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Analysis and interpretation of inflammatory fluid markers in Alzheimer's disease: a roadmap for standardization

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Abstract

Growing interest in the role of the immune response in Alzheimer's Disease and related dementias (ADRD) has led to widespread use of fluid inflammatory markers in research studies. To standardize the use and interpretation of inflammatory markers in AD research, we build upon prior guidelines to develop consensus statements and recommendations to advance application and interpretation of these markers. In this roadmap paper, we propose a glossary of terms related to the immune response in the context of biomarker discovery/validation, discuss current conceptualizations of inflammatory markers in research, and recommend best practices to address key knowledge gaps. We also provide consensus principles to summarize primary conceptual, methodological, and interpretative issues facing the field: (1) a single inflammatory marker is likely insufficient to describe an entire biological cascade, and multiple markers with similar or distinct functions should be simultaneously measured in a panel; (2) association studies in humans are insufficient to infer causal relationships or mechanisms; (3) neuroinflammation displays time-dependent and disease context-dependent patterns; (4) neuroinflammatory mechanisms should not be inferred based solely on blood inflammatory marker changes; and (5) standardized reporting of CSF inflammatory marker assay validation and performance will improve incorporation of inflammatory markers into the biological AD criteria.

Keywords Inflammation, Inflammatory markers, Alzheimer's disease, Biomarkers, Immune

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Introduction

Over the past decade, tremendous progress has been made in codifying the pathological changes and temporal sequencing of amyloid and tau deposition in Alzheimer's disease (AD), most recently resulting in the AT(N) research framework [1]. Reactive and degenerative glial changes have long been noted to accompany core neurodegenerative pathologies, and genome-wide association studies (GWAS) have associated multiple immune-related genes with AD. An immune response – which may reflect activation, dysregulation, or other processes – is now also considered a core component of AD risk/pathogenesis and has been incorporated into the AT(N) framework, denoted with an (I) for "inflammatory/immune mechanisms" [1]. This immune focus additionally touches on "brain maintenance" as operationally defined by NIH, with a large corpus of data demonstrating early alterations in immune cell function and inflammatory processes that change over the course of the disease. These findings present an opportunity to identify druggable targets in AD based on the expanding repertoire of FDA-approved immune-modulating therapies.

Growing interest in the role of the immune response in AD and related disorders (ADRD) has led to the prolific use of fluid inflammatory markers in research studies. Compared to the frequent usage of these markers as evidence for inflammation, discussion on correlation with pathologic (or at least independent) measures of neuroinflammation or measurement harmonization has been more limited. Biologically, the assumption that each soluble inflammatory marker only reflects one cell type, or one immune event is overly simplistic (see Fig. 1). This is compounded by increasing recognition that proteins with largely inflammatory roles in the blood can be secreted by neurons when found in the central nervous system, and neurons can further physiologically express receptors for these "inflammatory" mediators for presumably non-inflammatory effector functions. Moreover, endeavors to develop potential marker cut points or clear immune response staging have been largely unfruitful. In order to standardize the use and interpretation of inflammatory markers in AD research, we build on prior guidelines [2–4] to develop consensus statements and recommendations in this Roadmap paper to advance these markers' application and interpretation. This is not

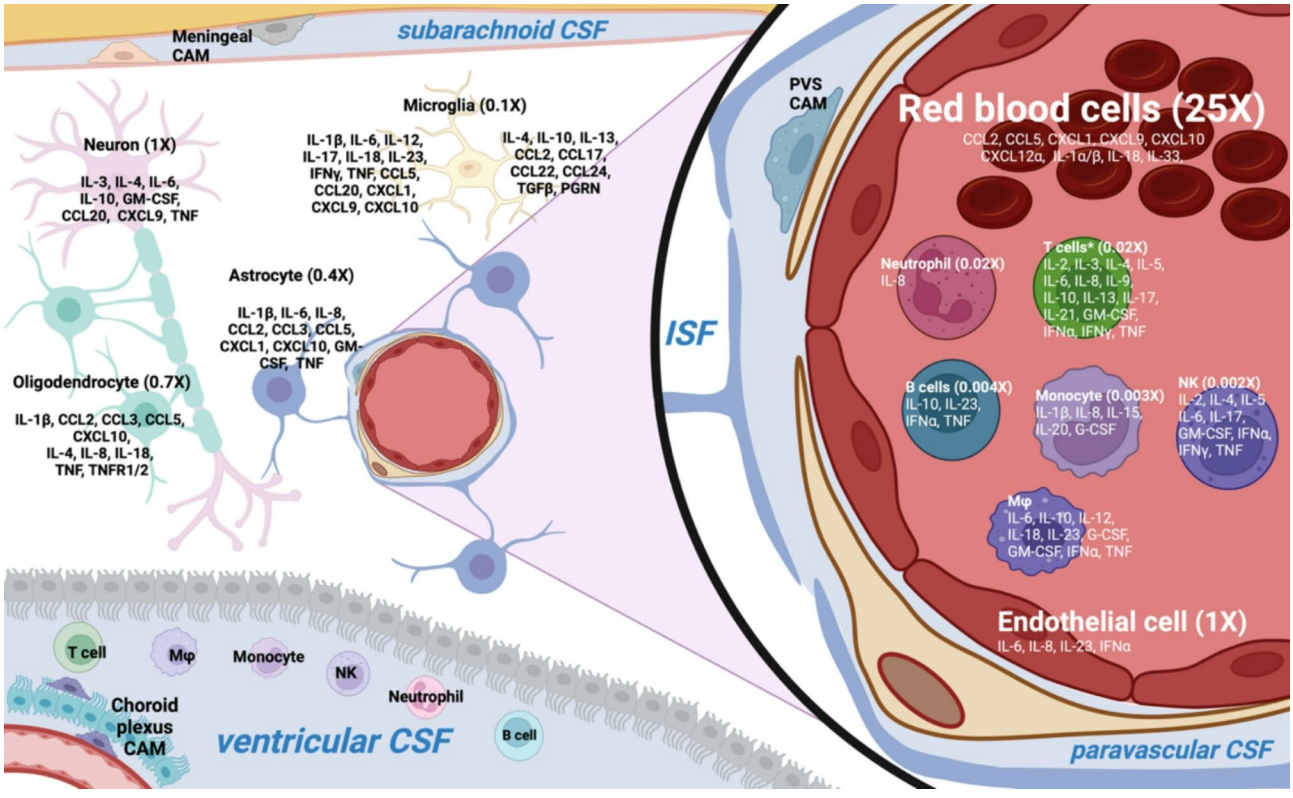

Fig. 1 Diversity of inflammatory markers and their cellular sources across central nervous system (CNS) compartments, including subarachnoid cerebrospinal fluid (CSF), ventricular CSF, interstitial fluid (ISF), and paravascular CSF. The schematic illustrates contributions of CNS-resident cells (neurons, astrocytes, oligodendrocytes, microglia) and peripheral immune cells (T cells, B cells, monocytes, natural killer cells, neutrophils, and red blood cells) to inflammatory responses, with currently known associated cytokine and chemokine ligands listed. This representation underscores the complexity of immune interactions in the CNS. Created in https://BioRender.com

