Research Strategy

A. Significance

Inflammation is an Influential and Modifiable Component of Alzheimer's Disease (AD). Pro-inflammatory processes represent a normal innate immune response to pathogen invasion that is critical for initiating tissue repair and maintaining homeostasis. However, sustained inflammation has deleterious effects on neurological functioning that can disrupt cognition and enhance neurodegeneration (1-3). Current literature on inflammation and neurodegeneration suggests that although inflammation was initially thought to be a secondary effect of aberrant protein accumulation, it is now considered an early event that interfaces with and potentially contributes to clinical manifestations of mild cognitive impairment (MCI) and AD (1, 4-6). MCI has been conceptualized as an intermediary diagnosis between healthy aging and dementia, with amnestic presentations most prone to later development of AD dementia (7). *Considering that inflammation may be modifiable and mitigated by therapeutic treatments, elucidating innate immune-associated biological factors in the early stages of AD pathogenesis is of great significance. Older adults with MCI represent an optimal, yet understudied, patient group for evaluating the mechanistic role of inflammation in cognitive decline*.

Inflammation Plays a Critical Role in Inhibiting Hippocampal Neurogenesis and Promoting Neuropathological Changes in Animal Models of Aging and AD. In the context of pro-inflammatory processes, the TNF-α and IL-6 pathways (8-10) and specific chemokine families have received attention as modulators of long-term potentiation (LTP) and memory. Specifically, Eotaxin-1 and monocyte chemotactic protein-1 (MCP-1) markers are members of the C-C chemokine family, which is clustered on the long arm of chromosome 17, and have been directly linked to memory impairment (11) and increased Aβ pathology (12), respectively, in animal models of aging and AD. Consistent with these findings, a study of aged and younger mice reported that increasing peripheral Eotaxin-1 levels *in vivo* led to decreased hippocampal neurogenesis and impaired learning and memory (13). Taken together, these results suggest that Eotaxin-1 and MCP-1 markers may provide an innate immune basis for cognitive decline in animal models of AD. *Translating these basic mechanistic discoveries of innate immune system function and investigating their associated processes in human MCI will provide a unique opportunity to assess the longitudinal relationships between peripheral inflammatory markers, brain structure, and cognition at one of the earliest stages of AD pathogenesis.*

Determining the Temporal Relationship Between Inflammation, Amyloid Pathology, and Cognitive Decline Will Provide Insights into the Etiology and Progression of Amnestic MCI. The progression of cognitive and clinical symptoms in amnestic MCI (aMCI) occurs at a variable rate, with estimates of 5-20% of MCI subjects converting to dementia annually (14, 15). Recent studies have reported higher blood levels of inflammatory markers in MCI subjects relative to both AD and control subjects (4, 10, 16-20), and found that baseline levels of inflammatory markers predict later conversion to AD (21). These studies tentatively suggest that inflammatory processes may render their most injurious effects **early** in the disease. Despite these observations, few studies have examined longitudinal changes in inflammatory markers together with changes in cognitive and neuroimaging outcome measures (20, 22-25), and studies have failed to examine the relationship between inflammation and AD pathology (as indexed by PET amyloid molecular biomarkers). *Identifying how AD molecular biomarkers interact with peripheral inflammation will offer a window into the clinical heterogeneity of aMCI trajectories.*

Evaluating Central Nervous System (CNS)-Derived Blood Exosomes in aMCI May Clarify the Mechanistic Role of Central Versus Peripheral Innate Immune Processes in AD Pathogenesis. Despite the robust association between inflammation and AD, the vast majority of studies have focused solely on peripheral, circulating markers of immune system functioning, making it difficult to determine how well these inflammatory signatures reflect potential underlying CNS alterations (26, 27). Exosomes are small vesicles (50–100 nm diameter) that are released from both CNS cells (CNS-derived) and peripheral cells (peripheral-derived) into the extracellular environment by exocytosis when multivesicular bodies fuse with the plasma membrane. Exosomes play key roles in cell-to-cell communication and cellular signaling, and accumulating evidence suggests that CNS-derived exosomes may serve as robust vehicles for the transport of a wide range of deleterious proteins and immune markers, thereby potentially initiating or exacerbating pathogenic processes in nearby cells by fusing with neurons (28-30). As such, the cargo associated with CNS-derived exosomes may serve as markers of underlying CNS immune system changes that occur in advance of changes in peripheral immune markers associated with circulating plasma. Indeed, a study in older adults indicated that high levels of P-T181-tau and Aβ1-42 in CNS-derived blood exosomes predict the later development of AD (31). Although that study establishes methodological feasibility and provides tentative support for the hypothesis that exosomes play a role in AD development, no study to date has examined

inflammatory processes associated with CNS-derived exosomes as potential regulators of cognitive decline in aMCI. *Given that changes in the CNS cellular milieu affect the protein content of CNS-derived exosomes, these vesicles and the cargo they harbor provide a critical opportunity to understand how immune-mediated CNS functions may underlie AD pathogenesis and phenotypes.*

Summary: Our goals are to rigorously examine links between established and novel peripheral inflammatory markers in circulating plasma, memory consolidation, and grey matter over time, to determine whether peripheral inflammatory markers synergize with amyloid pathology to accelerate clinical progression, and to determine whether immune cargoes from CNS-derived blood exosomes are better predictors of cognitive decline and cortical thinning when compared to total exosome markers. These goals are unexplored areas of research that have direct implications for the early diagnosis and treatment of AD. The short-term impacts of this work are that: **a) it will translate and evaluate the contribution of basic mechanistic discoveries of innate immune system function to clinical progression in aMCI; b) it will elucidate potential mechanisms underlying the deleterious connection between AD pathology and inflammation in predicting cognitive decline and structural brain changes in aMCI; and c) it will identify whether CNS- versus circulating markers of inflammation (or both) influence cognitive decline and cortical thinning**. The long-term impact of the study is that the **identification of a specific exosomal pathway will offer potential therapeutic modifications of CNS immune dysfunction in AD, thereby aligning our research investigations with an innovative and urgently needed transdisciplinary approach to understanding — and treating — immune dysfunction in AD.**

B. Innovation

Evaluation of CNS-Derived and Circulating Markers of Inflammation: An innovative feature of this prospective study is the characterization of inflammatory protein markers in circulating plasma as well as CNS-derived and total blood exosomes. One of the critical barriers to advancing the immune-AD field is the reliable, antemortem measurement of CNS inflammation. Although systemic inflammation measured in blood has been associated with brain structure and clinical outcomes in AD (32, 33), it remains an unresolved issue as to whether circulating inflammatory markers are reflective of the CNS milieu. One means of circumventing this issue is the use of cerebrospinal fluid (CSF); however, this method typically has a lower rate of ascertainment in observational studies due to its invasiveness and risk for post-lumbar puncture headaches. Utilization of exosomes surmounts the aforementioned issues as it allows us to capitalize on a relatively non-invasive method (blood) *in vivo* while isolating CNS-derived exosomes and the proteins they harbor. Moreover, this innovative technology facilitates additional specification of the originating cells in the CNS that shed the exosomes, including neuronal- and glial-derived exosomes. This will provide much needed information about the source of immune proteins and how they relate to cognitive decline and cortical thinning.

