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Review

Neuroinflammation Biomarkers in the AT(N) Framework Across the Alzheimer's Disease Continuum

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Abstract

In the past years, neuroinflammation has been widely investigated in Alzheimer's disease (AD). Evidence from animal, in vivo and post-mortem studies has shown that inflammatory changes are a common feature of the disease, apparently happening in response to amyloid-beta and tau accumulation. Progress in imaging and fluid biomarkers now allows for identifying surrogate markers of neuroinflammation in living individuals, which may offer unprecedented opportunities to better understand AD pathogenesis and progression. In this context, inflammatory mediators and glial proteins (mainly derived from microglial cells and astrocytes) seem to be the most promising biomarkers. Here, we discuss the biological basis of neuroinflammation in AD, revise the proposed neuroinflammation biomarkers, describe what we have learned from anti-inflammatory drug trials, and critically discuss the potential addition of these biomarkers in the AT(N) framework.

Keywords: *Alzheimer's disease, astrocyte, biomarker, microglia, neuroinflammation.*

Introduction

Since its first description in 1906, Alzheimer's disease (AD) has been consistently associated with amyloid-beta (Aβ) plaques and tau neurofibrillary tangles. The pathological potential of glial cells has also been described, but less mentioned, by Alois Alzheimer's original reports (1). Neuroinflammation was later associated with the disease's pathological process, likely playing a fundamental role in potentiating Aβ and tau pathologies in the brain (2).

In the last years, AD diagnosis shifted from a clinical construct to a biological definition, allowing for identifying pre-symptomatic AD stages – the so-called preclinical AD. The National Institute on Aging – Alzheimer's Associacion (NIA-AA) 2018 proposed a research framework based on biomarkers, independently from clinical presentations - the AT(N) system (3). It essentially defines AD according to the biomarker positivity for Aβ (A), Tau (T), and neurodegeneration (N). Moreover, this dynamic system may incorporate novel candidate biomarkers to provide information about additional pathophysiological mechanisms represented by other letters. Hampel and colleagues propose using the letter "X" to represent an additional group of promising pathological markers, with neuroinflammation being of high interest (4).

Neuroinflammation in AD is typically associated with glial changes in the brain. This complex process includes microglial cells and astrocytes associated with a cascade of inflammatory mediators and modulators (2). A growing body of evidence suggests that the inflammatory process may occur in the early stages of AD, potentiating the accumulation of insoluble Aβ and tau (5). It is thought that inflammatory changes are a response to Aβ and tau pathologies, but one cannot rule out that they are triggering the deposition of Aβ and tau in the brain (6-8). Also, early inflammation might protect against protein accumulation, which could explain why some studies have shown conflicting results (9, 10). Thus, biomarkers of neuroinflammation may become useful for early diagnosis, prognosis, and potential drug-target engagement in the secondary prevention of AD.

In this review, we explore the biological basis of neuroinflammation in AD, especially the roles of microglial cells and astrocytes, and provide detailed information on current biomarkers of neuroinflammatory changes. We also describe advances and contributions of anti-inflammatory drug trials and discuss the potential of adding biomarkers of neuroinflammation in the AT(N) framework.

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The biology of neuroinflammation

To understand the biology of neuroinflammation, we first need to discuss the basic concepts of inflammation. According to the Nature Portfolio, inflammation is "a biological response to harmful stimuli, such as pathogens, damaged cells or irradiation. It is a protective attempt by the organism to remove injurious stimuli and to initiate the healing process. It is characterized by pain, redness, heat, swelling and disturbance of function" (11). These cardinal signs of inflammation were described in the first century by the Roman encyclopedist Aulus Celsus. The absence of signs of inflammation in diseases of the central nervous system (CNS) was historically behind the dogma of the brain as an "immune-privileged" organ (12, 13). This idea changed in the last decades when histopathological studies identified neuroinflammatory changes in the brain. Currently, neuroinflammation refers mainly to the dynamic responses of microglia and astrocytes and has been associated with neurodegenerative disorders such as AD (14).