intended to be a comprehensive review of the inflammatory marker literature or a summary of pre-analytical factors that impact marker measurement, both of which have been reviewed elsewhere [5, 6]. Instead, we clarify specific challenges that have impacted the field's progression, particularly within the context of CSF inflammatory markers, and provide recommendations for developing standardized inflammatory marker approaches.

A challenge across all scientific fields is the use of common, agreed upon nomenclature that denotes specific biological processes. The study of inflammatory markers is no exception, as each group has their own conventions and nomenclature. Considering that terminology used is varied and often not aligned with each other, we first propose a glossary of suggested terms (in English, see Table 1) that reflects our broader recommendations without being prescriptive. There are two common

Table 1 Glossary of frequently used terms and suggested consensus terms related to the immune response

Process or indicator	Frequently used terms in publications	Suggested consensus terms for human fluid marker studies	Suggested consensus term rationale/qualification
Astrocytic Changes	Reactive astrogliosis; astrocytosis; astrogliosis; activated astrocytes; astrocyte reactivity; degenerative astrocyte changes	Reactive Astrogliosis or Reactive Astrocytes	The term is descriptive without assigning function. Recommended based on prior nomenclature consensus papers.
Immune System Changes	Immune response; immune dysregulation; immune activation; immune processes; immune cascades; immune pathways	Immune Response	Immune response is a neutral term that does not assign functional mechanism. Referencing "dysregulation" suggests a negative valence. However, changes in immune response could be to establish homeostasis (i.e., neutral).
Neuroinflammation	neuroinflammation; neuroinflammatory; CNS inflammation ; inflammatory brain changes	Neuroinflammation or Neuroinflammatory	These terms should only be used when referencing inflammatory processes in the central compartment, regardless of source.
Biomarkers	biomarker; marker; candidate biomarker; potential biomarker	Marker under investigation: Candidate Fluid Biomarker or Potential Fluid Biomarker	A biomarker should be linked to an underlying biological process; a measure should not be advanced to a 'biomarker' without validation.
Immune System Markers	Immune AD biomarkers; inflammatory proteins; inflammatory markers	Inflammatory Marker	The term "marker" is neutral and does not assume final validation. Recommend use of "inflammatory" in isolation of AD to avoid conflating context with mechanism.
Pro-inflammatory and Anti-inflammatory Processes	pro-inflammatory; anti-inflammatory	Inflammatory	Given that several inflammatory markers can reflect pro- and anti-inflammatory processes, and given that we are not directly measuring the underlying mechanism of action, it is recommended to use the term "inflammatory" in human fluid marker studies.
Chronic vs. acute Inflammation	chronic inflammation; inflammatory insult; acute inflammation	Acute or Chronic Inflammatory Effect	Recommend caution in defining the temporal course of inflammation in human studies. Considering what is measurable, should focus on the temporal course of the effect (i.e., acute vs. chronic inflammatory effect).
Localized Inflammation	localized/local inflammation	N/A	Recommend avoiding the term. We cannot confidently state in human fluid studies that an inflammatory process is purely localized. Inflammation that is initially localized may still yield global effects.
Inflammatory markers measured outside CNS compartments, often in blood	systemic inflammation; peripheral inflammation	Blood-based Inflammatory markers	To avoid invoking mechanism or specific biological functions, recommend stating strictly the measurement source.
Blood Brain Barrier Changes	BBB dysfunction; BBB dysregulation; BBB disruption; BBB failure	BBB dysfunction	If invoking a negative change to the BBB, we favor the term "dysfunction" to connote a physiological impairment. In contrast, "disruption" could reflect an anatomical or physical change, which we currently cannot measure.

Legend:

Process or Indicator includes a description of immune processes that are frequently evaluated in research studies

Frequently Used Terms in Publications includes terms/descriptions commonly applied to these immune processes in published papers

Suggested Consensus Terms for Human Fluid Marker Studies include terms/descriptions proposed by the authors for use when addressing these immune responses in published papers. These terms are used throughout this Roadmap paper, and we acknowledge that they are often less specific than commonly used terms in publications

Suggested Consensus Term Rationale/Qualification includes rationale for why we have suggested these terms/descriptions

themes in our nomenclature recommendations. First, we follow principles laid out by prior consensus papers that terms intended to distinguish abnormal from normal immune functions (e.g., degenerative, dysregulation) in neurodegenerative disorders should be reserved for when immune cells themselves are diseased. Second, a "biomarker" should reflect an underlying biological process – normal or abnormal. While there is a trend, including in many of our own prior published works, to refer to "biomarkers" as proxies for an umbrella process, we recommend that indicators should not be labeled as "biomarkers" until they have sufficient biological validation. The purpose of this table is not to recommend elimination of a specific term, but to highlight that less specific terms are favored in human research when we cannot specifically identify or measure a marker's context-dependent function. While terms listed in the "suggested consensus terms" column of Table 1 align with prior nomenclature consensus papers [2, 3], we acknowledge that more specific terms may be or become appropriate in some contexts when there is additional research support. We use the suggested terms throughout the manuscript as a means of highlighting the importance of the two common nomenclature themes described above.

In addition, given on-going discussions of incorporating "inflammatory/immune mechanisms" into the AT(N) framework, it is also worth considering how the AT(N) framework may or may not translate to inflammatory markers. Table 2 provides an overview of biomarker applications, while highlighting conceptual and practical differences in how AT(N) [1, 7] and inflammatory markers [7] are assessed and interpreted. We will follow this brief overview with in-depth discussions related to current understanding of inflammatory markers and recommend best practices to address key knowledge gaps.