Longitudinal Assessment of Established and Novel Inflammatory Markers: Most studies of inflammation and cognition have been cross-sectional (9, 20, 34). These studies are limited because they cannot address how changes in inflammation are related to clinical outcomes in aMCI subjects. We address this design challenge by conducting a rigorous longitudinal assessment of both established and novel inflammatory markers that have been linked to disrupted LTP and reduced hippocampal neurogenesis (13). If inflammation plays a pathogenic role in AD progression, then alterations in inflammatory markers should be observed over time in tandem with clinical changes over time. Our study will be the first to comprehensively assess longitudinal changes in multiple high priority inflammatory markers and their associations with clinical outcomes.

Utilization of Computer-Based Cognitive Neuroscience Measures: We will implement a combination of both standard and novel neuroscience measures of cognition, with the overall goal of being to evaluate sensitive measures of neuropsychological functioning that can be applied to a wide range of severity levels, thereby improving early detection of aMCI and prediction of AD dementia. Our proposed measures allow for greater variability in scores and reduce the likelihood of floor or ceiling effects. Given that we will be examining two time points of cognitive functioning (i.e. at baseline and at a one year follow-up visit), our inclusion of these novel measures is both innovative and critical for detecting meaningful changes in memory consolidation.

Evaluation of Inflammation in the Context of Multimodal Biomarkers: Our proposed study will be the first to examine circulating and CNS-derived markers of inflammation in the context of multimodal cognitive and imaging (structural and molecular) biomarkers. We propose a model in which inflammatory processes increase early in the AD clinical course (35), move from the CNS to peripheral systems via exosomal pathways, and, ultimately, exacerbate cognitive decline in older adults with aMCI (Figure 1). In order to address the relationships between inflammation and clinical outcome variables over time, our study will longitudinally

evaluate plasma biomarkers (including circulating plasma proteins, CNS-derived exosomes, and total exosomes), cognitive assessments, neuroimaging, and baseline amyloid PET. **This approach will allow us to better understand the mechanistic connections between inflammation, pathology, and clinical phenotypes**. Moreover, it will allow us to elucidate the *sensitivity* of specific CNS and peripheral immune pathways to AD pathology (as indexed by amyloid PET).

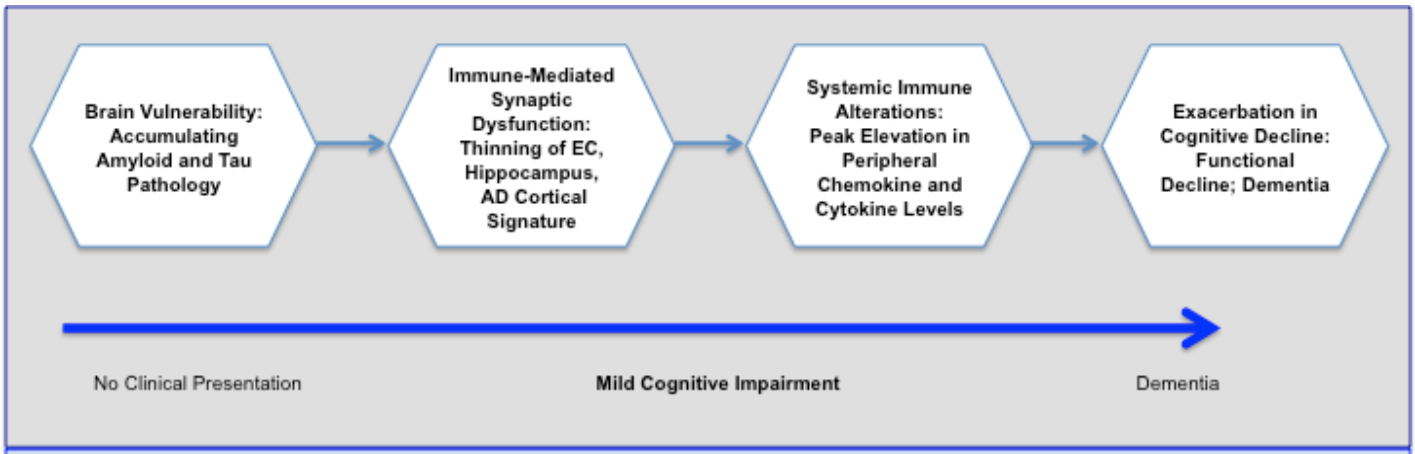

Figure 1. Early AD is associated with a chronic inflammatory process that involves early, cytotoxic innate immune responses, followed by dysregulated communication between CNS and peripheral blood through exosomal pathways. We hypothesize that the relationship between AD pathology and cognitive functioning is negatively modified by these inflammatory mechanisms. MTL=Medial Temporal Lobe. EC=Entorhinal Cortex

C. Approach

C.1. Overview of Study

Our proposed research study is a longitudinal evaluation (baseline and one year follow-up) of innate immune system-associated mechanisms of cognitive decline in aMCI. Data collection for this study will occur at the Rocky Mountain Alzheimer's Disease Center (RMADC) at the University of Colorado Anschutz Medical Campus to address three primary aims. **Aim 1: Evaluate longitudinal associations between markers of peripheral inflammation, cognition, and brain structure in aMCI; Aim 2: Examine how markers of peripheral inflammation impact the relationship between AD pathology and clinical progression of aMCI;** and **Aim 3: Delineate the role of CNS-derived exosomes as potential propagators of cognitive decline and structural brain changes in aMCI**. This study is extremely efficient in that it leverages established infrastructure and parent projects at the RMADC. Importantly, it contributes new multimodal data on immune aging in aMCI to this RMADC cohort that will answer our hypotheses and be made available to other researchers for future studies.

C.2. Preliminary Studies

1. Peripheral inflammation is deleteriously related to cognition and brain structure in typical aging.
Work from my laboratory focused on "inflammaging," has demonstrated that higher peripheral levels of inflammatory markers are related to worse memory and smaller medial temporal lobe structure (36), Directly relevant to our current study proposal and neuroimaging methods, preliminary data (n = 92) also suggests that **higher baseline peripheral levels of IL-6 predict smaller mean cortical thickness in the AD-signature at follow-up** (18 to 24 months post baseline; beta = -20.133, standard error [SE] = 9.604, p = 0.03), even after controlling for total cortical thickness (p = 0.01). Although this finding does not demonstrate causality, it does provide preliminary evidence that higher levels of inflammatory markers may precede critical AD-associated changes in brain structures.

