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14. ABSTRACT Traumatic brain injury (TBI) affects approximately 3.8 million people annually and costs the US more than \$48 million. Furthermore, TBI has become an increasingly common feature of modern military conflicts. It has been estimated that in the Iraq and Afghanistan conflicts following the terrorist attacks of September 11, 2001, the rate of TBI in military populations has dramatically increased to upwards of 10-20% of those serving, with over 250,000 soldiers exposed to some form of TBI (Source; DoD). The long-term consequences of TBI are multifaceted and include increased risk for AD. To date, mechanisms linking TBI to AD remain unclear. One of the earliest hallmark features of TBI is neuroinflammation, which is defined as the brain's innate immune response. Post-injury neuroinflammation includes activation of brain resident microglia, infiltration of peripheral monocytes due to disruption of the blood-brain barrier, and high level production of pro- and anti-inflammatory molecules. Although this initial response is thought to promote repair following TBI, exaggerated or persistent neuroinflammation can be detrimental. For example, TBI can trigger progressive neurodegeneration, brain atrophy, neuronal loss, and axonal degeneration for months to years after the initial insult and these events are often associated with neuroinflammation. We hypothesize that the TBI-induced neuroinflammatory response is critical in mediating AD-related pathology and specific inflammatory proteins can be used as post-injury biomarkers.					
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Introduction:

Little is currently known regarding the role of inflammation in the progression of traumatic brain injury induced neurodegenerative disorders. Alzheimer's Disease is a common outcome of patients who have experienced mild to moderate brain injury, as well as those who have suffered from repeated concussive injury. There is a persuasive body of evidence favoring a significant inflammatory component in AD. A large number of inflammatory cells including microglia, astrocytes, and infiltrating peripheral immune cells and inflammatory molecules are present at elevated levels in the AD brain. potential role of neuroinflammation in regulating AD pathologies as a result of brain trauma is supported by several observations. First, a recent set of experiments demonstrated that MAPT pathology temporally co-exists with gliosis following mild repetitive TBI in the hTau mouse model of MAPT pathology. Our preliminary studies supported by a previous DoD grant (W81XWH-14-1-0265) have confirmed that even a single TBI enhances accumulation of activated macrophages and phosphorylated MAPT in the hTau mouse model. Similar findings have been reported in wild-type mice after blast induced brain injury, as well as in a triple transgenic mouse model of AD following a single moderate TBI. Second, numerous reports have demonstrated activated microglia near the injury release several pro-inflammatory cytokines and chemokines and that these inflammatory components in turn can exacerbate MAPT pathologies. Third, post-injury neuronal accumulation of A β correlates with increased numbers of IL-1 α expressing microglia. Finally, our preliminary studies (DoD grant W81XWH-14-1-0265) show that a single moderate TBI induces expression of key pro-inflammatory cytokines at acute (3 days post-injury, DPI) time points in non-transgenic mice. Together, these studies suggest that brain injury induced neuroinflammation could be an initiating factor in AD-related pathologies and provides substantial rationale for studies aiming to identify and characterize post-injury inflammatory biomarkers associated with TBI and AD. The current study proposes to characterize inflammatory states in peripheral blood, plasma, and CSF of active and retired MMA fighters and boxers. We predict that our observations will add critical knowledge to the fields of neuroinflammation and traumatic brain injury as well as identify potential biomarkers of disease and novel therapeutic targets.

Keywords:

Alzheimer's Disease, Inflammation, Microglia, Monocytes, Neurodegeneration, TREM2, immunity, Traumatic Brain Injury

ACCOMPLISHMENTS:

What were the major goals of the project?

1. Approvals: HRPO and IRB approval for all studies; month 1-6; 100% complete
2. Staff training: Optimized procedures for sample collection, transfer, and processing; month 3-12; 100% complete
3. Preliminary study to detect biomarkers: Inflammatory biomarkers confirmed in peripheral blood; month 12-15; 30% complete
4. Blood sample collection: Collect blood from separate groups of active or retired profession fighters and age-matched controls; month 15-33; 15% complete
5. Complete imaging studies: Complete magnetic resonance imaging (MRI) and positron emission tomography (PET) amyloid imaging in a subset of retired fighters; month 18-33; 50% complete
6. Collect and process cerebral spinal fluid (CSF): Collect and process CSF for expression of inflammatory biomarkers in a subset of retired fighters; month 18-33; 30% complete
7. Data analysis: Complete data analysis and begin manuscript preparation; month 30-36; 10% complete
8. Manuscript publication: Submit manuscript for publication; month 33-36; 0% complete