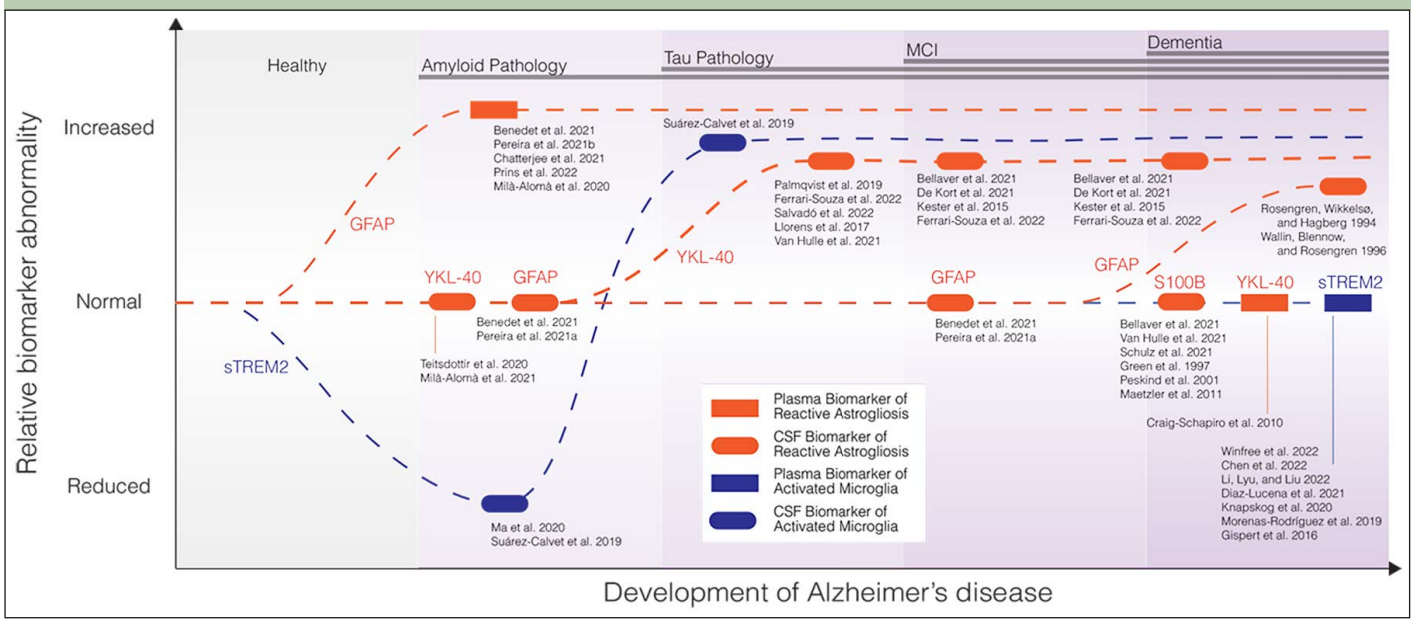
As for the brain, it is important to differentiate the adaptive immune response, a core feature of multiple sclerosis and autoimmune encephalitis, from the innate immune response, mostly as seen in neurodegenerative diseases (15). The first involves adaptive immune cells such as T and B lymphocytes and NK cells, along with myeloid cells like monocytes, invading the CNS through the blood-brain barrier (BBB) and directly provoking local modifications. On the other hand, the innate response involves mostly microglia and astrocytes becoming active/reactive in response to various stimuli. In an attempt to halt hazardous threats, this response happens by promoting local alterations in tissue homeostasis, phagocytosis and degradation of small aggregates, as well as the release of signaling and toxic molecules (16). Clinical evidence suggests that innate and adaptive immnune responses occur in AD and seem dependent on the disease stage (17).

Microglial cells, the brain-resident immune cells, are continuously active and change their phenotypes in response to inflammatory stimuli. Indeed, microglia play a complex role in the trajectories of AD, acquiring multiple phenotypes through the activation of different pathways (18). For a long time, microglial responses were considered exclusively detrimental to AD progression. However, more recently, protective subtypes of microglial responses were identified in AD (19). While there is still room for discussion about the protective/detrimental role of microglia in AD, it seems clear that its activation is stage-dependent and that multiple phenotypes may co-exist in particular disease stages. Activated microglia have been extensively studied in immune surveillance, debris phagocytosis, regulation of neuronal apoptosis, synaptic plasticity, and pruning. Depending on the original insult, microglia can recognize abnormal molecules, become activated into heterogeneous morphologies, and promote a context-specific response. This response usually involves internalizing and degrading molecules using different endocytic pathways and releasing pro-inflammatory factors, such as cytokines, chemokines, and reactive oxygen species (ROS). It is proposed that repeated exposure to hazardous stimuli might cause microglia to become aberrantly active, sustaining noxious conditions to the CNS and, eventually, leading to, or accelerating, neurodegeneration (20, 21).