Use of a single biomarker to describe a generalized neuroinflammatory state

Consensus Statement: **A single inflammatory marker (e.g., IL-6) is likely insufficient to describe an entire biological cascade, and multiple markers with similar or distinct functions should be simultaneously measured in a panel.**

Recommendation: **Studies should assess multiple CSF inflammatory markers to capture the complexity of neuroinflammatory processes (see also** Sect. 6).

Proteins and other markers can have variable temporal profiles through aging and disease. It is critically important to differentiate candidate fluid biomarkers that capture a dynamic, self-regulating network from those that appraise discrete and broadly unidirectional (i.e., monophasic) indicators of pathological burden (e.g., the AT(N) framework) [8]. While post-mortem examinations support the gradual accumulation of inflammatory cells alongside amyloid and tau neuropathology, ante-mortem studies have failed to demonstrate marked concentration differences in soluble inflammatory markers between those with and without AD. This alone is not unusual nor unexpected, given the homeostatic nature of immune cells and their effectors. However, it is unlikely that an inflammatory protein or panel would supplant soluble amyloid and tau levels as measures of core AD pathologies [9, 10].

Beyond diagnostic prediction, there have been numerous studies in the AD field using single inflammatory markers to shed light on the role of inflammation in disease onset and clinical progression. In a somewhat circuitous manner, these approaches have been validated by their inclusion in meta-analyses of individual markers to support the role of the immune responses in AD pathological cascades [11]. Ascertainment of single inflammatory markers has the advantage of being more scalable and having more pragmatic clinical utility than large-scale inflammatory panels. However, a single marker, especially one chosen based on biological theories and assay availability, is insufficient for characterization of immune function or dysfunction. For example, cerebrospinal fluid levels of interleukin-10 (IL-10) are highly correlated with levels of interleukin-6 (IL-6), even though these two proteins have been described as having opposing functions [12, 13]. If one were to measure IL-10 alone, an elevated level could be interpreted as an "anti-inflammatory" state. Conversely, an elevated IL-6 level could be interpreted as a "pro-inflammatory" state when elevated IL-10 is not simultaneously considered. Taking a step back, there are many other parallels for this concern in neurology and medicine broadly. For example, a brief 'serial 7' working memory test should not reflect the entire construct of "cognition." Similarly, blood C-reactive protein (CRP) level is neither sensitive nor specific for measurement of cognitive decline [14, 15]. Thus, a single measure (e.g., IL-6, GFAP) is unlikely to adequately describe the complex immune biology of a disease state, even if it proves useful in the future for selecting and monitoring specific therapies targeting IL-6.

Another challenge in applying the one marker/one mechanism template to inflammatory marker studies is the use of a single threshold. Whereas CSF p-Tau_{181} and Ab_{42} levels undergo monophasic changes in the presymptomatic and symptomatic phases of AD, the same cannot be said for inflammatory proteins. For example, aging is a significant mediator for many CSF inflammatory markers, and prior studies have shown nonlinear or U-shaped trajectories for these markers in aging as well as asymptomatic AD [16]. This phenomenon is not unique even in AD, as levels of Ab_{42} also rise with age before falling with AD onset. This makes a single threshold-crossing impractical, especially when inflammatory

Table 2 Contexts of use for inflammatory markers in AD

Context	Possible use of Inflammatory Markers	Current Use of AT(*N*) Markers	Caveats When Incorporating Inflammatory Markers
Diagnosis and Disease Confirmation	• Might address incongruence between AT(N) biomarkers and clinical outcomes.	• AT(N) biomarkers provide biofluid correlates of accumulating neuropathology from none to severe. • These biomarkers can be staged with relative independence from each other. • Relative specificity/usefulness of a single biomarker is dependent on the disease-associated effect size (after accounting for inter-individual variability).	• Clearly defined, gold-standard neuroinflammatory outcomes for biomarker development are lacking • To date, inflammatory markers have restricted pathologic correlates, limiting opportunities for validation. • Non-degenerative causes can temporarily or chronically alter inflammatory marker levels or profiles, which impact single time point utility in diagnosis.
Risk stratification/ Endophenotyping	• Might provide better risk stratification of people with similar AT(N) profiles. • Might identify subgroups useful for clinical trial design.	• AT(N) biomarkers have limited role in risk stratification beyond distinction from people with similar cognitive status and AT(N) biomarkers.	• There is no consensus on the temporal sequencing of neuroinflammation relative to AT(N). Inflammation is tightly regulated, which makes disequilibrium between markers more difficult to detect than level changes. Surrogate markers of neuroinflammatory processes need to be empirically defined. • Examination of disease-associated inflammatory profiles in the context of inflammaging is needed for risk stratification (similar to MRI measures of atrophy).
Prognosis	• Might identify rapid vs. slow decliners.	• Use of AT(N) biomarkers for prognosis is based on distinction from those free of neuropathology.	• Inflammation is tightly regulated which makes disequilibrium between markers more difficult to detect than level changes. Surrogate markers of neuroinflammatory processes need to be empirically defined. • Markers useful for predicting cognitive and/or functional decline are likely to vary according to disease stage.
Disease monitoring	• Might be useful in monitoring rates of disease progression and response to emerging therapeutics.	• AT(N) biomarkers are useful for measuring target engagement. • They may also be useful as surrogate markers of downstream treatment effects. • However, small longitudinal changes in AT(N) biomarkers limit their role in natural history studies.	• Longitudinal disease-associated changes need to be distinguished from effects of aging/inflammaging. • This may be useful for assessment of predicted inflammatory activation (e.g., monoclonal antibodies).
Safety	• Useful for assessing and predicting possible adverse effects linked to therapies (for patients) or exposure to environmental agents (in the general population). • These markers may be important for the identification of individuals for whom specific therapies should not be administered.	• None currently.	• Empirical and potentially distinct biomarkers will be needed for Adverse Events and Serious Adverse Events.

markers more dynamically undergo level changes according to disease state (asymptomatic AD vs. AD dementia), disease-modifying therapies (e.g., anti-amyloid monoclonal antibodies), and non-CNS conditions/disorders (e.g., vaccination, infection) than core A/T/N markers. It is also overly reductionist to use one marker's concentration from a single time point to dichotomize complex immune responses across the entire longitudinal disease spectrum. In other words, interpretation of inflammatory markers will need to be contextualized alongside baseline physiologic, superimposed pathological (i.e., AT(N) stratification; clinical severity), and other background (e.g., sampling time relative to seasonal allergies and vaccinations) indicators.