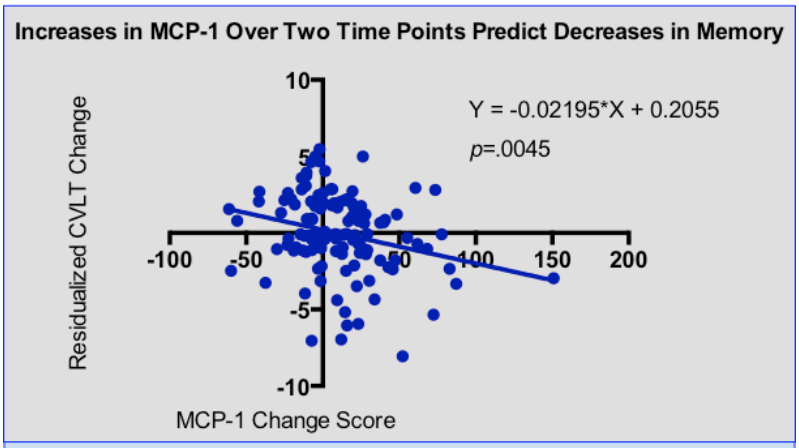

Figure 2. Displays an inverse association between MCP-1 change scores (Time 2-Time1) and memory change scores (California Verbal Learning Test [CVLT] Time 2-Time 1).

2. Increases in peripheral inflammation predict poorer memory and are associated with memory decline in typical aging. Basic science models have recently linked specific chemokines (MCP-1; Eotaxin-1) to reduced hippocampal neurogenesis and worse spatial memory consolidation in mice (13, 37, 38). To better understand how increases in inflammation are related to memory

performance, we examined these novel chemokines in healthy aging adults (n = 122). **Our results suggested that increases in MCP-1 over 18 to 24 months predict poorer memory performance at follow-up** (beta = -0.018; SE = 0.009; *p* = 0.046; controlling for baseline MCP-1 levels: *p* = 0.020). These increases in MCP-1 display **even stronger associations** with *decline* in verbal memory recall over the same time frame (controlling for demographics, *p* < 0.01; Figure 2).

3. Peripheral chemokine levels are related to memory and grey matter volume in aMCI and AD. Extending the reports in the literature regarding chromosome 17 chemokines (39) and our findings in healthy aging controls (HC), we examined pilot chemokine data relative to memory performance in HC, MCI, and AD-dementia subjects (32). Specifically, we hypothesized that higher levels of MCP-1 and Eotaxin-1 would be related to worse memory consolidation. Controlling for demographics, severity level, and *APOE* genotype, higher levels of MCP-1 were related to lower memory scores (n=171) (t=-2.52; *p*=.01), and analyses revealed an interaction between chemokines, such that **higher levels of both Eotaxin-1 and MCP-1 were associated with worse verbal and visual memory** (t = -3.02, *p* = 0.003; Figure 3: memory scores residualized for demographic variables). Inflammatory markers from other chemokine pathways were not related to memory function in AD, suggesting that MCP-1 and Eotaxin-1 levels are sensitive, and possibly specific to memory recall. A subsample of participants also obtained brain MRI (n=55). **Higher MCP-1 and Eotaxin-1 levels were associated with smaller left medial temporal lobes** (*p* = 0.03 for both chemokines) using FreeSurfer.

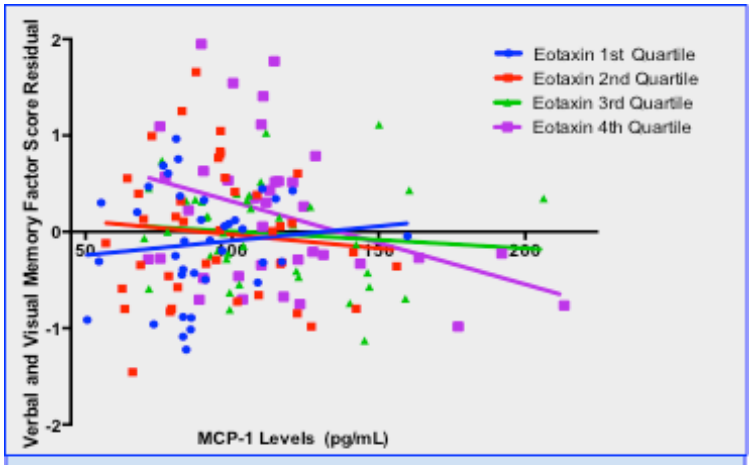

Figure 3. Inverse Relationship between MCP-1 Levels and Memory as a Function of Eotaxin

4. Baseline peripheral chemokine levels predict memory decline in aMCI.

To further elucidate the relationship between inflammation and memory decline, we examined chemokine levels in circulating plasma relative to memory consolidation in adults with aMCI. Pilot analyses revealed that **higher baseline Eotaxin-1 levels in aMCI subjects (n = 40) predict decline in recall on an episodic memory list-learning test (CVLT) over a 12-month period** (beta = -0.016, SE = 0.006, *p* = 0.017). These findings remained significant after controlling for baseline memory performance (i.e. delayed recall), demographics (i.e. age; sex), severity level (i.e. CDR of 0 and 0.5), and *APOE* status (i.e. presence or absence of the E4 allele), suggesting that pro-inflammatory factors may independently predict cognitive *change* over a one-year period. These findings provide support for our proposed model (Figure 1), in which alterations in peripheral inflammation play a significant role in clinical outcomes and progression of aMCI subjects, and also suggest that these changes can be detected in a one-year time span.

5. Inflammatory markers from CNS-derived exosomes are feasibly measured in early stages of AD.

In order to establish the feasibility of isolating neurally derived exosomes from plasma and measuring

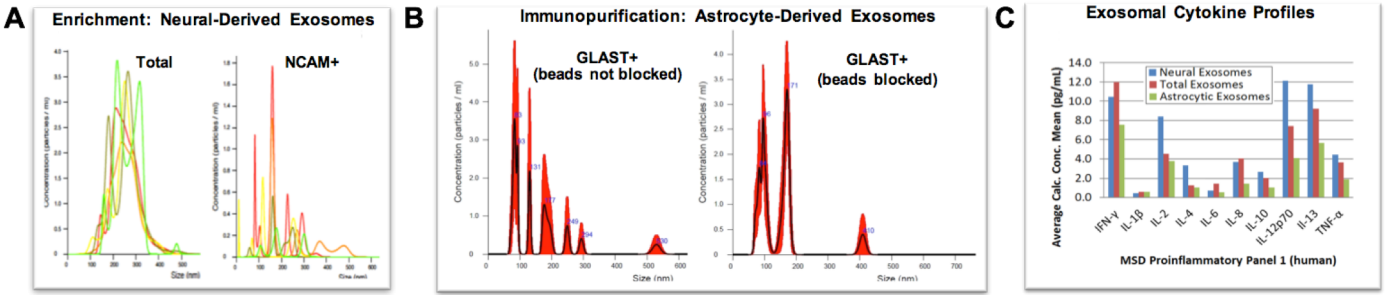

Fig. 4. Enrichment of Neural-Derived Exosomes, Immunopurification of Astrocyte-Derived Exosomes, and Analysis of Cytokine Protein Cargo from Neural-Derived, Glial-Derived, and Total Exosomes. A, Enrichment of neural-derived exosomes from total plasma exosomes using anti-NCAM antibodies. **B,** Immunopurification of astrocyte-derived exosomes using biotinylated anti-GLAST antibodies bound to streptavidin-conjugated magnetic beads with or without blocking of free streptavidin binding sites with biotin prior to incubation with total plasma exosomes. **C,** Cytokine profiles from enriched neural-derived, astrocyte-derived, and total exosomes detected using the MSD human Proinflammatory Kit.