Astrocytes, an abundant glial cell type in the human brain, carry many important physiological roles in the CNS, such as regulation of synaptic function, maintenance of the BBB, calcium signaling, energy supply to neurons, homeostasis of neurotransmitters and ions, and release of gliotransmitters (22). Along with these homeostatic roles, astrocytes can also respond to inflammatory stimuli by becoming reactive. Reactive astrogliosis refers to molecular, functional, and morphological changes that may impact the brain environment during pathological stimuli, such as hypoxia, cytokines, misfolded proteins, low glucose supply, neurotransmitter imbalance, and ROS (23-26). Reactive astrocytes have been reported in numerous diseases and are typically observed in post-mortem AD brains, colocalizing with Aβ and tau pathologies (27). While reactive astrocytes were initially considered a homogeneous population, nowadays, there is a consensus that astrocytes assume multiple phenotypes in response to different pathological stimuli (26, 28). The interplay between microglial cells and astrocytes deserves attention since microglia can trigger astrocyte reactivity, increasing the release of cytokines and chemokines and generating more ROS (29). In addition to this orchestrated response, in vitro studies demonstrated that astrocytes can independently react to inflammatory insults by activating classical inflammation-related pathways (30, 31). However, unlike microglia, astrocytes are not primarily inflammatory cells, and their repertoire of functions is broader. Thus, it is important to highlight that astrocyte responses are not always associated with inflammatory changes.

Interestingly, activated microglia and reactive astrocytes colocalize with Aβ plaques and tau tangles in post-mortem studies, suggesting that inflammation is associated with AD core pathological features. Additionally, it was shown that misfolded proteins act as danger-associated molecular patterns, triggering microglial activation and astrocyte reactivity via surface and toll-like receptors (32). In addition, the recent genetic risk factors described for AD are mainly from inflammatory pathways, strengthening the theory of an amyloid-inflammatory cascade in the disease pathogenesis (33). Another important hypothesis in the genesis of neurodegenerative diseases is inflammaging (34). It proposes that an impairment in any of the highly interconnected seven pillars of aging - stem cell

Figure 1. Temporal progression of neuroinflammation fluid biomarkers in Alzheimer's disease



Hypothetical curves of fluid neuroinflammatory biomarkers across the AD continuum. Plasma biomarker findings from previous studies are represented by rectangles, while CSF biomarker findings are represented by ellipses. The dotted lines show hypothetical relative biomarker abnormalities and are not based on previous studies. Curves are color-coded, with orange indicating astrocyte biomarkers and blue indicating microglia biomarkers. It's important to note that the magnitudes of other biomarkers presented in the graph are relative to their own normal range and should not be compared across different biomarkers. Abbreviations: AD – Alzheimer's disease; MCI – mild cognitive impairment; CSF – cerebrospinal fluid; sTREM2 – soluble triggering receptor expressed in myeloid cells 2; YKL-40 – chitinase-3-like protein 1; GFAP – glial fibrillary acidic protein; S100B – calcium-binding protein B

regeneration, metabolism, inflammation, proteostasis, macromolecular damage, stress, and epigenetics - affects all other pillars. The insidious disruption in this aging network builds up to a persistent state of low-grade inflammation, chronically activating the immune system and accelerating senescence. It is proposed that inflammaging byproducts in the periphery can enter the CNS and trigger proinflammatory states in glial cells, eventually leading to neurodegeneration (35, 36). Thus, the inflammatory component of AD cannot be neglected and deserves further investigation. To date, a few indirect fluid and imaging biomarkers of neuroinflammation have been investigated and provided the initial basis for understanding the role of neuroinflammation in AD.

Biomarkers of neuroinflammation in AD

Fluid biomarkers

Microglia

In the brain, the triggering receptor expressed on myeloid cells 2 (TREM2) is almost uniquely expressed by microglia and is mostly upregulated on by these cells around amyloid plaques in AD. Secretase shedding of the receptor ectodomain gives rise to soluble TREM2 (sTREM2), which can be detected in the blood and cerebrospinal fluid (CSF). An increase in sTREM2 proteolytic shedding seems directly related to decreased TREM2 activity (37). The activation of TREM2 in animal models has produced controversial results, either ameliorating pathological phenotypes (38) or exacerbating AD pathology's spreading (39).

The sTREM2 has been increasingly explored as a biomarker of AD, however, its performance in discriminating between clinical diagnoses is still under investigation. While some studies observed increased significant differences between cognitively unimpaired (CU) and AD dementia individuals' (40, 41) other reports found no alterations (42-48). However, CSF sTREM2 levels seem to fluctuate according to changes in the hallmarks of AD pathology. Specifically, CSF sTREM2 levels decrease in response to CSF Aβ1-42 (49, 50) but increase in response to CSF tau elevation (total tau or phosphorylated tau) (50), suggesting it is a stage-specific biomarker. Additionally, high levels of sTREM2 are associated with high levels of neurofilament light chain (NfL), and with an increased CSF/plasma albumin ratio, which indicates lower BBB integrity (42). Cross-sectional studies pointed to a protective effect of sTREM2 in mild cognitive impairment (MCI), as individuals with higher sTREM2 present a higher gray matter volume in the bilateral inferior and medial temporal cortices and precuneus as in the left supramarginal gyrus (48).