In place of the single marker/threshold/mechanism approach, assessing network-level change(s) in sufficient numbers as well as types of immune cells and/or inflammatory markers in a fit-for-purpose manner has greater potential of providing the window into a changing immune ecosystem which intersects or interacts with AD [17, 18]. More concrete recommendations from this group related to general vs. specialized inflammatory marker characterization will be detailed later in the current manuscript. In the meantime, while basic and

translational researchers continue to refine conceptual models of brain-resident and circulating immune cells as well as their interaction with non-immune cells and extracellular pathologies, we recommend to avoid measuring or interpreting any single inflammatory marker in isolation as an overall index of AD immune biology.

Assigning origins or functions of inflammatory markers

***Consensus Statement*: Association studies in humans are insufficient to infer causal relationships or mechanisms.**

***Recommendation*: Biomarker constructs and the biological role of individual markers should not be overgeneralized in application, and supporting studies (longitudinal, cellular, animal) are necessary to advance beyond statistical associations.**

As the primary defense against an isolated external pathogen (e.g., influenza or vaccination), the immune system's subacute activity waxes and wanes. In measuring inflammatory markers, often this activity is summarized as juxtaposed pro- and anti-inflammatory events. However, in conditions where there is no overall resolution of microglial or astrocytic activation such as in human AD, whether a particular inflammatory marker/event is pro- or anti-inflammatory can quickly become a matter of debate. For example, in human studies of AD and in the context of broader clinical measures, elevated levels of so-called "pro-inflammatory" markers have been associated with negative prognostic outcomes, including greater brain atrophy [19] and cognitive deficits [20, 21]. Although few CSF inflammatory markers or receptor cognates (such as IL-6, TNF, and IL-1β) [22] are typically included in such studies, levels of these markers do typically rise with age [23] and their association with all-cause dementia risk appears to support their role in AD/ADRD pathogenesis [24]. At the same time, interpreting these types of results as *mechanistic* support for pro-inflammatory markers in deleterious clinical outcomes has limited foundation, ignores the role of immunity in regulating many necessary neural functions, and invokes biological processes that were not directly measured.

To expand upon this concern, AD researchers often select a few CSF markers to model immune cell activation or recruitment based on experimental data in blood or in vitro, even though inflammatory markers are often measured in these other contexts to corroborate – not deduce – cellular changes. This can quickly lead to erroneous biological interpretation. For example, higher CSF levels of TH1-promoting cytokines (e.g., IFN-γ and IL-12) can be paradoxically linked to slower cognitive decline in older adults [25] despite their direct association with greater age-associated inflammatory responses. Thus, an important caveat when interpreting CSF inflammatory markers in aging studies is that the (a) origins (e.g., release of other inflammatory markers; membrane and soluble receptor binding; relative states of secreting and effector cells) and (b) multiple biological roles of inflammatory markers should be considered.

We will illustrate the importance of these caveats in assessing microglial activity. Microglia in neurodegenerative disorders have numerous roles and can initiate phagocytosis or modify synaptic processes [26, 27]. Prior data have shown exposure to effectors like IL-4/IL-13 can transition microglia in vitro from a homeostatic to a cytotoxic phenotype [28, 29]. Activation of the complement pathway via C3 can also prime microglia toward a cytotoxic state [30]. In a neurodegenerative context, measuring C3 and other complement proteins could then improve the interpretation of changing IL-13 and microglial marker levels. Conversely, in a non-pathologic state, the same handful of markers – elicited by neuronal activities or aging – can have harmful, protective, reactive, or bystander roles on neuronal/synaptic function. Applying the conceptual framework underlying measuring CSF C3 and IL-13 from neurodegenerative to healthy or non-degenerative neurological contexts may therefore conflate in vitro observations with abnormal aging [31–33].

Complicating matters more is the historical over-simplification of microglial activities into dichotomous good vs. bad and protective vs. harmful [34]. The parallel between this and the over-simplification of chemokine/cytokine as pro- or anti-inflammatory certainly has not escaped us. Recent data using human stem cell-derived microglia xenotransplantation models suggest that microglia display a heterogeneous range of states, including those described as homeostatic, cytokine response-1 and −2, interferon response, disease-associated response, and antigen-presenting response [35]. These distinct microglial clusters were noted to have differential responses to both specific pathologies and AD risk genes. Moving forward, consideration of microglial subpopulations/states and genetic drivers of these states will be critical to understanding whether, when, and how microglia might impact AD across the pathological continuum.

Beyond the microenvironment and broader contexts, analytic approaches must also account for inflammatory markers' pleiotropic functions at *different concentrations*. In vitro, IL-1β and IL-6 can induce and maintain long term potentiation, neural plasticity, brain homeostasis, plaque clearance via activated microglia, and tissue repair, but these effects diminish at higher concentrations [36–40]. How these in vitro effects – where inflammatory markers are added or measured in isolation – translate into bulk soluble levels of the same markers far removed from the brain remains unclear. As such,

classic "pro-inflammatory" mediators should also be interpreted with caution due to their pleiotropic nature at concentrations which can be magnitudes apart in vitro and interstitially.

It is worth noting that parenchymal glia secretome is likely insufficiently and non-uniformly captured by bulk CSF assays. For example, one of us (WH) led a group of investigators to modestly extrapolate CSF IL-9 levels to brain parenchyma immunohistochemistry and gene expressions [41]; however, the CSF marker-neuropathology correlation cannot be assumed even for long measured CSF proteins like YKL-40, as an autopsy-based study failed to show a relationship between CSF and brain YKL-40 [42]. Development of a CSF inflammatory marker panel must thus proceed in parallel with neuropathological studies to better characterize the biological processes they represent in vivo.