inflammatory markers from these extracellular vesicles at the RMADC, we conducted a pilot study of 10 participants with early stages of AD. Using the methods detailed in section C.4.e, we extracted neurally derived exosomes and found that the ratio of pTau (Thr231) to Total Tau was 3.27 times higher in neurally derived exosomes as compared to total plasma exosomes. Importantly, we were able to analyze inflammatory markers using the Meso Scale Discovery Pro-Inflammatory Plate (see Figure 4) and obtained detectable levels for the normalized markers. These findings highlight that inflammatory markers from neurally derived exosomes are feasibly measured and detectable in early stages of clinical AD presentation, which is critical to our **Aim 3**.

Preliminary Studies Summary: Preliminary data from Dr. Bettcher's ongoing K23 grant and recent collaborative studies at the RMADC collectively indicate that associations between inflammatory markers, cognition, and brain structure are evident, and that these relationships can be feasibly assessed in aMCI patients over a one-year period. In addition, our collaborator, Dr. Michael Graner, has an established research program focused on evaluating the relationship between exosomes and immune markers as a function of brain tumor biology (28, 40, 41). Given the breadth of his laboratory's experience and expertise on exosome methodology, our laboratory is uniquely positioned to examine this novel mechanism in the context of aMCI.

C.3. Participant Recruitment

C.3.a. Recruitment Sources

HC and aMCI participant recruitment will occur in collaboration with the RMADC, primarily from the parent project *"Longitudinal Biomarker and Clinical Phenotyping" study (Bio-AD; PI: Bettcher)*, which is a multimodal longitudinal cohort study (enrollment in past six months, n = 52), and the newly initiated Amyloid PET project (PI: Bettcher), both of which enroll HC and aMCI participants. Participants in these studies receive a comprehensive evaluation, including a neurological and physical examination by a geriatric neurologist, cognitive assessment, neuroimaging, and a blood draw, and individuals with HC and MCI are eligible for an amyloid PET scan as part of the Amyloid PET protocol. In addition to the parent projects, we will also directly recruit from the *longitudinal standard of care MCI-AD database*, which currently has enrolled XX subjects in the past year (MCI, n = 75). Final recruitment sources for aMCI participants will be the *Memory Disorders Clinic, the University of Colorado Hospital*, and the *Seniors Clinic,* which have collectively evaluated more than 1,200 MCI and AD patients in the past two years. To increase enrollment efforts and feasibility, we will work with the Department of Neurology's recruitment specialist, Ms. Nikola Haakonsen (effort paid by department), to maximize clinic outreach and streamline the recruitment pipeline. Based on current clinic flow and participant study consents, we can expect to recruit two to three participants per week; considering the projected 10% attrition over the follow-up period, we plan to recruit 138 aMCI and 56 HC to reach our two-time point goal of 125 aMCI and 50 HC subjects (ages 60–90 years). All participants will be enrolled at the RMADC.

Table 1. Proposed Annual Enrollment of MCI and HC

Baseline	Study Yr 1*	Study Yr 2*	Study Yr 3*	Study Yr 4	Total N
MCI	46	46	46	-	138
HC	19	19	18	-	56
1-Year Follow-Up		**Study Yr 2**	**Study Yr 3**	**Study Yr 4**	**Total N**
MCI	-	42	42	41	125
HC	-	17	17	16	50

*Baseline visits will target 10% additional participants to account for projected attrition rates at follow-up visits.

C.3.b Participants

Healthy Aging Control (HC): The parent projects (*Bio-AD; Amyloid PET)* utilize a multi-step approach to the identification and screening of HC subjects, including a 10-minute phone screen and an in-person visit to the RMADC. The phone screen addresses basic demographics and history, and also serves to identify a participant study partner. Potential participants who pass this screen undergo subsequent in-person cognitive evaluation and mood assessment. In addition, the participant's study partner is interviewed regarding functional abilities. All potential participants are then reviewed by a consensus team, including a geriatric neurologist and a neuropsychologist, to ensure that all study inclusion criteria are met.

Inclusion criteria for HC subjects are as follows: a) Montreal Cognitive Assessment (MoCA) score of > 26; b) Clinical Dementia Rating score of 0; c) normal neurological exam; and d) no informant report of decline during the prior year. Of note, recruitment of healthy older adults for the proposed study will be based on *clinical* screenings conducted by an interdisciplinary team. Literature indicates that AD pathology may be present in functionally normal adults decades before a diagnosis of MCI or AD (42, 43) . Given that we are interested in determining how inflammation may influence the relationship between AD pathology and cognitive decline, this will provide important heterogeneity in our sample.

Amnestic Mild Cognitive Impairment (aMCI): A similar multi-step approach will be used for the identification and screening of aMCI participants, including a phone screen and an initial oral consent to review medical records. Medical records will subsequently be reviewed for each participant, and, when available, a clinical

MRI will be examined to rule out other neurological conditions (e.g. tumor; large vessel infarct; see exclusion criteria below) *prior* to the participant's first research visit. Participants will subsequently be brought into the RMADC for an in-person visit with a study partner, and all assessments will be reviewed by a consensus team.

Criteria for the diagnosis of aMCI will be based on the National Institute on Aging and Alzheimer's Association workgroup (44) criteria, and will include the following clinical determinations: a) cognitive complaint that reflects a decline in cognition; b) objective neuropsychological impairment in one or more domains, including memory; c) independence in functional activities; and d) not meeting criteria for a dementia. Patient diagnoses will be determined by a multidisciplinary team in a consensus conference at the RMADC.