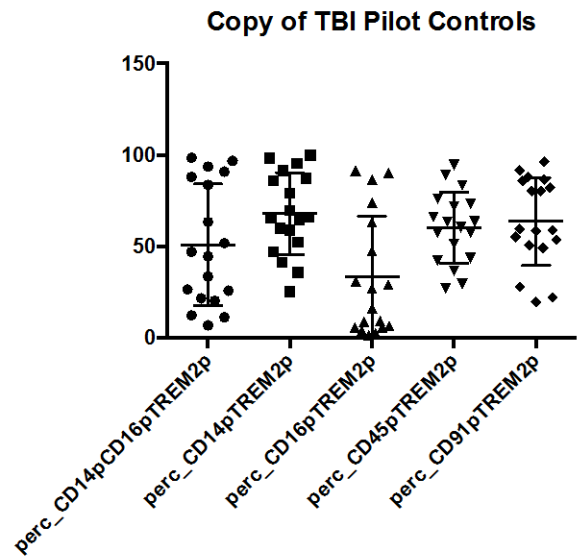
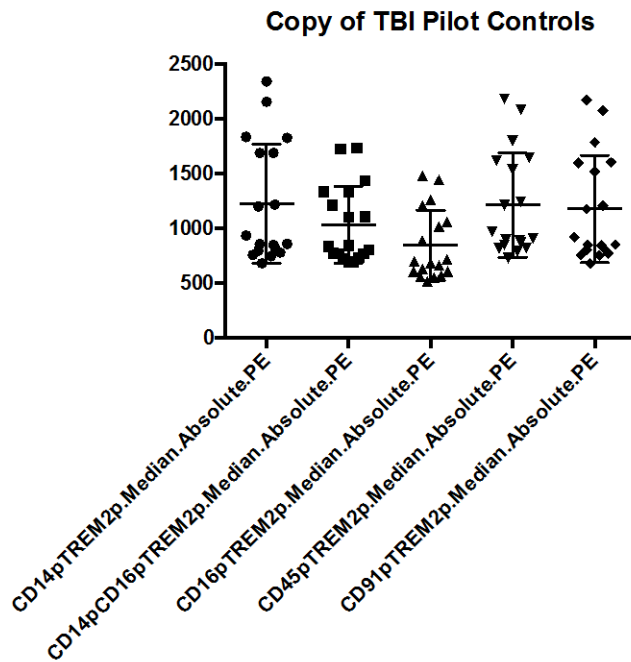
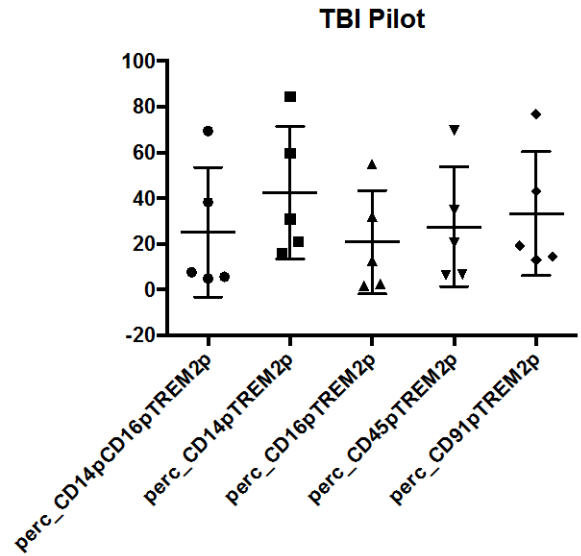
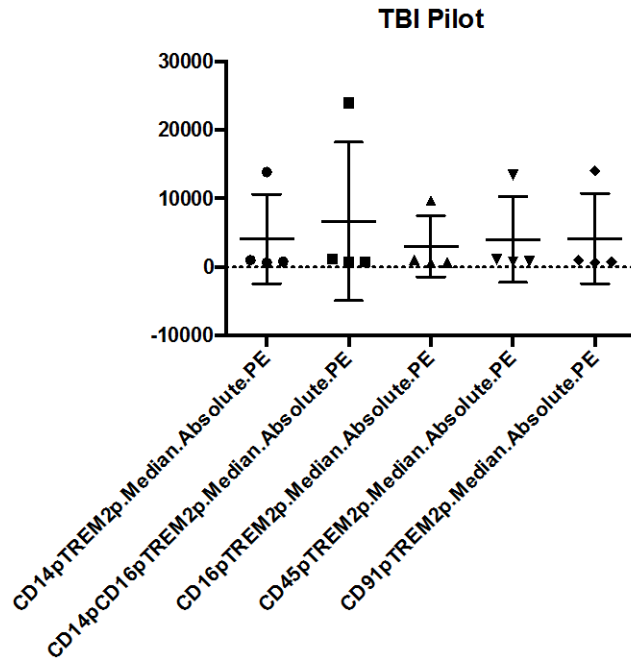
What was accomplished under these goals?

Over the past year of the award, we have made significant progress on a number of items which had previously caused major setbacks. As mentioned in our previous progress report, we have restructured the project personnel and have added Drs. Shane Bemiller and Nipun Chopra who have quickly gotten up to speed and have progressed all aspects of the project. **1 and 2)** We have completed all of our required regulatory elements in order to process and analyze samples at all participating institutions, including IRB exemption and HRPO approval at the IU School of Medicine. Secondly, we have been in extensive contact with Ms. Heather Rodney our point of contact within the Federal Interagency Traumatic Brain Injury Research informatics system (FITBIR). We have successfully gained access to the database, which has required Drs. Lamb, Bemiller and Chopra to create personal FITBIR accounts and create an active study. Our FITBIR account is now online and ready to impute GUID and pseudoGUIDs for our existing subjects. We are currently working with UNLV and the CCF to setup patient IDs as well as inputting experimental data. Third, we have established a pipeline for the processing, shipping, and analysis of plasma, CSF, and blood samples from UNLV to the IU School of Medicine which has taken sufficient time and effort to organize. Furthermore, **3)** blood plasma and serum have been collected and banked at baseline visit for nearly 400 fighters, and will be utilized for correlations and associations with blood TREM2 protein levels once adequate blood samples have been analyzed for TREM2 expression. Dr. Kinney at UNLV will be running half of the plasma samples for the following analytes: **GM-CSF Eotaxin IFN- α IP-10 IFN- γ MCP-1 IL-1 β MIG IL-1 RAMIP-1 α IL-2 MIP-1 β IL-2R RANTES IL-4 IL-5 IL-6 IL-7 IL-8 IL-10 IL-12 (p40/p70) IL-13 IL-15 IL-17 TNF- α** . Additionally, we have purchased the MSD ultra-sensitive multiplex plates in order to run parallel chemokines and cytokines: **CRP, Eotaxin, Eotaxin-3, FGF (basic), Flt-1/VEGFR-1, GM-CSF, ICAM-1, IFN- γ , IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-8**

(HA), IP-10, MCP-1, MCP-4, MDC, MIP-1 α , MIP-1 β , PlGF, SAA, TARC, Tie-2, TNF- α , TNF- β , VCAM-1, VEGF-A, VEGF-C, VEGF-D.

There will be some overlap temporarily with respect to the inflammatory protein analysis until we have sufficiently characterized the relative abundance and reproducibility of these assays moving forward, site to site. We predict that 50% of the samples required for analysis have been collected, with the remaining 50% to be collected at the time of TREM2 blood processing within the next year. **4)** With respect to the TREM2 blood studies, we have successfully completed a series of pilot experiments (Fig. 1) examining the efficacy of processing and shipping isolated leukocyte samples from Dr. Kinney's lab to the IU School of Medicine. We have demonstrated the feasibility and reproducibility which is necessary to continue running these assays on-site at the IU School of Medicine. **5)** We have included Dr. Sarah Banks, who will be spearheading operations at the CCF Lou Ruvo Center for Brain Health, and whom is involved with several related collaborative studies including COBRE. The addition of Dr. Banks has enabled much faster turnaround and organization of samples which will be used across institutions. Additionally, Dr. Banks will be continuing collection of neurological imaging and cognitive assessment of fighters in Las Vegas and has already obtained baseline MRI and PET data from nearly 200 patients associated with these studies, and which will be used to correlate blood levels of TREM2 and fluid biomarkers. We have formed a workgroup which meets monthly to organize efforts across institutions, which has eased much of the logistical issues previously encountered. **6)** CSF samples are actively being collected from a subset of fighters, and is currently being banked at the CCF Lou Ruvo Center for Brain Health in Las Vegas until the time of analysis due to limited sample size and challenges recruiting subjects willing to donate CSF. **7 and 8)** We predict with the frequency of samples which will be immediately arriving that we will have sufficient sample size to proceed with statistical data analysis and interpretation followed by preparation and submission of our manuscript within the next year.