In summary, pro-inflammatory markers are not inherently "bad" [43], just like anti-inflammatory markers are not necessarily "good". Due to the context-dependent functions of these markers (perivascular space vs. interstitial space; with vs. without AD pathology; free vs. bound to soluble decoy receptors) [44, 45], a network-based (or at a minimum, not multiple regression-based) approaches are necessary to advance neuroinflammatory analysis beyond cursory log2-fold comparisons.

Interpretation of neuroinflammatory CSF marker concentrations in aging and AD as dynamic processes

***Consensus Statement*: Neuroinflammation displays time-dependent and disease context-dependent patterns.**

***Recommendation*: Investigators should specify context of use, clarify timing in relation to disease state/staging and co-pathology, and incorporate independent measures of neuroinflammation when appropriate.**

Whereas the previous section distinguished between inflammatory proteins' theoretical and microenvironment-specific functions, the macro- or systemic environment also impacts interpretation of inflammatory marker levels. We have thus far proposed that inflammatory proteins should be sufficiently correlated with specific biological or pathological phenomena in the brain. One new challenge in interpreting their levels is that coupled or uncoupled changes in CSF inflammatory markers can be seen *across a range* of neurological disorders with unknown specificity. Significant gaps in our understanding of inflammatory processes in AD/ADRD are beginning to be filled by appropriate animal models and human cellular studies (e.g., FACS, scRNASeq), although it is important to highlight that historically, much of our knowledge regarding inflammatory processes has come from patients who die from discrete insults such as traumatic brain injury and strokes (see Table 3 for non-degenerative disorders with reported inflammatory marker changes, such as isolated seizures [46], epilepsy [47], systemic lupus erythematosus [48], normal pressure hydrocephalus [49, 50], and migraine [51–53]. Depending upon the neurological disorder, the immune response (and the interpretation of CSF markers of inflammation) may reflect highly specific biological processes or non-specific responses to injury [54].

As noted in the previous section, in AD, microglia can phagocytose and compact amyloid, with plaque-associated microglia demonstrating different phenotypes – including transcriptional profiles of secreted markers IL-6, TNF, IL-1a, CCL2, and CCL3 – from non-plaque associated microglia [55]. One might thus surmise greater time-dependent changes in these inflammatory proteins early during or before plaque deposition, which are further modified by specific *APOE* alleles [56] and other AD-related genetic variants [35]. In contrast, studies in Parkinson's disease have not shown similar transcriptional changes associated with microglial activation states [57, 58]. The number, activation status, location, and surrounding neuropathology of microglia thus all influence measured inflammatory protein levels with different biological implications.

Another good example is Triggering receptor expressed on myeloid cells 2 (TREM2). TREM2 pathogenic variants are risk factors for AD, yet – like TNF – TREM2 exists in a functional transmembrane form and the measured cleaved form (sTREM2). While function of the measured sTREM2 remains unclear [59], its levels appear to change in a non-monotonic manner across AT(N) pathological staging [18, 60], with these nonlinear changes across time evident long before AD symptom onset [61, 62].

As has now been well established, neuroinflammatory processes may also *precede* AD pathology. So called "inflammaging" [63, 64] is a well-known process in aging and age-related brain diseases that is tied to increased innate immune activation coupled with immunosenescence. For example, activity of the NLR family pyrin domain containing 3 (NLRP3) inflammasome in the periphery has been tied systemically to type 2 diabetes and in the brain to tau accumulation that often precedes AD [65, 66]. Inactivation of NLRP3 in mice reduces age-associated innate immune activity while lowering hyperglycemia and motor deficits [67]. Like many inflammatory processes, inflammaging is associated with increased expression of classically inflammation-related cytokines in mouse brains [68]. In other instances, the senescence-associated secretory phenotype (SASP) can activate and recruit immune cells to drive inflammation via the cardinal proinflammatory cytokines IL-6, IL-1β, and TNF [69, 70].

Table 3 Examples of non-neurodegenerative neurological disorders with localized inflammatory response

Disorder	Time-dependent neuroinflammation	Region-dependent neuroinflammation	Disease context-dependent neuroinflammation
Isolated seizures [46]	Inflammatory response is usually short-lived.	Increased IL-1β, IL-6, and TNF in brain regions where epileptogenesis and signal spreading occur.	Neuroinflammation can be due to infection, vascular causes (such as ischemia or vasculitis), neoplastic disease, trauma, and severe neurodegeneration.
Epilepsy [47]	Neuroinflammation can differ between ictal and non-ictal status.	Changes in catecholaminergic and serotonergic activity can occur in temporal lobe epilepsy, when increased monoamines and monoamine metabolites in spiking temporal cortex and cerebrospinal fluid may be found.	Epilepsy can be a complication of severe dementia, with traditionally established markers according to the dementia syndrome.
Systemic lupus erythematosus [48]	Neuroinflammation is more intense during neurologic episodes.	Neurological manifestations can be a result of vasculitis or regional inflammation of the brain.	Central nervous system involvement is mostly characterized by seizures, psychosis and movement disorders, with low C-reactive protein but high erythrocyte sedimentation rate in less than half of patients, and abnormal cerebrospinal fluid findings (elevated proteins, lymphocytic pleocytosis, oligoclonal bands) associated with poor prognosis.
Normal pressure hydrocephalus [49, 50]	Neuroinflammation is usually more intense when there is more interstitial oedema.	Mechanisms are common regardless of the etiology, with initially raised cerebrospinal fluid pressure followed by ventricular enlargement and decreased absorption of cerebrospinal fluid at the transcapillary or transvenular level, with interstitial oedema and ischemic damage of the white matter along with further normalization of cerebrospinal fluid pressure.	Normal pressure hydrocephalus can result from trauma, intracranial hemorrhage, meningitis (infectious or not), venous sinus thrombosis, or vasculitis. There has been evidence of a link between normal pressure hydrocephalus and systemic lupus erythematosus, in which the insidious inflammatory process that develops in the meningeal tissues (with deposition of IgG, IgA, IgM, C3 and C1q on the dural vessels) or the vasculitis itself may cause the volume of cerebrospinal fluid to increase. It is also known that patients with normal pressure hydrocephalus with moderate to severe AD burden are significantly less likely to respond to shunting.
Migraine [51, 52]	Neuroinflammation differs in the prodromal phase, during the migraine attack, and in the postdrome phase.	Immune response and inflammatory signaling pathways are triggered at the focus that affects cortical function, and contribute to progression of migraine.	No association with white blood cell-based inflammation markers, though genome-wide analyses of blood gene expression have shown significant associations with immune response and inflammatory signaling pathways.