Exclusion criteria for both groups will include: major psychiatric disorder (e.g. schizophrenia; bipolar disorder; major depression within past two years); neurological or autoimmune conditions affecting cognition (e.g. Parkinson's disease; epilepsy; multiple sclerosis; head trauma with loss of consciousness greater than 30 min; large vessel infarct); systemic medical illnesses (e.g. cancer, renal failure); substance abuse or dependence (DSM-V criteria); a Hachinski score greater than 4 (i.e. ruling out significant white matter disease); current medication use likely to affect CNS functions (e.g. long active benzodiazepines); current depression (defined as Geriatric Depression Scale (45) score > 15); and factors that preclude MR imaging (e.g. pacemaker).

C.4. Standard Procedures

Identification and recruitment of participants for the baseline visit will span the first 36 months of the study (see Table 2), and participants will sign a new informed consent form to complete the proposed study. One-year follow-up evaluations will occur beginning in Year 2 and will conclude at the end of Year 4.

All participants in the RMADC parent projects receive comprehensive neurological and physical examinations by a geriatric neurologist, and they also undergo cognitive, neuroimaging, and functional assessments at the CU Clinical and Translational Research Center (CTRC) and the Clinical and Translational Research Imaging Center (C-TRIC). This data is entered into a relational database and will be available to the PI and the project team. We will incorporate demographics (i.e. age; education), functional (i.e. Clinical Dementia Rating, CDR), behavioral (i.e. Geriatric Depression Scale, GDS), medication use (i.e. non-steroidal anti-inflammatory drugs, NSAIDs), cardiovascular risk metrics (i.e. body mass index, BMI; blood pressure; history of hypercholesterolemia), *APOE* genotype, and diagnostic information that will be routinely collected as part of standard RMADC practice. In addition, we will incorporate and expand upon routinely collected cognitive measures (e.g. MoCA and neuropsychological assessment).

Table 2 Research Activities

Aim 1 Research Activity	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**
Participant Recruitment/Evaluations	X	X	X	X	
Process MRI T1 Scans	X	X	X	X	
Process Circulating Blood Markers		X	X	X	X
Statistical Analyses			X	X	X
Manuscript Preparation			X	X	X
Aim 2 Research Activity	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**
Conduct and Process Amyloid PET	X	X	X		
Process Circulating Blood Markers		X	X	X	X
Statistical Analyses			X	X	X
Manuscript Preparation			X	X	X
Aim 3 Research Activity	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Year 5**
Process Exosomes		X	X	X	X
Statistical Analyses			X	X	X
Manuscript Preparation			X	X	X

C.4.a. Health History Measures

In addition to the aforementioned health history information obtained as part of standard RMADC procedures, we will also collect study-specific health information variables as they relate to immune function. Specifically, we will ascertain information on: recent infections and illnesses; surgical procedures in the past year; and autoimmune conditions that are not detailed in the exclusion criteria (e.g. Crohn's; irritable bowel syndrome; arthritis).

C.5. Aim 1 Methods: Evaluate longitudinal associations between markers of peripheral inflammation, cognition, and brain structure in aMCI.

The objective of Aim 1 is to elucidate the temporal relationship between established and novel markers of peripheral inflammation, memory consolidation, and grey matter cortical thickness over two time points: baseline and one year later. This aim will provide critical information regarding how immune dysregulation relates to clinical progress of aMCI. Note that although the term 'inflammation' may encompass a range of beneficial and detrimental aspects of innate immune system function, inflammation will be operationalized in our study as an elevation in the levels of pro-inflammatory cytokines and chemokines associated with innate immune activation in plasma.

C.5.a. Assessment of Inflammatory Markers in Circulating Plasma (Peripheral Inflammation):

To limit the impact of acute illness on blood levels of inflammation, participants will be queried on the day prior to their laboratory assessment regarding recent illness (e.g. cold; flu within the past two weeks). Their blood draw will be rescheduled to another date if they report any recent illness, no later than four weeks after their assessment and neuroimaging scan. Fasting blood will be collected into EDTA tubes and left to clot at room temperature for at least 30 min, but no more than 60 min. The blood will then be centrifuged at 2,500 rpm (1,300-1,800 x g) at room temperature for 15 min. Plasma will be stored at -80°C until analysis. **To minimize the likelihood of methodological errors (e.g. batch effects), we will use Meso Quickplex SQ 120 V-plex plates** (Meso Scale Discovery, MSD; Rockville, MD) for analyses, which have been shown to have lot-to-lot consistency, for these analyses. For the chemokine/cytokine assays, each aliquot will be slowly thawed on ice before diluting with sample diluent and loaded directly onto the multiplex array in duplicate. Remaining samples will be run on standard human panels, including Proinflammatory and Chemokine V-PLEX plates, and read on a Meso Quickplex SQ 120. Notably, MSD Proinflammatory and Chemokine plates have the capacity to measure 10 different analytes each. Our primary analyses will focus on markers previously associated with pathological aging and AD (i.e. IL-6; TNF-alpha) as well as on recent, novel indices of hippocampal neurogenesis (i.e. MCP-1; Eotaxin-1); however, given the limited literature on the longitudinal progression of inflammation, we will examine the remaining pro- and anti-inflammatory analytes in exploratory analyses. Finally, we will also measure Beta-2 microglobulin and Alpha 1-antichymotrypsin (ACT) in all participants, as they are strong markers of AD pathology, pathological aging, and clinical outcomes (37, 38, 46, 47) . These markers will be measured using a human ELISA platform from R&D systems.

C.5.b. Cognitive Measures:

A comprehensive cognitive protocol will be administered to all participants, including measures of global cognitive functioning, episodic memory (verbal and visual), language, executive functions, and visuospatial ability, as part of the standard RMADC protocol; however, considering that our hypotheses relate to episodic memory function, we will primarily utilize a combination of standard and novel cognitive neuroscience indices of verbal/visual memory (see Table 3). Traditional measures of episodic memory are cognitively multifactorial; as such, an individual may do poorly on these measures due to cognitive difficulties unrelated to memory consolidation. A novel component of this proposal is our prospective collection of both established and novel measures of memory function that will facilitate more specific assessment of medial temporal lobe function. See Table 3 for cognitive outcome variables used in analyses.

Table 3 Cognitive Outcome Variables

Focus	Measures	Variables
Recall/Recognition	CVLT	20' Delay, Total Correct
		Recognition, D-Prime
	Benson Figure	15' Delay, Total Correct
Pattern Separation	MST	Proportion Correct
Long Term Consolidation	Story Recall	1-Week Delay, Total Correct

Standard Memory Measures: Standard measures of memory will include a list-learning measure (California Verbal Learning Test-II; CVLT) (48) and a figure recall (Benson Figure) (49) . Both measures have been validated in MCI for detecting memory recall deficits, and the Benson Figure is currently part of the National Alzheimer's Coordinating Center (NACC) neuropsychological protocol.