Figure1) Preliminary analysis of TREM2 protein levels from 4 active pilot fighters and control individuals randomly selected from previous studies demonstrates a reduction in the levels of detectable TREM2 in peripheral blood via flow cytometry. Additionally, Mean fluorescent intensity of TREM2 is also altered between TBI fighters and healthy control populations.



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

Funding from the current project has been utilized to enhance the professional development of two key members of the Lamb Lab, namely Drs. Shane Bemiller, and Nipun Chopra. Drs. Bemiller and Chopra were able to attend and present at the Society for

Neurosciences Local Chapter Conference in Indianapolis, IN, May 2017. Additionally, salary support was granted for Dr. Chopra as he recently joined the lab. Additionally, these funds were utilized to help train Dr. Chopra to utilize flow cytometry, as well as for continued professional development.

How were the results disseminated to communities of interest?

Nothing to report.

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

We are now positioned to receive large batches of samples from UNLV/CCF Lou Ruvo Center for Brain Health after completing our pilot study which provided important information regarding fixing, and shipping samples to the IU School of Medicine. All samples which are being screened will have fresh plasma/serum samples isolated concurrent to isolating PBMCs in order to correlate the exact same biological samples from the same time. During the next reporting quarter, we will be running samples and working on the initial blood analysis. Additionally, we will be using baseline plasma samples to optimize our experimental protocols with regards to inflammatory biomarkers.

Impact:

Nothing to report.

Changes/Problems:

Our new investigators have made significant advances in coordinating the study and communicating with FITBIR and the collaborating institutions. We have resolved the majority of the communication issues among institutions through the addition of key collaborators associated with related studies. We are confident that we will be able to collect 50-75% of the required subset of blood samples (N=100) within 6-8 months in order to complete the major aims of the current project. We do not anticipate any further or additional problems that would arise to further delay our study.

Changes in approach and reasons for change:

We have decided after careful consideration that we will be examining CSF and plasma that are associated with the specific blood draw analyzed via flow cytometry. We have taken this course of action in order to avoid the confounds of serial blood draws, stage of potential disease, etc. Therefore, we will be banking CSF and plasma until we have recruited the full number of patient participants.

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

As mentioned in our previous quarterly reports, we have experienced significant and unforeseen delays due to coordinating sample transfers among the 3 participating institutions. We have therefore formed a subcommittee to address these issues moving forward. The committee includes Drs. Chopra, Lamb, Kinney, Bernick, Banks, and Lamb. We have scheduled monthly teleconferences to coordinate logistics and to provide data and general project updates. Further, Drs. Lamb and Bemiller have scheduled a face-to-face meeting for a project update which will take place in February in Las Vegas at the Cleveland Clinic Center for Brain Health.

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:

Nothing to report.

Products:

- The role of TREM2 in regulating Alzheimer's Disease pathology. Bemiller SM., January, 2017 Invited talk at Ashland University
- *Peripheral Roles for TREM2 and CD33 in Alzheimer's Disease*. Bemiller SM., Ransohoff R., Bekris L., Leverenz J., Saykin, A, Lamb BT. SFN Greater Indianapolis Chapter Anniversary Symposium, November 11th 2016, Indianapolis, IN.
- Bemiller SM, Mourany L, Jay T, Cheng P, Coteleur A, Formica S, Xu G, Lee M, Ransohoff RM, Rao S, Pillai J, Bekris L, Landreth GE, Leverenz J, Lamb BT. Peripheral TREM2 in Alzheimer's Disease (Under review *JCI* 2018)

Participants and collaborating organizations:

What individuals have worked on this project?

Name:	Sarah Banks
Project Role:	PI
Nearest person month worked:	1
Contribution to Project:	Dr. Banks is working along with the Bernick Group to facilitate sample transfers and imaging.

Name:	Jefferson Kinney
Project Role:	PI
Nearest person month worked:	12

Contribution to Project: Dr. Kinney and his group are isolating blood cells, and shipping to IUSoM

Name: Shane Bemiller

Project Role: Postdoctoral Fellow

Nearest person month worked: 9

Contribution to Project: New project manager coordinating efforts between IU School of Medicine and UNLV/CCF. Dr. Bemiller has completed all trainings required by the DOD and IU SOM.

Name: Nipun Chopra

Project Role: Postdoctoral Fellow

Nearest person month worked: 6

Contribution to Project: Assisting with data collection and interpretation

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

What other organizations were involved as partners?

Cleveland Clinic Lou Ruvo Center for Brain Health, Las Vegas, NV, USA

- Facilities, Collaboration, and personal exchanges

University of Nevada Las Vegas (UNLV)

- Facilities, Collaboration, and personal exchanges

Special Reporting Requirements:

None

Appendices:

Increased Peripheral TREM2 in Alzheimer's Disease

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1) Indiana University School of Medicine- Stark Neurosciences Research Institute, Indianapolis, IN; 2) The Cleveland Clinic- Lou Ruvo Center for Brain Health, Cleveland, OH; 3) Kent State University, Department of Biological Sciences, Kent, OH; 4) Biogen IDEC- Cambridge, MA 5) Indiana University School of Medicine, Department of Biostatistics, Indianapolis, IN 6) Case Western Reserve University, Department of Neurosciences 7) University of Vermont College of Medicine, Burlington, VT 8) Emory University, Department of Neurology, Atlanta, GA 9) Indiana University School of Medicine, Department of Neurology, Indianapolis, IN

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Keywords: Inflammation, Alzheimer's Disease, TREM2, Neuroinflammation, Peripheral Blood Mononuclear Cells, Tau, A β , Peripheral Infiltration

Abstract

Recently identified genetic variants of the innate immune regulating receptor Triggering Receptor Expressed on Myeloid Cells-2 (*TREM2*) confers increased risk of developing late-onset Alzheimer's disease (AD). Gene expression studies revealed increased peripheral *TREM2* in patients with mild cognitive impairment (MCI) and AD compared to cognitively normal individuals. Using flow cytometry, we characterized levels of *TREM2* protein on classically defined subsets of human monocytes and demonstrated increased percentages of CD14⁺CD16^{dim}*TREM2*⁺ inflammatory monocytes in MCI and AD patients compared to healthy control individuals. Additionally, significant inverse correlations were found between monocyte *TREM2* protein expression and CSF levels of total tau and p-tau.