In addition to considering time-dependent contexts with AD staging, interpretation of CSF inflammatory markers is also affected by the presence of co-pathology. Co-pathology is commonly noted with advancing age [71], even in the absence of frank cognitive impairment [72], with individuals in their 80s and 90s typically showing evidence of multiple pathological processes [73]. Although there has been some evidence to suggest that different neurodegenerative diseases may show distinct CSF inflammatory signatures (e.g., bvFTD vs. AD [74]), disentangling the relative contribution of each pathology to CSF inflammation continues to be a challenge given the lack of in vivo biomarkers for many neuropathological processes. Although outside the scope of this Roadmap paper, many other chronic health factors may also exacerbate inflammatory pathways and result in changes in CSF inflammatory markers.

Collectively, interpretation of CSF inflammatory markers is affected by the timing of disease, the presence of co-pathology, and the presence of co-morbid health conditions. As a result, it can be challenging to link inflammatory markers with specific biological or pathological phenomena if the broader context is not fully considered. It is important to contextualize CSF inflammatory marker results in relation to these factors and understand that these markers may be influenced by different physiological and pathological variables.

Relationship between blood-based and CSF inflammatory markers

***Consensus Statement*: Neuroinflammatory Mechanisms Should Not Be Inferred Based Solely on Blood Inflammatory Marker Changes.**

***Recommendation*: Blood inflammatory markers provide important insights into peripheral immune system and are associated with AD-related pathological and clinical outcomes; however, in isolation, they should not be interpreted as proxies for*neuroinflammation*.**

The contribution of neuroinflammation, as measured by CNS inflammatory markers, to neurodegenerative diseases is complex and dependent on multiple factors. Additional challenges to the interpretation of fluid markers have been introduced with blood inflammatory markers, as neuroinflammatory mechanisms have frequently been attributed to results from blood-based assays.

As context, several important key studies may be conducted with blood inflammatory markers in the context

of AD, including (but not limited to) studies examining contributions of non-CNS systems to disease pathogenesis and progression, studies aimed at understanding crosstalk between peripheral and CNS compartments, and studies designed to identify therapeutic targets in the periphery. While there is clear rationale and purpose for evaluating blood-based inflammatory markers in the context of AD, it is important to avoid misinterpreting these markers as definitive indicators of neuroinflammatory mechanisms without clear evidence of CNS immune involvement. Such misinterpretation overlooks the contribution of other health conditions and peripheral processes. For example, many blood-based cytokines and interleukins reflect general or non-specific inflammation processes [75] and are produced by different tissues/organs in the periphery. Isolating the source of a blood-based inflammatory marker is thus difficult in human studies.

Moreover, although blood inflammatory markers are associated with aging and AD-related outcomes, their associations with central (e.g., CSF) inflammatory markers using paired blood-CSF samples are often poor (although higher correlations are seen with advancing age) [76]. This does not negate their potential role in AD, but it does suggest that blood inflammatory markers are insufficient proxies for neuroinflammation when interpreted in isolation.

A challenge to this assertion, however, has come with blood GFAP. Blood GFAP is a commonly used proxy marker of reactive astrogliosis in the AD field and was recently codified in the AD diagnostic criteria as the primary indicator for "I" (inflammation/immune mechanisms). Although increases in GFAP are seen in a range of conditions, including head injury and other neurological diseases, blood GFAP shows greater increases in AD compared to non-AD neurodegenerative syndromes [77]. GFAP is also less affected by age than neurofilament light chain protein (NfL), and is linked with longitudinal cognitive decline in asymptomatic and symptomatic AD [78]. The association between GFAP and AD pathology is also stronger for blood than CSF, which is unusual for a putative CNS marker and raises questions regarding the underlying biology and mechanisms by which this marker is released into the blood stream. Although several biological explanations have been offered, it has also been suggested that differences in GFAP stability between blood and CSF could be one of the factors contributing to this discrepancy [79]. Salient to our prior comments regarding the need for CNS immune validation, recent data do suggest a correlation between blood GFAP and astrocyte reactivity in post-mortem tissue [80]. In parallel, astrocytes in some brain regions, such as the hippocampus, have demonstrated higher GFAP content than other regions [81]. Dissecting the potential mechanisms by which GFAP might enter the blood stream at higher concentrations than in CSF are outside the scope of the paper but do warrant further consideration regarding interpretation.

First, it is possible that changes in blood GFAP reflect processes other than reactive astrogliosis (including other astrocyte-related processes). Peripheral contributions to circulating blood GFAP levels have not been adequately considered when interpreting this marker. In addition, external factors, such as glucocorticoid drugs and physical activity, can also elevate GFAP levels [82, 83]. Whenever a blood inflammatory protein increases, researchers should always raise the possibility of concomitant reasons for this - such as tissue damage, inflammation, and external stimuli altogether. Thus, while (a) increases in blood GFAP are likely to be maladaptive and (b) decreases in GFAP in response to an intervention might be beneficial, more research is needed to establish the extent to which these changes primarily reflect reactive astrogliosis, the mechanisms of crosstalk [84], and whether changes in blood GFAP are predictive of disease progression or remission. Collectively, several of the authors on this consensus paper have published research on the role of GFAP as a promising marker for AD-related outcomes; nonetheless, we feel that it is premature to consider GFAP a definitive indicator of *neuroinflammation*.

Another blood marker that is typically associated with neuroinflammation is soluble Triggering receptor expressed on myeloid cells 2 (sTREM2). Blood levels of sTREM2 have changed over many physiological processes. However, CSF and blood levels of sTREM2 in AD individuals have demonstrated contradictory findings [85, 86]. sTREM2 is also released by peripheral cells like leukocytes, so its use as an index of microglia activation should be cautiously interpreted. A recent preclinical study has suggested that TREM2-activating antibodies may enhance microglial function, potentially triggering a microglia-responsive state [87]. It is noteworthy that this study used both in vivo and in vitro models to measure cytokines and different microglial states to track the pathological process of interest.