Longitudinal studies were key to better understanding the dynamic changes in microglial states. In line with this, it was demonstrated that higher baseline levels of sTREM2 are associated with slower gray matter volumetric loss in parahippocampal gyrus, left fusiform cortex, left middle temporal gyrus, and left lateral occipital cortex (51). Baseline CSF sTREM2

levels predicted longitudinal memory decline but not longitudinal worse executive functioning (42, 43, 52). Importantly, individuals with presymptomatic AD with a steeper longitudinal increase in sTREM2 presented a slower rate of cognitive decline (53). Similarly, higher sTREM2 levels are associated with slower clinical decline in the dementia stage of AD (54, 55). A higher annual increase in sTREM2 is associated with a diminished annual rate of Aβ accumulation in presymptomatic carriers of pathogenic variants (53) and individuals across the AD continuum (56). The temporal changes of microglial activation fluid biomarkers in AD can be seen in Figure 1.

Astrocytes

The glial fibrillary acidic protein (GFAP) is an intermediate filament protein of astrocytes. Because it has been considered a canonical marker of reactive astrocytes for a long time, it was the first astrocyte protein explored as a biomarker in AD. Initial studies on CSF GFAP in AD date from the 90s and have already pointed to a significant increase in GFAP levels in patients with AD dementia compared to CU controls (57, 58). GFAP in the CSF seems to have diagnostic value and correlated with disease severity (59). However, CSF GFAP does not seem sensitive enough to detect the early phases of dementia, as no differences were found between CU and MCI Aβ-positive (60, 61). However, an association was observed between CSF GFAP and Aβ pathology in CU individuals, as assessed by PET and CSF biomarkers (62, 63). It is important to consider that an age-related increase in CSF GFAP levels was observed in CU individuals, contributing to the idea that GFAP is an unspecific marker (57, 64). Additionally, CSF GFAP levels are increased in other neurodegenerative conditions, such as frontotemporal lobe dementia (65, 66), Creutzfeldt-Jakob disease (67), Parkinson's disease (65), and vascular dementia (58).

The recent development of ultrasensitive techniques allowed the detection of brain-derived proteins in the blood in low concentrations. Surprisingly, plasma GFAP seems to better predict AD pathology than CSF GFAP (60, 61, 65), detecting amyloid load before symptoms onset. Part of the explanation of plasma GFAP's better performance compared to CSF relies on sample stability. Specifically, GFAP in the blood is less vulnerable to freeze-thaw cycles, making it a better matrix than CSF (68). Specifically, studies in CU older individuals at risk for AD (i.e., Aβ-positive) showed that plasma GFAP associates with Aβ (69, 70). Additionally, individuals with autosomal dominantly inherited familial AD (mutation carriers) have higher levels of plasma GFAP, even in the presymptomatic phase, compared to non-mutation carriers (71). Plasma GFAP is associated with Aβ but not tau pathology (61, 72), which might explain why plasma GFAP is a better marker of early AD pathology. It was demonstrated that plasma GFAP increases the ability of other plasma biomarkers to distinguish between Aβ-negative and Aβ-positive individuals (69). Increased plasma GFAP levels are also associated with other biological findings in AD, such as decreased cortical thickness (72, 73), decreased hippocampal volume (73), and white matter hyperintensity (72, 74).

Longitudinal studies also point the prognostic utility of plasma GFAP. Initial findings showed that plasma GFAP measures in MCI can predict conversion to AD within a five-year window (75). Plasma GFAP can also be combined with other AD plasma biomarkers to add predictive value in detecting clinical progression (76). A recent study demonstrated that plasma GFAP levels were associated with a greater risk of clinical AD incidence more than a decade before diagnosis (77). Higher baseline GFAP levels were associated with a steeper rate of decline in cognitive domains (76, 78). Like CSF GFAP, plasma GFAP seems to increase as a function of aging (69).