Introduction

Recent identification of risk variants in genes *TREM2*, *TREML2*, *CD33*, *CLU*, *CR1*, *EPHA1*, *MS4A4E/MS4A6A*, and *PTK2B* have ignited new interest in the role innate immunity in modifying Alzheimer's disease (AD) pathology¹⁻⁴. We recently reported an increase in *CD11b⁺CD45^{hi}TREM2⁺* cells detected in the brains of *APP^{PS1}* mice correlating with progression of A β pathology utilizing IHC and flow cytometry⁵. These cells, which accumulate around dense deposits of A β , are largely absent upon heterozygous or homozygous deletion of *Trem2*^{5,6}. A number of recent studies have identified associations that suggest involvement of peripheral blood mononuclear cells in modifying AD^{5,7-10}. Furthermore, monocytes have also been shown to express *TREM2*⁷. Taken together with recent genetic association studies, these data suggest a key role for monocytes in regulating AD pathology^{4,7}. Expression of *Trem2* on peripheral monocytes is clear, however, questions remain such as whether *TREM2* is expressed by inflammatory *CD14⁺CD16^{dim}*, patrolling *CD14^{dim}CD16⁺*, or *CD14⁺CD16⁺* intermediate monocytes, in addition to whether these cells are directly or indirectly influenced by, or involved in disease pathogenesis. In this study, we address these questions by interrogating peripheral *TREM2* expression levels from existing data sets, and characterizing *TREM2* expression on classically defined subpopulations of monocytes in healthy control individuals, and those with neurodegenerative diseases.

Results and Discussion

Peripheral *TREM2* transcripts are selectively upregulated in AD

Previous studies in the APPPS1 mouse model of AD suggest the recruitment of peripheral myeloid cells into the diseased brain which increase in number throughout pathology^{5,11}. In order to explore the relationship between AD pathology and peripheral *TREM2* expression, we analyzed a cohort of 4-month APPPS1 and C57BL6/J (B6) non-transgenic mice for levels of *Trem2* mRNA using qPCR. We detected a significant increase in transcript levels of *Trem2* in isolated CD45+ circulating leukocytes (Figure 1a) in APPPS1 compared to B6 control mice.

To explore the translatability of these findings to human disease we began by interrogating the UCLA Imaging and Genetic Biomarkers for AD (ImaGene) study data which contains longitudinal imaging, cognitive, biofluid, and transcriptomics data from research subjects with mild cognitive impairment (MCI), Alzheimer's disease (AD) dementia, Parkinson's disease, and cognitively normal healthy control (HC) individuals (Table 1). Serial blood draws were analyzed for *TREM2* transcript levels between cognitively impaired (combined MCI and AD) and HC individuals. A significant increase in *TREM2* was detected in the combined MCI and AD group (Figure 1b; $4.5\% \pm 1.3$; $P=0.011$). Given the strong association between the high risk apolipoprotein E4 (*APOE4*) allele and worsened AD pathology¹²⁻¹⁵, we next compared *TREM2* levels among AD and HC *APOE4* carriers and non-carriers. This comparison revealed trends towards increased *TREM2* transcript levels within combined MCI/AD patients, and transcripts were notably increased in *APOE4* carriers (Figure 1c; $P=0.07$)

Next, we stratified patients into MCI and AD dementia groups to assess how *TREM2* expression was altered across different stages of disease. We detected a significant increase in *TREM2* mRNA ($5.6\% \pm 1.5$) in MCI patients compared to HC ($P=0.005$) and modest non-significant increases between MCI and AD dementia patients (Figure 1d; $P<0.15$). Further stratification based on *APOE4* genotype revealed trending increases in *TREM2* mRNA which were most elevated in MCI *APOE4* carriers (Figure 1e; $p=0.06$). Taken together, these data suggest that *TREM2* is selectively upregulated in the periphery in early-mid stages of disease and is modestly influenced by *APOE4* carrier status.

CD14⁺CD16^{dim}TREM2⁺ inflammatory monocytes are increased in AD

Given the increases in peripheral *TREM2* detected in AD mice and human patients, we next used flow cytometry to characterize *TREM2* protein expression on peripheral blood mononuclear cells (PBMCs) isolated from whole blood that was collected from subjects diagnosed with probable AD, MCI, or HC. To identify monocytes, isolated leukocytes were initially gated on live, single-cells (Figure 2a). Side and forward scatter properties were utilized to isolate the PBMC containing population followed by gating on pan-leukocyte surface marker CD45. We next analyzed *TREM2* surface expression among individual monocyte populations and determined that inflammatory CD14⁺CD16^{dim} and intermediate CD14⁺CD16⁺ monocyte populations contain significantly higher percentages of *TREM2*⁺ cells ($P<0.001$ and $P<0.01$) than do CD14^{dim}CD16⁺ monocytes regardless of disease status (Figure 2a-c). We detected a significant increase in the percentage of CD14⁺CD16^{dim}*TREM2*⁺ cells ($P<0.05$) in

combined AD and MCI populations ($72.7\% \pm 3.0$) compared to HCs ($61.2\% \pm 3.4$; Figure 2d). Similar to our RNA expression analysis, we next stratified our groups based on *APOE4* carrier status. We detected significantly increased TREM2⁺ monocytes in MCI and AD *APOE4* non-carriers ($77\% \pm 3.3$) compared to HC *APOE4* non-carriers ($61.4\% \pm 4.7\%$; $P < 0.05$) and HC *APOE4* carriers ($60.99\% \pm 6.0$; Figure 2e). After stratification based on disease status, we detected a significant increase in TREM2⁺ inflammatory monocytes in AD patients ($73.0\% \pm 3.9$) compared to HCs ($61.2\% \pm 3.4$), and trending increases between MCI ($71.7\% \pm 5.8$) and HCs ($P = 0.09$; Figure 2f) which we attribute to our relatively low N for MCI patients within the Cleveland Clinic patient cohort. Finally, no statistically significant differences were detected between *APOE4* genotypes among individual MCI, AD, and HC groups, although very similar trends were detected between mRNA and protein levels among the 2 patient cohorts (Figure 2g).