Pattern Separation: In order to establish a more sensitive measure of medial temporal lobe functions in aMCI, we will employ a computer-based measure of pattern separation that strongly correlates with hippocampal functioning, specifically the dentate and CA3 regions, and is less vulnerable to floor or ceiling effects than traditional memory measures (50-52) . This measure assesses the extent to which participants orthogonalize overlapping or similar stimuli to create discrete memory traces. We will utilize a task developed by Kirwan & Stark (50) called the Mnemonic Similarity Task (MST), which is freely available at: http://faculty.sites.uci.edu/starklab/mnemonic-similarity-task-mst/. In brief, this task entails administration of a recognition memory test that incorporates both a study period and a test period. Individuals are initially shown a series of pictures (study period) and are asked to make dichotomous judgments about the stimuli using a keyboard (i.e. "Are they outdoor or indoor items?"); after a brief delay, participants are shown another set of pictures, and they have to determine whether the stimulus is "old" (i.e. exactly like an object they saw before), "new" (i.e. a new object, not shown before), or "similar" (i.e. similar to an object they saw before, but not identical). The MST has been well validated for use in healthy aging as well as in individuals with MCI (50) .

Long Term Consolidation: We will also incorporate a measure of long-term forgetting, which entails learning to a set criterion and incorporates a one-week delayed recall. *The combination of these two features equilibrates*

the initial acquisition of verbal material, while also allowing for detection of memory consolidation deficits over a protracted delay period. The latter has been shown by us and by others to be particularly sensitive to early stages of memory dysfunction in aMCI (53). More specifically, this task involves reading a brief story over several trials, until the participant reaches the established criterion (90%; maximum of 10 trials for cessation of task). Participants are then asked to recall the story 30 min later and one week later.

C.5.c. MRI Acquisition and Processing:

In order to delineate the longitudinal relationship between inflammation and changes in brain structure, structural neuroimaging (i.e. to evaluate cortical thickness) will be conducted.

Structural MRI Acquisition Parameters: Whole brain MRI scans will be obtained on a 3.0 Tesla Siemens (Iselin, NJ) Skyra scanner equipped with a 20-channel head coil located at the CU Imaging Center, and acquired using an established CU magnetization prepared rapid gradient-echo sequence (MPRAGE; TR/TE/TI = 2300/2.45/900ms; matrix= 256x256; 176 slices, 1mm slice thickness) and T2 Flair.

Image Processing: The T1 MPRAGE structural MR images will be analyzed using the FreeSurfer 5.1 image analysis suite (54-56). FreeSurfer processing includes removal of non-brain tissue, segmentation of subcortical white matter, white matter lesions, and deep gray matter structures, tessellation of the gray/white matter boundary, and surface deformation following intensity gradients to place the gray/white and gray/CSF borders. Cortical thickness, calculated as the closest distance from the gray/white boundary to the gray/CSF boundary, is generated using intensity and continuity information from the 3D MR volume. Estimated total intracranial volume (ICV) is calculated via an atlas normalization procedure. The surfacing algorithm uses intensity and continuity data, and corrects topological defects to generate a continuous cortical ribbon that is used to calculate gray matter volume and thickness (57-59). This cortical surface is then inflated and registered to a spherical atlas and parcellated into regions of interest (ROIs) based on gyral and sulcal structure (60). With regard to longitudinal processing, to extract reliable thickness estimates, images will be automatically processed with the longitudinal stream in FreeSurfer (61). Specifically an unbiased within-subject template space and image is created using robust, inverse consistent registration (62). Several processing steps, such as skull stripping, Talairach transforms, and atlas registration are then initialized with common information from the within-subject template, significantly increasing reliability and statistical power (63).

Structural Imaging Region of Interest (ROI) Analysis: FreeSurfer 5.1 image analysis suite (64) and the Desikan atlas (60) will be used to analyze ROIs from the established cortical signature of AD (65, 66) , namely the medial temporal gyrus, inferior temporal gyrus, temporal pole, middle frontal gyrus, superior frontal gyrus, superior parietal lobule, supramarginal gyrus, and precuneus. For the purposes of data reduction, we will examine mean cortical thickness of all AD cortical signature regions.

C.5.d Aim 1 Expected Outcomes:

We will employ novel measures of both peripheral inflammation and cognition to test our hypotheses that higher baseline cytokine and chemokine levels in circulating plasma will predict declines in memory consolidation and decreases in AD-signature cortical thickness **(Hypothesis 1a)**, and that greater increases in cytokines and chemokines will be associated with greater declines in episodic memory and cortical thickness **(Hypothesis 1b)**. The rationale for this Aim is that while there is growing consensus that inflammation is related to cognition, the longitudinal relationship between circulating inflammation, episodic memory function, and the AD cortical signature remains unclear and relatively unstudied (1). Knowledge gained from this Aim will advance our understanding of the role of inflammation in cognitive decline, and inform our conceptualization of innate immune-mediated biological factors in the early stages of AD pathogenesis.

C.5.e Aim 1 Analytical Plan:

Reminder for Aim 1a: outcomes: decline in memory (change in 1-year) and change in cortical thickness (change over 1-year). covariates for Aim 1 – cytokines and chemokines (i.e. IL-6; TNF-alpha; MCP-1; Eotaxin-1; Beta-2 microglobulin; and ACT). I have to adjust for age and sex and anything else you tell me needs to be adjusted for. For example how will we handle the known associations between inflammation, cardiovascular risk, immunological health history conditions, and *APOE* genotype, correlations between the health measures (i.e. BMI, history of hypercholesterolemia, NSAID use; immune-related health conditions) (67, 68) and inflammation variables, as well as t-tests using *APOE* genotype will be conducted. Are these confounders???

C.5.f. Aim 1 Potential Barriers and Alternative Approaches:

An important consideration is that higher levels of inflammatory markers in circulating plasma may reflect acute illness. Although participants will be asked about recent illnesses, we will also closely examine all inflammatory markers to identify possible outliers in data that might reflect impending illness. Individuals who have spikes in multiple inflammatory markers greater than two standard deviations outside the sample mean will be flagged as a potential outlier and further investigated.

The potential noise in single cognitive outcome measures is a possible barrier to the analysis of cognitive data. In this case, we will evaluate whether data reductive techniques (e.g. principle component analysis) to create a single robust memory measure is warranted. In terms of barriers to analyzing the neuroimaging data, it is possible that changes in isolated regions or volumes will be more highly related to inflammatory markers than our a priori large ROI of cortical thickness. In this case, we will utilize whole-brain voxel-based morphometry (**VBM; family-wise error corrected**) methods to elucidate associations not fully captured in our hypotheses.

C.6. Aim 2 Methods: Examine how markers of peripheral inflammation impact the relationship between AD pathology and clinical progression of aMCI.