YKL-40 is a secreted glycoprotein expressed in several tissues and involved in immune system activation. In the brain, it is mainly produced by reactive astrocytes during neuroinflammatory conditions (79). Through numerous studies, CSF YKL-40 levels are increased in MCI and AD (80-82), but its role in AD pathophysiology is still poorly understood. Notably, only ~10% of astrocytes express YKL-40 in the human cortex and hippocampus (79). Thus, YKL-40 measures likely reflect a more specific astrocyte population than GFAP. Additionally, temporal analysis of AD biomarkers shows that CSF YKL-40 levels increase later than other biomarkers, which might indicate that this biomarker changes in response to AD pathology (i.e., Aβ and tau) (83). However, it was estimated that in individuals with familial AD, CSF YKL-40 levels start to increase at least 15 years prior to symptoms onset (84). These apparently contradictory findings might highlight important pathological differences between sporadic and familial AD cases.

In addition, CSF YKL-40 levels increase with age, with a steeper elevation observed in at-risk APOE ε4 carriers (85-87). Although neuropathological studies have identified YKL-40–positive astrocytes in clusters near Aβ plaques, its levels in the CSF are more related to tau (either fluid or PET biomarkers) than Aβ pathology in CU and cognitively impaired (CI) individuals (79, 88-90). Interestingly, CSF YKL-40 seems to positively associate with hippocampal atrophy and cognitive impairment in individuals in the AD continuum (88); however, in CU individuals, CSF YKL-40 was associated with higher grey matter volumes and [^{18}F]Fluorodeoxyglucose ([^{18}F]FDG) metabolism (91), which might represent a transient astrocytic response to AD pathology in the early stages of the disease. Longitudinal studies corroborate cross-sectional findings demonstrating the YKL-40 association with brain atrophy and cortical thickness (92). Baseline levels of both YKL-40 in MCI predicted progression to AD with a hazard ratio of 3 (82).

CSF YKL-40 might be altered in other neurodegenerative conditions, as already demonstrated in frontotemporal dementia (93), and Creutzfeldt-Jakob disease (79). Additionally, in individuals with behavioral variant FTD, the presence of positive AD biomarkers is associated with higher levels of YKL-40 compared to those with negative AD biomarkers (87). Similarly, it was observed that YKL-40 is only increased in Lewy body dementia presenting AD as a co-pathology (47), suggesting that the increase is driven by AD-related neurodegeneration.

YKL-40 is significantly produced outside of the brain (e.g., macrophages, chondrocytes, vascular smooth muscle cells, and some types of cancer cells), which might make it difficult to use plasma YKL-40 as a proxy of brain neuroinflammation. Indeed, studies observed only a modest correlation between CSF and plasma YKL-40 levels (94, 95). Differently from CSF, studies diverge about the utility of plasma YKL-40 for distinguishing between CU and AD individuals. Additionally, no correlation between plasma YKL-40 and Aβ42, tau, p-tau181, or cortical amyloid load was observed (95). Finally, because plasma YKL-40 is increased in several non-neurodegenerative diseases, adding numerous confounding factors, its utility as a plasma AD biomarker might be limited.

In the brain, S100B is mostly expressed in astrocytes with a minor expression in other glial cell types, such as oligodendrocytes (96). Despite the fact that S100B biological functions are still not precisely described, it is known that under non-pathological conditions, small amounts of this protein are released and present neurotrophic effects. However, its increased release by reactive astrocytes seems to enhance neuroinflammation (97). The S100B expression is not confined to the CNS, and because many non-neural cell types produce and release S100B, the use of blood measures of this biomarker as a proxy of brain pathology is limited. In this context, only a moderate correlation between plasma and CSF S100B was observed (98). Thus, the CSF measures of S100B may represent a more reliable measure of reactive astrogliosis.

However, findings regarding CSF S100B in AD are contradictory (90, 98-101). A few studies demonstrated a moderate increase in CSF S100B in AD, especially in patients with mild to moderate AD (CDR = 1-2) (99, 100). However, no differences between CU and AD were observed in later studies (101, 102). In fact, our recent meta-analysis synthesized the literature findings about changes in CSF S100B levels in AD and found no significant differences compared to healthy controls (80). Additionally, no associations between S100B and CSF $A\beta_{1-42}$ levels were observed in AD patients (103), and no difference was found in S100B levels between CU Aβ-negative and CU Aβ-positive individuals (104). Finally, as observed for other astrocyte biomarkers, levels of CSF S100B were increased in other neurodegenerative diseases, such as Lewy body dementia and Parkinson's disease (98). The temporal changes in fluid biomarkers of reactive astrocytes across the AD continuum is depicted in Figure 1.