Peripheral TREM2 correlates with cerebrospinal fluid (CSF) tau levels

TREM2 protein levels have been examined in CSF samples and levels of full-length, as well as the soluble TREM2 protein (sTREM2) are increased in MCI and AD patients compared to HC and are significantly correlated with levels of CSF tau and phospho-tau¹⁶⁻¹⁸. This suggests that TREM2 expression both in the periphery and CNS may be subject to disease stage specific regulation. To interrogate the underlying biology of increased peripheral TREM2 and determine if increased expression correlated with commonly used biomarkers of disease, we performed correlations with CD14⁺CD16^{dim}TREM2⁺ monocyte percentages with CSF total-tau (t-tau), phospho-tau

(p-tau), A β 42, and CSF amyloid/tau ratios (ATI), and clinically administered Montreal Cognitive Assessment scores (MOCA) within MCI and AD patients. Significant inverse correlations were detected among CD14⁺CD16^{dim}TREM2⁺ monocyte percentages and total tau (t-tau) levels (n=47; r= -0.267; P<0.05) as well as phosphorylated tau (p-tau181) levels in CSF (Figure 3a,b; n=47; r= -0.297; P<0.05). No associations were detected between CSF A β 42, ATI, or MOCA scores and CD14⁺CD16^{dim}TREM2⁺ monocytes. Notably, in support of these findings within the Cleveland Clinic cohort, no significant correlations were detected among *TREM2* transcript levels and CSF A β 42, or cognitive outcomes within the UCLA cohort (data not shown). CSF data was unavailable for the ImaGene cohort. These data further support our previous findings that *TREM2* RNA is increased in a disease stage specific manner which is in line with reports demonstrating disease stage specific effects in *TREM2* deficient mouse models of amyloidosis and tauopathy^{19,20}.

Peripheral TREM2 upregulation is specific to early AD pathology

To determine whether peripheral TREM2 upregulation occurs in other neurodegenerative disorders, we analyzed a parallel cohort of Parkinson's disease (PD) patients (Table 1) within the ImaGene database (n= 45), as well as within the Cleveland Clinic experimental cohort (n= 28). No significant differences were detected between PD patients and HC in *TREM2* mRNA or TREM2 protein levels suggesting that this phenomenon is specific to the pathogenesis of AD, although this finding warrants further exploration with larger group sizes (Figure 3c,d).

Taken together, our data suggests that TREM2 is upregulated in early to middle stages of pathology by a population of peripheral blood mononuclear cells (PBMCs), namely the CD14⁺CD16^{dim} inflammatory monocytes, which abates at later stages of disease (Figure 3e). This idea is supported by our findings that *TREM2* transcripts are upregulated specifically within MCI patients compared to healthy controls and that TREM2⁺ monocyte percentages are increased in both AD dementia and MCI populations, specifically within *APOE4* non-carriers. It could be plausible that *APOE4* non-carriers is due to these non-carriers being in the earlier stages of MCI and AD, stages of the disease where we found TREM2 was most highly upregulated. However, our data suggests that there is likely a modest independent effect of *APOE4* genotype on TREM2 expression, which aligns with recent studies examining the interactions of *APOE4* and TREM2 signaling in mouse models of neurodegeneration²¹⁻²³. MCI patients harboring the *APOE4* allele have the highest detectable levels of *TREM2* mRNA comparatively. Furthermore, TREM2⁺ monocyte percentages inversely correlate with CSF tau levels which are known to be lower at less advanced disease stages.

Further studies are required to understand the mechanisms and roles these TREM2⁺ monocytes play throughout disease, the signal that is responsible for *TREM2* upregulation, as well as the effect of *TREM2* risk variants on these peripheral cell populations in the context of disease. Together, our findings lend further support to an expanding body of literature which points to peripheral immunity as a key player in AD pathogenesis.

Materials and Methods

Human subjects

The UCLA ImaGene study cohort has been extensively characterized previously ²⁴⁻²⁶. In brief, existing data from the UCLA ImaGene study were analyzed and interrogated for levels of TREM2 RNA and associations among available fluid biomarkers. All subjects were assessed to meet the inclusion or exclusion criteria set by the ImaGene study. ImaGene has enrolled 106 mild cognitive impairment subjects, of whom, 32 converted to AD dementia, and 50 cognitively normal HC. A cohort on Parkinson's disease subjects were later recruited as a neurodegenerative disease control subjects. These subjects were enrolled through 1) referring UCLA and outside neurologists and 2) the UCLA Alzheimer's Disease Research Center (ADRC) ongoing longitudinal database study. All subjects provided informed consent after very careful detailed explanation of study procedures and all associated risks by the study physician. Diagnosis for each subject was based on a consensus by all UCLA ADRC neurologists, the ADRC neuropsychologists, psychometricians, and other key study personnel. Diagnosis of MCI required a score of 1.5 standard deviations below the age- and education-adjusted norms on neuropsychological tests and intact functional abilities. NC scored within expected limits as compared to their peers. All studies were approved by the UCLA Institutional Review Board.

Human bio-specimens were obtained from the Cleveland Clinic Lou Ruvo Center for Brain Health Aging and Neurodegeneration Biobank and were approved by the institutional review board of the Cleveland Clinic Foundation. Subjects included cognitively HC (MMSE>26; n=41), MCI (MMSE=22-26; n=18) and probable AD patients (MMSE<21; n=66). Subjects were enrolled through 1) the Lou Ruvo Center for Brain Health and 2) referral from Cleveland Clinic and outside neurologists. A subset of HC was recruited from a parallel Cleveland Clinic study examining high-risk families. All subjects included met internationally determined criteria for biomarker studies, and were free from generalized inflammatory conditions. All subjects provided informed consent

after very careful detailed explanation of study procedures and all associated risks by the study physician.

