins and nucleic acids in the same brains from a well characterized patient cohort (see **Figure 1**) to better understand the late structural and molecular substrates of TBI-related neurodegeneration.

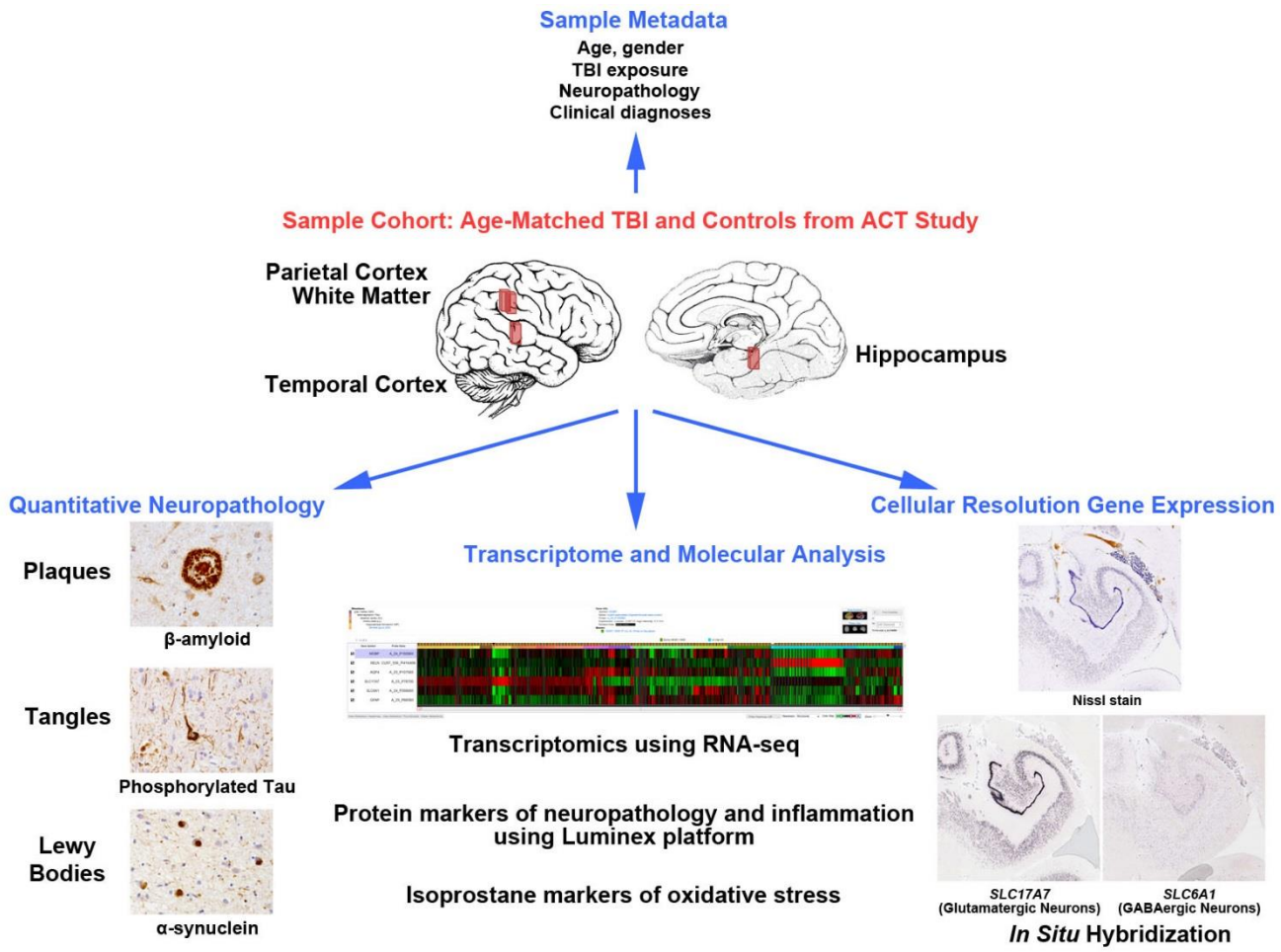

Figure 1. Overview of the Aging, Dementia and TBI Project data modalities.
Age-matched control and TBI subjects from the Adult Changes in Thought (ACT) study were used. This cohort consented to brain donation and have extensive medical histories available. Formalin-fixed paraffin-embedded (FFPE) and fresh frozen tissues from each subject were isolated from the temporal neocortex, parietal neocortex, parietal white matter, and the hippocampus and systematically analyzed for three major data types. As detailed below, these included a quantitative neuropathologic analysis, an extensive molecular analysis including whole genome RNA-seq analysis, and analysis of the distribution of different cell types in each tissue sample.