Other fluid biomarkers of inflammation

Other inflammatory fluid biomarkers measured in AD are non-cell and non-disease specific such as cytokines, chemokines, and growth factors. Cytokines are a heterogeneous group of proteins that can be synthesized and secreted by most cells in the human body. Cytokines can be classified as pro- or anti-inflammatory according to their response to a foreign threat. The coordinated and time-limited action of these inflammatory mediators is key to eliminating the invading pathogens and re-establishing the body's homeostasis. Although several pro-inflammatory proteins [e.g., interleukin (IL) 6, IL-1β and transforming growth factor beta (TGF- β)] were already found around Aβ plaques in the AD brain (105), their changes represent unspecific alterations of the immune system. Because numerous peripheral factors might affect CSF cytokine levels, studies measuring these proteins in body fluids are heterogeneous. Their use as a biomarker in AD seems limited due to several confounding factors (106). Finally, the lack of longitudinal studies evaluating cytokine expression/release undermines the interpretation of these cytokines as prognostic markers in AD.

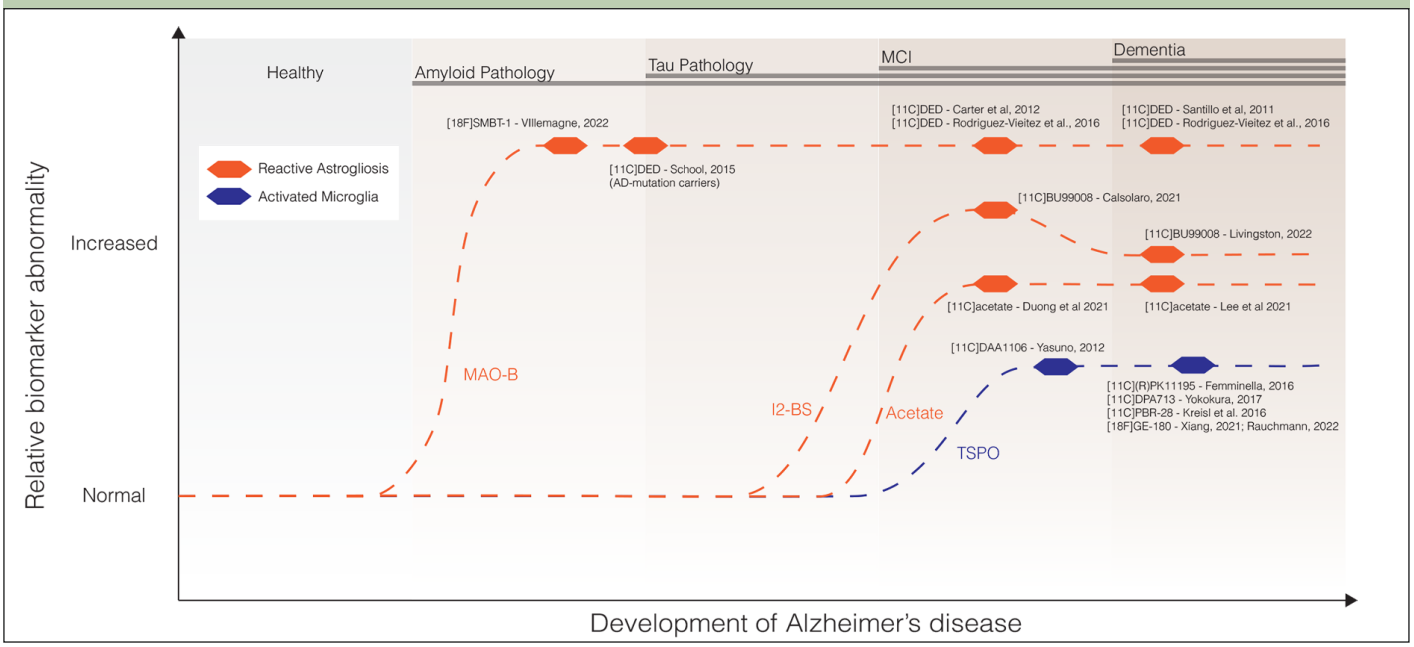
PET Biomarkers

Microglia

Due to its response to pathological alterations in the AD brain, especially in the early stages, microglial changes are promising imaging biomarkers of neuroinflammation. PET imaging targeting microglia has been a challenge because of the complexity of microglial responses and the inability of the exams to capture different microglial phenotypes (107, 108). Currently, a few molecular targets have been proposed to assess microglial alterations using PET: 18kDa translocator protein (TSPO), colony-stimulating factor-1 receptor (CSF1R), cannabinoid receptor type 2 (CB2R), P2Y12 receptor (P2Y12R), P2X7 receptor (P2X7R), and TREM1 and TREM2. From the ones described above, the most widely investigated PET microglial target is the TSPO.