Experimental Mice

APPPS1-21 (termed APPPS1) mice (provided by M. Jucker, Professor; German Center for Neurodegenerative Diseases [DZNE], Tübingen, Germany) express human APP with the Swedish (K670M/N671L) and PSEN1 L166P mutations under control of the Thy1 promoter²⁷. This mouse was backcrossed a minimum of 6 times and maintained on the B6 background, which were used as controls for this study.

Mice were housed in the Cleveland Clinic Biological Resources Unit and the Jackson Laboratory, facilities fully accredited by the Association and Accreditation of Laboratory Animal Care. All experimental procedures were approved by the Institutional Animal Care and use Committee at each respective institution.

Flow Cytometry

Fresh blood samples collected in sodium heparin coated 5mL tubes were immediately (within 2 hours maximum of the blood draw) spun at 600 G and leukocyte containing buffy coat collected. White blood cell suspensions were lysed for 60s in a hypotonic solution followed by light fixation using 2% EM grade PFA (Polysciences) for 15 minutes with light vortex performed halfway through. Fixed single cell suspensions were blocked using human FC blocking reagent (Miltenyi Biotec) for 30 minutes and stained with antibodies CD45 (EBiosciences; eFluor® 450 Clone: HI30; 1:50) CD14 (Becton Dickinson; FITC Clone: MφP9; 1:25) CD16 (EBiosciences; APC Clone: eBioCB16; 1:25) TREM2 (R&D Systems; Human/Mouse Phycoerythrin; Clone 237920; 1:50) and CD 33 (Becton Dickinson; Phycoerythrin; Clone WM53; 1:50). Samples were run through a BD Fortessa flow cytometer housed and maintained by the flow cytometry core at the Cleveland Clinic Lerner Research Institute. Data were processed and analyzed using FlowJo v9 (FLOWJO, LLC). Axes were normalized and standardized using SPHERO rainbow calibration beads (BD Biosciences) to allow for longitudinal comparisons.

RNA Expression Analysis

Mice were perfused with PBS, and their brains were removed, snap frozen, and stored at -80°C until use. Tissue was homogenized in 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1:100 protease inhibitor cocktail in PBS. RNA was isolated using chloroform extraction and was purified using Purelink (Life Technologies). cDNA was prepared from 1.5 µg using a QuantiTect Reverse Transcription kit (QIAGEN), and real-time PCR was performed for 40 cycles with the StepOne Plus real Time PCR system (Life Technologies). All primers and TaqMan probe were purchased from the Life Technologies database. Relative gene expression was determined using the $\Delta\Delta C_T$ method.

The complete transcriptomic ImaGene data analysis protocol has been previously described ²⁸. Briefly, total RNA was extracted from each serial blood draw using the PAXgene blood RNA kit (Qiagen) using a semiautomated extraction system (Qiagen Qiacube) and stored at -80 °C until analysis. RNA quantity was assessed with Nanodrop spectrophotometer, and quality was checked with Agilent Bioanalyzer Nanochips. RNA with RIN values <8.0 were not used for experimental analysis. Total RNA (200 ng) was amplified, labeled, and hybridized on Illumina Human BeadChips, querying the expression of ~ 24K RefSeq-curated gene targets including TREM2. The slides were processed and scanned with Illumina BeadStation platform. The raw data was loaded in the statistical software R. For low-level quality control several indices were utilized including inter-array Pearson correlation and clustering based on variance. Data were normalized using quantile normalization.

APOE genotyping

Genotyping for *APOE4* was performed using allelic discrimination as previously described ²⁹ using commercially available TaqMan genotyping assays for rs429358 and rs7412 (Thermo Fisher).

CSF Bioanalysis

CSF samples obtained from consenting patients were sent to Athena Diagnostics for analysis of Aβ42, t-Tau, and p-Tau levels.

Montreal Cognitive Assessment (MOCA)

MOCA was performed in a standardized manner consistent with published protocol³⁰ on patients presenting the Lou Ruvo Center for Brain Health. Cases were classified as AD, MCI or normal after review of clinical, laboratory, imaging and neuropsychological data in regular consensus meetings.

Diagnostic criteria used in the ImaGene study to determine patient disease classification were as outlined in Ramirez et al. 2016.

Statistical Data Analysis

Data are presented as mean \pm SEM unless otherwise noted. ImaGene Subjects were seen annually up to 6 visits. Mixed effects models with repeated measures were used to compare the levels of TREM2 by the various diagnoses and diagnoses/*APOE4* presence. Diagnoses were classified as Dementia, MCI and NC. In addition, Dementia and MCI were collapsed to compare against NC. Both sets of diagnoses were further split into 6 and 4 categories by stratifying by *APOE4* allele presence. Models were run adjusted for age at diagnosis, gender and education. Models were adjusted for multiple comparisons using Bonferroni correction. Multiple group comparison, or multiple comparisons were analyzed using one or two-way ANOVA or MANOVA followed by Bonferroni post hoc test where appropriate. Pearson correlations were performed on CSF samples and analyses was performed using Prism GraphPad, R, or SPSS software. Significance was determined at $P < 0.05$ *, $P < 0.01$ **, and $P < 0.001$ ***.

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The authors declare no competing conflicts of interest.