The objective of Aim 2 is to explore the relationship between inflammatory marker levels in circulating plasma, AD pathology, and cognitive decline. The rationale for this Aim is that recent studies have reported more pronounced levels of inflammatory mediators in early MCI compared to AD subjects and controls, suggesting that inflammatory processes may render their most injurious effects during this earlier stage; however, no studies to date have examined the association between inflammation and clinical outcomes in view of underlying AD pathology. As such, it is unclear whether inflammation is solely a function of the aging brain, or whether it relates to accumulating disease. **Given the fact that amyloid accumulation is presumed to occur decades before initial clinical symptoms** (42) **, it is likely that inflammatory processes reach their peak levels after this initial pathogenic development.** To assess how systemic inflammation synergizes with amyloid deposition to predict clinical outcomes in aMCI, we will assess blood inflammation (see C.5.a) and memory (see C.5.b) over two time points, and relate it to amyloid deposition using amyloid PET imaging at baseline.

C.6.a. Amyloid-PET Acquisition and Processing:

An integral component of this study is the baseline assessment of AD molecular markers (to provide a quantitative proxy for underlying amyloid deposition) in the context of longitudinal inflammation.

Florbetapir-PET Acquisition: PET will be performed on a Phillips Gemini TF PET/CT scanner operated by the CU Department of Radiology, which is located at Building 400, a 5-min walk from the CU CTRC. A 10 mCi (±10%) 18F-florbetapir injection will be followed by a 50 minute incorporation period and a 20 min acquisition period of 4 x 5-min frames. Image reconstruction, standardization and quality assurance procedures will adhere to ADNI protocols (http://adni.loni.ucla.edu/).

Florbetapir-PET Quality Control (QC): QC at the level of the scanner will be managed by the CU Department of Radiology using standard methods appropriate for this Phillips scanner. Further image quality assessment will be done after acquisition and reconstruction to include: (1) motion assessment through coregistration of frames and realignment; (2) total count and count rate assessment; (3) assessment of artifacts related to reconstruction or motion between emission and transmission/CT; and (4) assessment of field of view so that no part of the brain is cut off.

Florbetapir-PET Analysis: Individual subject florbetapir frames will be realigned and spatially warped to the subject's structural MRI in FreeSurfer (FS) space, as previously described (70) . A whole cerebellum reference region will be generated by FS subcortical parcellation. Vertex-wise standard uptake value ratios (SUVR) will be generated by dividing uptake in each vertex with mean uptake in the cerebellum reference region. Mean regional values will be extracted from the FS atlas gray matter regions in native space (60) . A global measure of cortical tracer uptake will be derived by calculating mean SUVR in typical amyloid accumulating regions, including lateral and medial frontal, anterior and posterior cingulate, lateral parietal, and lateral temporal FS regions. The FS-based approach yielded nearly identical values to those derived from a PET-only stream in the florbetapir pivotal clinical trial (71) in which *in vivo* PET scans were compared to amyloid burden at autopsy (72) . Global SUVR in that study was highly correlated with post-mortem Aβ burden assessed with immunohistochemistry (72) . The SUVR threshold of 1.10 had a sensitivity of 97% and a specificity of 100% for identifying individuals with clinically significant amyloid pathology (defined as moderate to frequent neuritic plaques by CERAD criteria) (73) . We will use global SUVR in our analyses, and will consider a dichotomous (amyloid 'positive' or 'negative') outcome as an alternative analytic strategy.

C.6.b. Aim 2 Expected Outcomes:

We will test our hypothesis of an interaction between peripheral levels of inflammation and amyloid deposition, such that the presence of both significant amyloid deposition and elevated peripheral inflammatory markers will be the **strongest** predictors of memory decline and decline in AD-signature cortical thickness over a one year period. This stems from our hypothesis that there is an exaggerated inflammatory response that contributes to AD pathological processes and is more pronounced and pervasive than in non-AD aging. Results gleaned from this Aim will provide new insights into the relationship(s) between inflammation and underlying amyloid pathology in predicting clinical progression.

C.6.c. Aim 2 Analytical Plan:

Reminder Aim 2a: outcome is amyloid deposition and cortical thickness and covariates are inflammatory markers. As in Aim 1 we need to control for age and sex. Also confounders??

Reminder Aim 2b: outcome is clinical progression variables (change in memory—see study design for measures), covariates are amyloid deposition (and cortical thickness) and inflammatory markers. I want to know if cytokines and chemokines modify the association between amyloid deposition (or cortical thickness) and clinical progression. Confounders?

C.6.d. Aim 2 Potential Barriers and Alternative Approaches:

A potential barrier to any study involving participants with aMCI is that they may ultimately not have elevated levels of amyloid deposition. We believe that using amyloid deposition as a primary variable rather than clinical diagnosis in this analysis represents a strength of the study and incorporates important natural variability into our statistical analyses. We will enrich the likelihood of amyloid positivity in aMCI patients by confirming that they are amnestic when they enter the study. In terms of alternative approaches, two will be considered with regard to amyloid imaging. 1) Although we have proposed using a continuous SUVR variable for amyloid PET, we will examine the utility of incorporating a dichotomous variable for amyloid positivity using recent cutoffs described in the literature (73). Given that a strong linear relationship between PET and outcomes has been debated, this alternative approach will allow us to account for a possible non-linear and indirect relationship between variables. 2) We have proposed using Florbetapir-PET for our analysis, given the use of this molecular marker at our center and its clinical availability; however, our radiologists have successfully made and used Pittsburgh Compound B (PiB) (see letters of support from Drs. Kwak and Miao; approved FDA-IND application by Dr. Bettcher), and we will consider this option during the initial stages of our study.

C.7. Aim 3 Methods: Delineate the role of CNS-derived exosomes as potential propagators of cognitive decline and structural brain changes in aMCI.

The objective of Aim 3 is to investigate the association between CNS-derived blood exosome markers of inflammation and circulating total exosome markers of inflammation to determine the role of central immune contributions to cognitive decline in aMCI. The rational for this Aim is that despite the robust association between inflammation and AD, the vast majority of studies have focused solely on peripheral markers of innate immune functioning, making it difficult to determine how well these inflammatory signatures reflect underlying CNS alterations. Exosomes are membrane-bound extracellular vesicles that carry cellular proteins to neighboring cells, and they are shed by both CNS and peripheral cells into the blood. Importantly, accumulating evidence suggests that CNS-derived blood exosomes may serve as potent vehicles for the transport of a wide range of proteins and immune markers, thereby potentially initiating or exacerbating pathogenic processes in nearby cells. Given that changes in the CNS cellular milieu directly impact the contents of CNS-derived exosomes in the blood, this population of exosomes provides a critical opportunity to understand how immune-associated CNS processes may underlie disease pathogenesis and clinical phenotypes in AD. Moreover, CNS-derived exosomes provide an opportunity to understand the relationship between CNS and 'systemic' inflammatory processes. We will examine whether levels of inflammatory markers from CNS-derived exosomes are higher in aMCI relative to HC. Moreover will elucidate whether inflammatory markers from CNS-derived exosomes (baseline and longitudinal) are stronger predictors of memory decline (C.5.b) and cortical thinning (see C.5.c) than total exosomes.