This study examines individuals that had experienced 1-3 TBIs in their lifetime, with a loss of consciousness ranging from a few seconds to an hour. These subjects are matched to "control" subjects of the same age, sex, year of death, and post-mortem intervals, resulting in a single best match for every case (see **Table 1**). Subjects are from a cohort of individuals that consented to brain donation, and who had been members of the ACT study. As members of the ACT study, subjects have extensive medical histories available to researchers and have undergone longitudinal neuropsychological testing to assess for dementia.

Table 1. TBI and control cohort characteristics.

	TBI Cohort	Control Cohort
Number of cases	55	55
Age	89.0 +/- 6.3	89.0 +/- 6.2
Sex	32 male / 23 female	32 male / 23 female
Year of death	2008.4 +/- 3.8	2008.7 +/- 3.4
Post mortem interval (hours)	4.6 +/- 1.5	4.7 +/- 2.0

To assess potential effects of mild-moderate TBI, tissue was isolated from the temporal neocortex, parietal neocortex, parietal white matter, and the hippocampus and analyzed using a suite of qualitative and quantitative methods (see **Figure 1**). Fixed tissue was stained by immunohistochemistry (IHC) and histochemistry to assess β-amyloid, tau, and α-synuclein deposition in plaques, tangles, and Lewy bodies, respectively using formalin-fixed paraffin-embedded (FFPE) preparations standard in the field. To more accurately correlate local pathology and transcriptome variation as they relate to aging, dementia and mild to moderate TBI, it was also important to measure pathology and RNA expression in the same tissue blocks. Fresh frozen tissue blocks were therefore processed both for histological assessment (to allow for a comparison to the neuropathology information obtained from the fixed tissue) and RNA sequencing. In addition, this fresh frozen tissue was used for *in situ* hybridization (ISH) for canonical marker genes for major cell classes to gain insight into the cellular makeup of the tissue; to measure the concentrations of various proteins, including b-amyloid, tau, and alpha-synuclein, chemokines, cytokines, and interleukins by Luminex; and to measure oxidative stress in the temporal and parietal regions by assessing levels of f2-isoprostanes. For IHC of FFPE tissues, tissue blocks were taken from parietal cortex and temporal cortex from the same side of the brain that was sampled during the rapid autopsy for frozen tissues. FFPE hippocampus for IHC was taken from the opposite side as the entire hippocampus hemisphere was used for frozen tissue processing (see accompanying Tissue Collection whitepaper In Documentation).

Datasets

The principal data modalities of the Aging, Dementia and Traumatic Brain Injury (TBI) Project include:

1) **Demographic and clinical information:** Since this project cohort is derived from the larger ACT cohort (see ACT Cohort whitepaper in Documentation), significant information exists about the subjects included in the study (initially 55 TBI subjects and 55 matched control subjects—data from 3 subjects failed to meet quality control criteria and are excluded from the final project data set). Included in the demographic and clinical information is exposure to TBI, including the number of TBI with loss of consciousness, duration of the loss of consciousness, and the age at which the first TBI occurred. Additionally, age (which is presented linearly through age 89, then binned thereafter into 5 year blocks to protect subject anonymity), post-mortem intervals, clinical diagnoses including dementia status and dementia diagnosis, and a variety of neuropathology indicators, including Braak and CERAD stages and NIA-Reagan diagnosis are provided. As this cohort was chosen to match donors with and without TBI, it is important to consider selection bias when conducting analyses that examine risk factors other than TBI (see "Weighted Analysis" whitepaper in Documentation). Additional information, both pre-and post-mortem, are available. Data available from this project web site do not require any additional Institutional Review Board (IRB) approval or permissions. Linking this project data with other ACT study or Kaiser Permanente Washington data requires additional review, initiated by contacting KPWA.actproposals@kp.org.