In the brain, TSPO is present in the outer mitochondrial membrane of microglial cells, astrocytes, and endothelial cells. Under pathological conditions, TSPO is upregulated in microglial cells. Thus, TSPO-PET has been proposed as a marker of microglial activation (109). Indeed, the TSPO-PET signal is increased in vulnerable regions of AD. The [^{11}C](R)PK11195-PET, the first TSPO tracer, presents high binding in AD-related brain regions such as frontal, temporal, parietal, and occipital cortices and hippocampus in dementia stages (110). Fan et al. (2015) (111) observed a persistent increase in the TSPO density at

Figure 2. Temporal progression of neuroinflammation imaging biomarkers in Alzheimer's disease



Hypothetical curves of imaging neuroinflammatory biomarkers across the AD continuum. Neuroimaging biomarkers from previous studies are represented by polygons. The dotted lines show hypothetical relative biomarker abnormalities and are not based on previous studies. Curves are color-coded, with orang indicating astrocyte biomarkers and blue indicating microglia biomarkers. It's important to note that the magnitudes of other biomarkers presented in the graph are relative to their own normal range and should not be compared across different biomarkers. Abbreviations: AD – Alzheimer's disease; MCI – mild cognitive impairment; TSPO – 18 kDa translocator protein; I2-BS – imidazoline2 binding sites; MAO-B – monoamine oxidase B.

different stages of the disease in a 17-month longitudinal study.

The second-generation of TSPO tracers also identified increased binding in AD patients. AD participants that underwent [^{11}C]DPA713-PET imaging presented a widespread increase in binding potential (BPND) values compared to young and older healthy controls, whereas [^{11}C](R)PK11195 binding increase was restricted to only a few regions in the same patients (112). In another study, higher [^{11}C]DAA1106 binding was observed in MCI individuals that developed dementia within 5 years. However, it was unable to predict the clinical outcome in terms of the type of dementia due to similar patterns of [^{11}C]DAA1106 uptake in AD and Lewy body dementia converters (113). Conversely, [^{11}C]PBR-28-PET had conflicting results. Kreisl et al (2016) (114) observed higher binding in cortical regions of AD patients. However, in a similar study (115), AD patients displayed displayed a trend for increased uptake in some brain regions, but they were not significant. It should be noted that a common single-nucleotide polymorphism (rs6971) in exon 4 of the TSPO gene is responsible for a significant variation of binding affinity of the second generation TSPO radiopharmaceuticals, which may undermine its use without genetic testing. A new generation of TSPO radiotracers has been recently developed aiming to avoid the need of genetic testing (116). The [^{18}F]GE-180, a third-generation TSPO tracer, showed increased PET signal in cortical areas compared to healthy controls, supporting the hypothesis of increased microglial activation in AD (40, 117). The [^{11}C]ER176 presents favorable kinetics (118), high signal-to-noise ratio, and volume of distribution stability (119), however, no data with AD patients is available yet. In summary, TSPO tracers have been useful to identify TSPO overexpression in the human brain, but the lack of specificity for microglial cells is a limiting factor that should be carefully considered and debated (120, 121).

The selectivity of microglial PET tracers has been a matter of debate, with multiple targets being explored. In this context, the CSF1R is only expressed on the cell surface of microglia and macrophages (122). At the moment, only a few CSF1R tracers have been developed, such as [^{11}C]CPPC and [^{11}C]AZ683. The [^{11}C]CPPC-PET consistently identified higher binding in the cortex, hippocampus, and cerebellum of a mouse model of amyloidosis (16-month-old APP mice), suggesting its capability of detecting microglial changes (123). No human PET studies with these tracers have been conducted so far, but histological analysis in post-mortem tissue confirmed increased CSF1R density in the AD brain (124, 125).

The CB2R is a key player in the endocannabinoid system but is very little expressed in in the homeostatic brain. The upregulation of C2BR occurs in microglial cells as a response to immune activation. However, the [^{11}C]NE40, a novel PET tracer for CB2R, identified overall lower CB2R availability in AD mouse model brains, which remains to be better explored. The authors suggest that CB2R plays an important role in microglial functions

related to initiation, maintenance, and removal of Aβ plaques (126).