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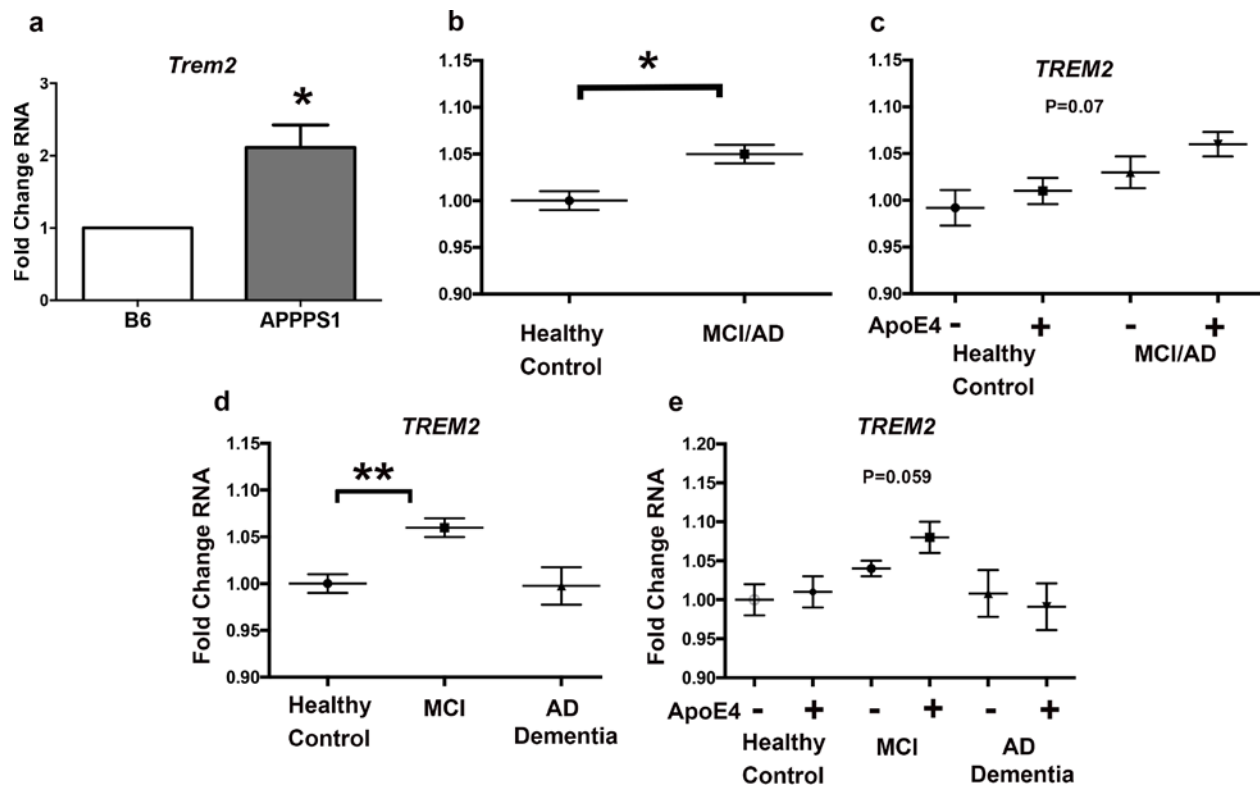


Figure 1. *TREM2* is Significantly Upregulated Throughout Alzheimer's Pathogenesis. (a) qPCR expression analysis on isolated CD45+ cells from C57BL6/J mice (n=3; 2M, 1F) and APPPS1-21 A β mice (n=3; 2M, 1F) reveals significantly upregulated peripheral *Trem2* transcript levels (P<0.05) at 4-months of age in diseased mice. (b) Microarray transcriptomic analysis of peripheral leukocyte *TREM2* levels collected longitudinally from the UCLA ImaGene study reveals significantly upregulated *TREM2* transcripts in combined MCI and AD patients (MCI/AD) (n= 160; n=351 blood draws) compared to cognitively normal healthy control individuals (H.C.) (n=84; n=255 blood draws). (c) Stratification by *APOE4* genotype reveals no statistically significant differences within *APOE4* carriers and non-carriers between patient groups, although increased trends were observed within MCI/AD groups compared to healthy control ApoE4 non-carriers. (d) Post-hoc analysis reveals highly significant *TREM2* transcript levels in MCI patients compared to HC and AD groups (Student's *t*-test; P<0.001 and P<0.05). (e) Further stratification of *APOE4* genotypes reveals upregulated *TREM2* transcripts in *APOE4*+ MCI individuals. Error bars represent SEM.