Like concerns noted in section two, it is unlikely that a single blood marker will reflect an entire biological system. Taking a broader lens, we recommend caution in conflating blood inflammatory markers with CNS inflammatory markers or neuroinflammation, even in situations where blood inflammatory markers robustly predict AD pathology or clinical outcomes. For an in-depth prospectus of how to advance our understanding of the central-peripheral immune and inflammation crosstalk in AD and ADRDs the reader is referred to a prior roadmap paper [84].

Challenges in *harmonizing inflammatory markers in AD/ADRD*

***Consensus Statement*: Standard reporting of CSF inflammatory marker assay validation and performance will improve incorporation of inflammatory markers into AT(N) scheme.**

***Recommendation*: A minimum set of CSF inflammatory markers should be measured by established laboratories for accuracy and precision using assays from manufacturers committed to transparency and continuous improvement. Preanalytical factors and assay performance should be routinely assessed and reported.**

A necessary aspect of candidate biomarker discovery is its eventual application in pre-clinical and clinical settings, which often represents more of an engineering than a biological problem. For example, CSF AD biomarkers over the past 30 years were only empirically testable when there was the complete or near-complete automation of their measurements. While there are few established CSF AD biomarkers, there are hundreds for plasma or CSF inflammatory proteins. Because of these inflammatory proteins' abundance in blood circulation, most have been extensively interrogated in non-neurological disorders. In isolation or in combination, these markers may reveal latent disease mechanisms and translate themselves into clinical diagnostics. Unfortunately, there are limited attempts at replication or head-to-head comparisons in either compartment (blood; CSF). The goal should be to encourage data democratization and open science by independently replicating markers of interest. There is a need for standardization and quality control of pre-analytical and other measurement factors, and indeed there have been considerable efforts to develop certified reference methods and materials, along with published recommendations regarding known pre-analytical factors that impact AT(N) biomarkers [5, 6]; however, it is unlikely that we will be able to 'copy-paste' and apply the standard AT(N) biomarker approaches for quality control to inflammatory markers for the reasons previously discussed. Reasons for this include the many folds greater and non-redundant effector proteins, greater number of assay and synthetic standard manufacturers, and lagging efforts to determine effects from pre-analytical variables.

What would this initiative entail? Reconciling concerns raised above across neurological disciplines will require large-scale efforts involving researchers, vendors, and funders in AD/ADRD, neuroinflammation, neuroinfectious diseases, and immunology. We first propose a *pre-regulatory* expert panel to objectively evaluate assay rigor, specificity, reproducibility, and validity for individual inflammatory markers and, more likely, targeted proteomic panels. Because of expected proprietary technology involved, this must be accompanied by a public-private partnership with sufficient flexibility between trade secrecy and data transparency. A prescriptive model outlining sample size, concentration range, and technical caveats will also likely be outpaced by emerging experimental design and assay techniques. Thus, a set of guiding principles on minimum and recommended thresholds for validation may be more appropriate to couple technical novelty with scientific rigor. For example, accuracy/precision thresholds for a new CSF cytokine assay panel could include:

- Minimum Threshold: manufacturer-reported spike-recovery in the intended biological matrix (in this case, CSF), measured at two distinct time points;
- Recommended Threshold: blinded assay of 15–30 samples (intended matrix) for correlation with results generated from previously validated assays requiring greater matrix volume, assay time, or operator experience;
- High Threshold: two experienced laboratories having participated in research activities described in Minimum and Recommended thresholds, or having completed independent testing of assay accuracy and precision.

To be successful, this initiative will need the appropriate incentive structure involving assay manufacturers, investigators, funders, and publishers. With appropriate support from funders in the likeness of ADNI or MODEL-AD, it will require little guesswork to identify a core group of vendors and scientists for whom this initiative is long overdue. Vendors whose assays achieve the Minimum Threshold will be able to distinguish themselves from their peers who have only word-of-mouth reviews, and only assays with Recommended or High Thresholds should be considered in research consortiums.

We further recommend a set of CSF markers (Table 4) to be prioritized for harmonization across large national studies (e.g., ADNI) or regional studies. These include proteins which are commonly postulated to reflect microglial activation, reactive astrocytes, synaptic pruning, neuroprotection, among other biological processes. In keeping with earlier discussions on the dynamic and regulated nature of neuroinflammation, we propose the inclusion of CSF markers which model feedforward and feedback loops (e.g., TNF, sTNFR1, sTNFR2; IL-1b and sIL-1ra), CSF markers sharing cell surface receptors (IL-4, IL-13, and YKL-40; TGFb1, b2, and b3), and CSF markers which form parts of a shared pathway (C3, C5, C4, C1q).

In this list, we distinguish between soluble signaling proteins with presumed biological activity, soluble

Table 4 Recommend set of CSF markers to be prioritized for harmonization across large studies

CSF Marker	CNS sources	CNS cells with corresponding receptors[c]
TNF (TNFSF2)[a, b]	Microglia, astrocytes, neurons [88]	Myeloid cells, all glia, neurons, [89] endothelial cells, area postrema, circumventricular organs
sTNFR1 (TNFRSF1A)[b]	Ubiquitous	Same
sTNFR2 (TNFRSF1B)[b]	Myeloid cells, B/T cells, neurons, [89] oligodendrocytes [90], endothelial cells	Same
sTREM2[b]	Microglia & macrophages, monocyte-derived dendritic cells	same
IFN-g (IFNG)[a]	Th1 [91] and other immune cells, microglia, endothelial cells, astrocytes [92]	Microglia, neurons [93]
sIL-1ra (IL1RN)[a, b]	Microglia [94]	Endothelial cells, neurons [95]
IL-1b[a]	Microglia, [96] astrocytes, oligodendrocytes, neurons [97]	Endothelial cells, neurons [95]
IL-6[a]	Microglia, astrocytes, neurons [98]	Astrocytes, microglia, neurons [99]
IL-8 (CXCL8)[a]	Microglia, possibly neurons [100]	Microglia, astrocytes, neurons [101]
IL-9[a]	Th9 and other immune cells, oligodendrocytes, neurons	Astrocytes, OPCs, oligodendro- cytes, microglia, neurons [102]
IL-10[a]	Multiple immune cells, activated microglia, reactive astrocytes	Microglia, astrocytes, oligodendrocytes, neurons
IL-12p40/p70[a]	Dendritic cells, lymphocytes, microglia	Microglia, neurons [103]
IL-4[a]	Mast cells, microglia, Th2, neurons [104]	Endothelial cells, lymphocytes, all glia [105], neurons [106]
IL-13[a]	Th2 cells and other immune cells	Lymphocytes, neurons (IL-13Ra1) [107]
YKL-40	Astrocytes, some in microglia, vascular smooth muscle cell, macrophage	Macrophages, dendritic cells (IL-13Ra2)
TGFb1/2/3[a]	Microglia, astrocyte, neurons (TGFb2/3) [108]	Astrocytes, microglia, endothelial cells, neurons (TGFbR3) [109]
IL-17A[a]	Astrocytes, microglia, other immune cells, endothelial cells	Endothelial cells, astrocytes, microglia, neurons [110]
IL-18[a]	Microglia, dendritic cells, and other immune cells	Neurons [111]
IP-10 (CXCL10)[a]	Monocytes, endothelial cells	Lymphocytes, microglia, neurons [112]
MCP1 (CCL2)[a]	Macrophages, microglia, endothelial cells	Astrocytes, microglia, endothelial cells, neurons [113]
MCP4 (CCL13)[a]		
RANTES (CCL5)[a]	Microglia, astrocytes	Glial cells, neurons [114]
Eotaxin 2 (CCL24)	Microglia	Astroyctes, microglia
Eotaxin 3 (CCL26)	Endothelial cells	
GM-CSF (CSF2)	Microglia, astrocytes, neurons	Microglia, astrocytes
EGF[a]	Macrophages	Neural stem cells, [115] neurons,[116] astrocytes, microglia
C3,[a] C5[a], C4, C1q[a]	Astrocytes, microglia, neurons [117]	Microglia, astrocytes, neurons [118–120]