2) **RNA-Seq data:** The transcriptome was analyzed by RNA sequencing in tissue isolated from four brain regions: neocortex from the posterior superior temporal gyrus and the inferior parietal lobule, white matter underlying the parietal neocortex, and the hippocampus. Tissue from these regions was collected by manual macrodissection. Using an annotated Nissl-stained section as a guide, samples were excised and RNA was isolated and processed for RNA sequencing, producing a minimum of 30 million 50bp paired-end clusters per sample. Raw read (fastq) files were clipped of adaptors, and then sequentially aligned to the transcriptome by Expectation-Maximization (RSEM)[10], the GRCh38.p2 human genome (current as of 01/15/2016) using Bowtie[11], and finally ERCC sequences. Resulting gene expression values (RPKM) were scaled for RNA quality which was quite variable across the sample cohort. For more information about the RNA-Seq data generation and analysis, see the Quantitative Data Generation whitepaper in Documentation.

3) **Histochemistry, immunohistochemistry (IHC) and *in situ* hybridization (ISH) data:** This data set consists of one data set from fresh frozen tissue, and one data set from FFPE tissue. For more information about this data, see the Tissue Processing whitepaper in Documentation.
    a. **Fresh frozen tissue**: Histological staining consisted of Nissl staining (for anatomical reference) and Thioflavin-S (for visualization of plaques and tangles). Immunohistochemical staining was

used to visualize amyloid, tau, and alpha-synuclein pathology within the fresh frozen tissue. In addition, a colorimetric, digoxigenin-based ISH method for labeling target mRNA was used to detect expression for canonical marker genes for astrocytes (*GFAP* and *AQP4*), oligodendrocytes (*MOBP*), and neuronal subtypes (*SLC17A7* (excitatory cells), *SLC6A1* and *RELN* (interneuron subpopulations)).

b. **FFPE tissue:** Tissue was processed for standard neuropathology and stained by IHC and histochemistry to assess β-amyloid, tau, and α-synuclein deposition in plaques, tangles, and Lewy bodies, respectively as well as markers for activated astrocytes (GFAP) and microglia (IBA1). In addition, tissue sections were stained with hematoxylin and eosin/Luxol Fast Blue to visualize cells and myelin.

4) **Quantitative neuropathology using automated image analysis:** Pathology in both fixed and fresh frozen tissue was measured with IHC and quantified using automated image analysis to generate proxy values for the severity of pathology. To generate quantitative image metrics for the IHC on fresh frozen tissue, the macrodissection sites (for the samples for RNA-Seq) as delineated on the Nissl images were used to guide the identification and annotation of equitable regions of interest (ROIs) on each of the near-adjacent IHC images. For quantitative image metrics for the IHC on fixed tissue, ROIs for image analysis were selected on images of IBA1 stained tissue, using the same criteria as what was used for determining macrodissection sites on fresh frozen tissue. The expression density, defined as the percentage of area within the ROI that was occupied by IHC reaction product, was then assessed algorithmically. For more information about the image analysis, see the Quantitative Data Generation whitepaper in Documentation.

5) **Protein measurements of disease pathology and inflammation:** Protein molecular changes in tau and phospho-tau variants, Aβ species, α-synuclein, inflammatory mediators (cytokines and chemokines), neurotrophic factors, and other targets were determined in fresh frozen parietal lobe, temporal lobe, and hippocampus for multiplexed Luminex assays. Tissue was processed through sequential extraction and centrifugation and the optimal extract was selected for the individual assays. Following the Luminex assay, fluorescence was analyzed using the LiquiChip Workstation (Qiagen) and the concentrations of samples were determined from the standard curve. For more information about the Luminex assays, see the Quantitative Data Generation whitepaper in Documentation.

6) **Measurements of oxidative stress:** Free radical injury in fresh frozen parietal cortex and temporal cortex was determined using gas chromatography mass spectrometry (GC/MS) quantitation of isoprostanoids. Isoprostane assays were conducted using the procedure detailed by Montine *et al*.[12] and Milatovic *et al*[13]. GC/MS analysis was conducted using a 6890N Agilent gas chromatograph coupled to a 5973 quadrupole mass spectrometer in the negative-ion mode. Areas under peaks for *m/z* 569.5 and 573.2 (internal standard) were manually integrated to quantify both analytes. For more information about the isoprostane assays, see the Quantitative Data Generation whitepaper in Documentation.

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