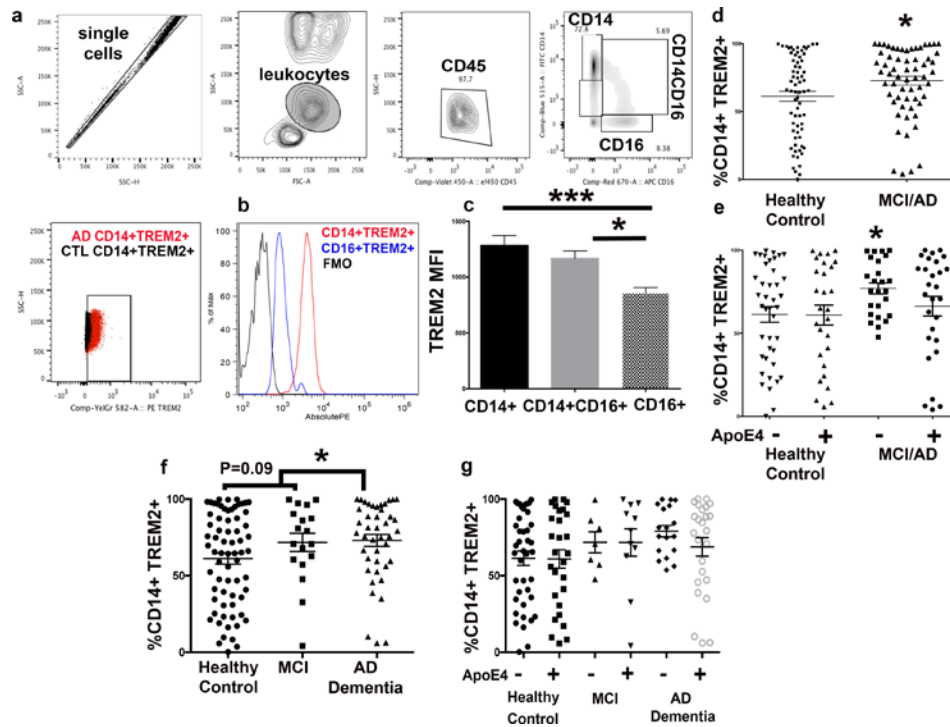


Figure 2. Increased CD14⁺CD16⁺TREM2⁺ Monocytes in AD (a) General gating strategy for flow cytometry analysis of PBMCs isolated from human AD patients, Parkinson's disease patients, and healthy control individuals. Isolated single cells were initially gated based off of side scatter and forward scatter properties to identify the peripheral blood mononuclear cell fraction. Cells were then gated on CD45 positivity, and subdivided into CD14⁺CD16^{dim}, CD14⁺CD16⁺, and CD14^{dim}CD16⁺ populations. (b,c) TREM2 protein expression was examined on individual subpopulations of monocytes using Median Fluorescent Intensity which demonstrates that CD14⁺CD16^{dim} monocytes and CD14⁺CD16⁺ express significantly higher levels of TREM2 than do non-classical CD14^{dim}CD16⁺ monocytes (n=153; ANOVA P<0.001). (d) Combined AD and MCI patients (n= 61) have a higher percentage of detectable TREM2⁺ CD14⁺CD16^{dim} (inflammatory) monocytes than healthy controls (n=66) healthy controls (2-way ANOVA with Bonferroni adjustment for multiple comparisons; P<0.05). (e) Significantly increased percentages of TREM2⁺ inflammatory monocytes were observed in ApoE4⁻ MCI/AD patients compared to HC groups (n= 20 MCI/AD APOE-ε4⁺, 21 MCI/AD APOE-ε4⁻, 37 Control APOE-ε4⁺, 29 Control APOE-ε4⁻; P<0.05.) (f) Post-hoc analysis of individual disease states reveals significantly increased percentages of TREM2⁺ inflammatory monocytes in both MCI (n=18) and AD (n=43) groups compared to HC (n=66) (P<0.05). (g) Comparisons of individual disease groups stratified by ApoE4 genotype reveals strong trends towards increased percentages of TREM2⁺ monocytes in MCI and AD groups irrespective of ApoE genotype which are further increased in the ApoE4⁻ AD group. Error bars represent SEM. All experiments were performed in duplicate.

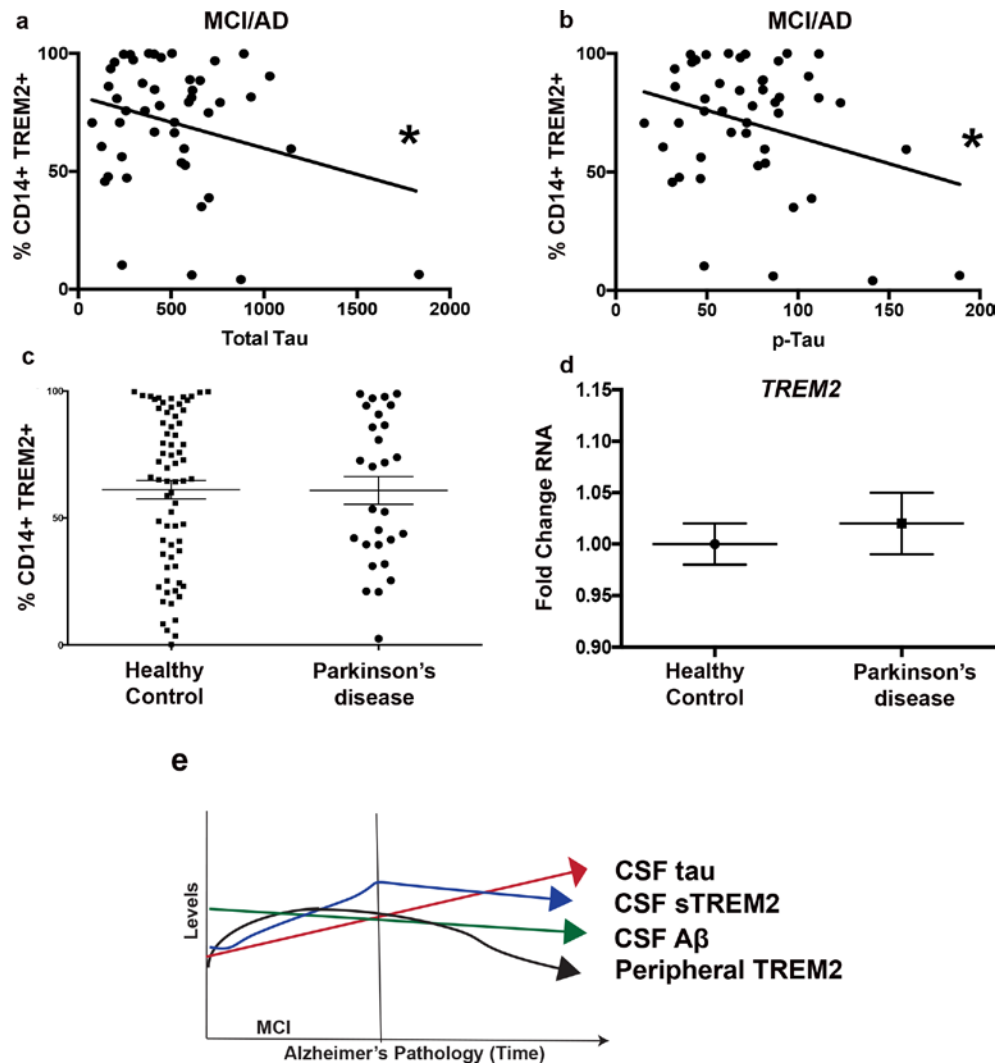


Figure 3. TREM2 upregulation (a) Significant Inverse correlations were found between percentages of TREM2⁺ CD14⁺CD16dim inflammatory monocytes and CSF total tau (Pearson Correlation; n=47; r= -0.267; P<0.05) and (b) p-tau levels (n=47; r= -0.297; P<0.05). (c) No statistically significant differences were detected between a cohort of PD patients and healthy controls (n=66) with respect to TREM2⁺ monocytes (n=28 PD; n=66 HC) or (d) *TREM2* transcript levels (n=45 PD; n=84 HC). Error bars represent SEM. All experiments were performed in duplicate. (e) Schematic demonstrating the initial upregulation and eventual recession of peripheral TREM2 levels compared to common CSF biomarkers of disease and CSF soluble TREM2 (sTREM2). All experiments were performed in duplicate.

Table 1. Patient populations from ImaGene and Cleveland Clinic Lou Ruvo Center for Brain Health cohorts.

ImaGene Cohort	Alzheimer's Disease	MCI	Parkinson's Disease	Healthy Controls
N	32	74	45	50
Median Age	77	69	71	68
Male/Female	12, 21	45, 61	26, 19	28,22
APOE ε4 +	18	48	N/A	22
APOE ε4 -	14	58	N/A	28
CCF Cohort	Alzheimer's Disease	MCI	Parkinson's Disease	Healthy Controls
N	43	18	28	66
Median Age	66	69	66	63
Male/Female	21, 22	9, 10	18, 10	37, 29
APOE ε4 +	25	11	7	30
APOE ε4 -	18	7	21	36