[a] Soluble proteins with neuronal receptors

[b] Soluble versions of cell surface proteins/receptors

[c] For sTNFR1, sTNFR2, and sTREM2, cells which express membrane-anchored forms of the same receptor

(cleaved) cell surface proteins whose function may differ or even oppose membrane-anchored versions of the same proteins, and proteins demonstrated to be secreted by inflammatory as well as non-inflammatory cells. While information included in the table is not meant to represent an exhaustive review of published evidence, we do highlight that most of these CSF proteins and their receptors have been found to be expressed by neurons especially in neurological disorders. Thus, concentration changes in these CSF proteins in the setting of neurodegeneration (including most if not all ADRD) or neuronal distress should be interpreted as evidence for neuroinflammation with caution as these markers are positioned at the intersection between inflammation and neuronal signaling.

While we acknowledge the usual logistic challenges in harmonizing these CSF measurements related to time, cost, and reproducibility, the availability of reliable targeted proteomic platforms with large numbers of targets at relatively low cost per sample (compared to traditional immunoassays) is creating ample opportunities for collaboration. Compared to similar experiments conducted across multiple experienced centers before 2010, there is an approximately 100-fold reduction in relative cost – ten times more CSF markers measured at one-tenth the cost. Success in advancing the application of CSF inflammatory markers towards true biomarker status will also hinge on funders supporting these endeavors. A coordinated effort across international researchers, high throughput proteomics service providers, and funders

may thus accomplish for neuroinflammatory therapies what core AD biomarkers in ADNI have for anti-amyloid therapies.

Conclusion

Increased use of inflammatory markers in AD research has shed light on several challenges in the field, particularly the standardization of use and interpretation of these markers in studies. In this Roadmap, we have identified core principles and suggested a glossary of terms related to inflammation and the immune response. We have further identified standardization concerns and offered both consensus statements and recommendations for how to address these gaps. We acknowledge that this is an evolving field, and thus proposed terminology, consensus statements, and recommendations will need to be updated based on new empirical work to maintain best practices over time.

Abbreviations

CSF	Cerebrospinal fluid
CAM	Central nervous system-Associated Macrophages
Mφ	Macrophage, CCL and CXCL = families of chemokine ligands
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
IFNγ or IFNα	Interferon gamma or alpha
IL	Interleukins
ISF	Interstitial Fluid
NK	Natural Killer cells
PGRN	Progranulin
TNF	Tumor Necrosis Factor
TNFR1/2	Tumor Necrosis Factor Receptor 1 or 2
TGFβ	Transforming Growth Factor beta

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Author contributions

This is a review and roadmap paper in which all authors made substantial contributions to the conception and design of the work; drafted the work and revised it; and have approved the submitted version. BMB and WTH oversaw and finalized the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

Not applicable.

Consent for publication

Not applicable.

Competing interests

MGT is co-inventor of Xencor's XPro1595, a soluble TNF-specific biologic under clinical development by INmune Bio for neurological indications. MGT is a consultant/advisor INmune Bio, Merck, Cellestial Therapeutics, Forward Therapeutics, Jaya, NysnoBio Longevity Biotech, Longeveron, iMetabolic Pharm, NovoNordisk, IMMvention, Ventus. MGT has served as an advisor/member on the Medical and Scientific Advisory Group of the Alzheimer's Association, Weston Family Foundation of Canada, Parkinson's Foundation, Parkinson's UK, World Parkinson Coalition, MJ Fox Foundation for Parkinson's Research, and the Alzheimer's Disease Cooperative Study Intervention Selection Committee (ADCS-ISC), and the International Linked Clinical Trials (iLCT) of the Cure Parkinson's Trust. MGT has served on grant review panels for NIH, MF Fox Foundation, Weston Family Foundation, Alzheimer's Association, Bright Focus Foundation, Alzheimer's Drug Discovery Foundation (ADDF), Parkinson's UK. MGT has served as Editor-in-Chief for Nature Partner Journal Parkinson's Disease, Associate Editor for Science Advances, Alzheimer's & Dementia: Translational Research and Clinical Interventions (AE), and Journal of Neuroinflammation. WTH has received research support from NIH, Robert Wood Johnson Foundation, Fujirebio Diagnostics Inc, Atlanta Family Foundation, and TMCity Foundation; has consulted for Apellis Pharmaceuticals, Beckman Coulter Diagnostics, Biogen, Fujirebio Diagnostics Inc; has patents on CSF-based diagnosis of FTLD-TDP, prognosis of MCI due to AD, and prognosis of spinal muscular atrophy on gene therapy; and has copyright on Mandarin-based cognitive assessments (licensed to Linus Health).

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