C.7.a. Assessment of Inflammatory Markers in CNS-Derived Blood Exosomes:

In order to evaluate a CNS-derived marker of inflammation, we will evaluate exosomes in plasma. Given that CNS-derived exosomes may offer a window into inflammatory processes that change *prior to* circulating levels

of inflammation, this is a novel and innovative aspect of the proposed study. To extract total exosomes from plasma, we will employ Qiagen's exoEasy Maxi Kits, due to increased efficiency and improved particle extraction reproducibility. To enrich for CNS-derived exosomes starting with total plasma exosomes, we will pre-incubate streptavidin-conjugated magnetic beads with biotinylated anti-human NRI-L1CAM (CD171) antibodies, which is a neuronal cell adhesion molecules, followed by incubation with an aliquot of total exosomes. Using an automated nanoparticle analyzer, the Malvern NanoSight NS300, nanoparticles such as exosomes can be characterized in solution, and by utilizing automated Nanoparticle Tracking Analysis (NTA), the nanoparticles can be characterized as ranging from 10–2,000 nm in diameter. NTA allows each particle to be individually, yet simultaneously, analyzed by direct observation and measurement of diffusion events using a particle-by-particle methodology that provides high-resolution results for both particle size distribution and concentration. To further immunopurify neuronal-derived nanoparticles from total plasma exosomes, after attaching the biotinylated antibodies to the magnetic streptavidin-conjugated beads, we will then block unbound streptavidin sites with biotin prior to incubation with the aliquot of total plasma exosomes. This additional blocking step differs from other published enrichment protocols. It has proven to be especially important for our work, since we have discovered that other nanoparticles can bind non-specifically to free streptavidin sites on the magnetic beads when blocking is not performed.

Although we will prioritize our analyses on total (i.e. CNS-derived and peripheral-derived exosomes) and neuronal (i.e. CNS-derived) exosomes, we will also use this more stringent immunopurification approach to isolate astrocyte-derived exosomes by using streptavidin-conjugated magnetic beads pre-incubated with biotinylated antibodies against the astrocyte-specific membrane protein GLAST (GLutamate ASpartate Transporter), with or without blocking of unoccupied streptavidin sites with biotin prior to incubation with the aliquot of total plasma exosomes.

Following immunopurification of exosomes, we will evaluate their respective population characteristics and analyze their protein cargo. We will lyse aliquots of total, neuronal, and astroglial exosomes and perform proteomic analyses using two different kits for the electrochemiluminescent Meso Scale Discovery (MSD) System (see C.4.b Methods above for MesoScale description and target inflammatory markers).

C.7.b. Aim 3 Expected Outcomes:

We will test our hypothesis that levels of cytokines and chemokines from CNS-derived exosomes will be higher in aMCI relative to HC subjects **(Hypothesis 3a)**, and we will clarify whether inflammation from CNS-derived exosomes mirrors protein levels of total derived exosomes. We will also test whether higher levels of inflammatory markers from CNS-derived exosomes and increases in these levels better predict memory decline and decreases in the AD-signature on neuroimaging **(Hypothesis 3b)** relative to total exosomes. Information obtained from these analyses will provide critical insights into the role of CNS immune dysregulation in predicting clinical changes in aMCI subjects.

C.7.c. Aim 3 Analytical Plan:

We will prioritize neuronal exosomes as our primary CNS-derived exosome marker; however, exploratory analyses will be conducted for each analysis below using astroglial-derived exosomes as a primary predictor. To elucidate the association between CNS-derived exosome inflammatory markers and diagnosis in **Hypothesis 3a**, we will conduct general linear models to determine whether CNS-derived exosome inflammatory markers differ between aMCI and HC subjects. We will then conduct regression analyses with CNS-derived exosome inflammatory markers as predictors and total exosome inflammatory markers as outcome variables, controlling for demographic nuisance variables; this will allow us to determine what proportion of the variance in total exosomes is explained by CNS-derived exosome cargo. Given the limited research on CNS-derived exosomes in HC or aMCI subjects, we will then include a term for diagnosis, allowing us to delineate differences in the magnitude of this relationship between HC and aMCI subjects. We will conduct additional linear regressions with CNS-derived exosome inflammatory markers as predictors, and baseline memory measures as outcome variables. We will repeat this analysis with change in memory measures and change in AD-signature cortical thickness to test **Hypothesis 3b.** To determine whether CNS-derived exosomes are better predictors of these clinical outcomes than total exosomes, we will compare the effect sizes between the two exosome populations. As noted in Aims 1 and 2, we will control for cardiovascular markers, immune health history variables, and *APOE* genotype in these analyses as appropriate.

C.7.d. Aim 3 Potential Barriers and Alternative Approaches:

Although we expect that aMCI subjects will show a stronger innate immune profile in CNS-derived exosomes than HC subjects, it is possible that other chemokines and/or anti-inflammatory markers will be more salient

predictors in aMCI subjects. Given the number of analytes available from the MSD plates, we will examine associations among analytes and determine whether a composite score of inflammation robustly differentiates between these two groups. In terms of additional alternative strategies, we have chosen to focus our analyses on CNS-derived exosomes and total exosomes in circulating blood; however, to further parcellate CNS- versus peripheral-derived exosome inflammatory markers, we will also consider enriching for peripheral-derived exosomes using a peripheral marker of monocyte lineage cells (i.e. CD45) and/or a marker of T-cells (i.e. CD3) during early stages of the study.

D. Power Analysis and Additional Statistical Considerations

Based on existing clinic flow and on enrollment at the RMADC, a reasonable final sample size is projected to be 125 aMCI and 50 HC subjects. We will enroll 137 aMCI and 55 HC to allow for a 10% attrition by one year follow-up. This is the sample size I am thinking of. Can you either justify this sample size. Note these two groups will be combined for analysis. I am recruiting from both populations so that I get a diverse representation of cytokine levels and outcome levels.

If you do your calculation and want me to recruit more people, just let me know.

Also, I know we have a lot of cytokine/chemokines so I am expected you to adjust for multiple comparisons some how. I just wanted you to know that I won't be surprises to see an alpha<0.05. Just tell me what you think we should do.

E. Data Sharing and Future Directions:

Our proposed study will contribute new prospective data to the existing RMADC data management program. This study will provide a critical platform for future investigations of CNS exosomal pathways as potential therapeutic targets, and will facilitate new studies of early innate immune alterations as potential biomarkers of MCI due to AD and, ultimately, of the pre-clinical stages of AD. Integral to scientific rigor and replication of findings, results from the proposed experiments will inform a larger new study to a) replicate and validate findings and b) build predictive models to develop cut points for clinical use.

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