Purinergic receptors are also explored as potential targets for imaging microglial cells with PET. P2Y12R is a cell-surface protein expressed by microglia in the brain, its expression in other brain cells remains unclear (127). Under neuroinflammatory conditions, activated microglia overexpresses PRY12R. To date, three PET tracers have been synthesized, but their BBB permeability needs to be improved. Similarly, P2X7R is expressed in microglial cells, oligodendrocytes and Schwann cells (128). In brain autoradiography experiments, a P2X7 analogue, [^{11}C]SMW139, showed no difference between AD and healthy individuals (129). Finally, although no clinical study has used PET imaging with TREM tracers, a few advances have been made using novel TREM1 and TREM2 tracers in animal models of neurodegenerative disorders (124, 130). The temporal changes in imaging biomarkers targeting microglial activation in AD can be seen in Figure 2.

Astrocytes

Although considered the brain's homeostatic cells, astrocytes can also play important roles in neuroinflammation. Astrocyte reactivity has been evaluated using different molecular targets such as monoamine oxidase B (MAO-B), imidazoline2 binding sites (I2-BS), acetate metabolism, and the organic anion transporter 1C1 (OATP1C1).

The first astrocyte-enriched target to have a PET tracer developed was MAO-B. This enzyme is located in the outer mitochondrial membrane and is mainly responsible for catalyzing the oxidative deamination of amines. In the brain, MAO-B is primarily found in astrocytes and radial glia, but also, in a smaller amount, in monoaminergic neurons (131, 132). MAO-B seems to be upregulated in reactive astrocytes (133), but it is important to note that this does not seem to be the case for all reactive astrocytes, but rather for a subpopulation of these cells (134, 135). Using tritiated MAO-B inhibitors, Saura et al., found MAO-B increased activity – up to three-fold – in cortical and hippocampal plaque-associated astrocytes (136).

The first radiotracer for MAO-B used in AD research was [^{11}C]Deuterium-L-Deprenyl ([^{11}C]DED). [^{11}C] DED was first developed as [^{11}C]-L-Deprenyl and later improved with the addition of deuterium, which reduces the tracer rate of trapping and improves sensitivity to changes in MAO-B. L-Deprenyl is an irreversible inhibitor oxidized by MAO-B to produce a reactive intermediate that covalently binds to the enzyme (137). Initial autoradiographic and PET studies using L-Deprenyl or [^{11}C]DED seemed to point to a significant and widespread increase in MAO-B in the brain of AD patients compared to healthy controls. However, subsequent, more consistent evidence has suggested that this increase occurs earlier in the disease course, in prodromal AD stages. For example, an autoradiographic study in AD patients using [3H]-L-Deprenyl identified a significant increase in MAO-B activity in gray matter regions, such as the frontal and temporal cortices, basal ganglia, thalamus, and white matter in comparison to controls (138). On the other hand, a much more recent autoradiographic study using [11C]-L-Deprenyl only identified an increase in its binding in AD versus age-matched controls in the temporal cortex and white matter (139).

A similar outcome was also observed in PET studies. In the first PET work (140), a higher [^{11}C]DED binding was observed in the parietal, temporal, and medial temporal lobe in AD patients compared to healthy controls. It is important to note that this study used [^{11}C]DED slopes/ intercept rate values as a measurement of radiotracer binding (considered a suboptimal measure) and that the group of AD patients analyzed included moderate to severe dementia (MMSE Score = 14.4 ± 2.07), different from subsequent studies, which used mild dementia cases (MMSE Score = 24.4 ± 5.7). Cross-sectional studies from Prof. Nordberg's group using an optimized PET analysis did not find differences between AD and controls. But, more interestingly, they identified an increased [^{11}C] DED binding in the prodromal stages of AD. A peak in [^{11}C]DED binding was observed in MCI Aβ-positive patients (141) and presymptomatic autosomal dominant Alzheimer's disease (ADAD) mutation carriers (142). Moreover, a longitudinal work (over a mean period of 2.8 ± 0.6 years) observed an initial higher [^{11}C]DED binding in presymptomatic stages of ADAD, which declined as amyloid load increased. The same decline in MAO-B levels was not seen in sporadic AD patients, which increased in MCI Aβ-positive patients and remained unchanged during the study (143). These findings suggest that (i) the astrocyte response in ADAD and sporadic AD may be distinct; or (ii) may result from a short study follow-up period since it has been already observed that ADAD might have an accelerated rate of evolution in comparison to sporadic AD (144).

Despite being, by far, the most used PET radiotracer to investigate changes in astrocytes in AD, [^{11}C]DED findings still have to be reproduced in larger and more diverse AD populations. One advancement that would potentially help is the development of an equivalent radiotracer using the [^{18}F] isotope. Several radiotracers have been developed with this purpose, such as DL-4-[^{18}F]fluorodeprenyl (145), [^{18}F]fluororasagilineD2 (146) and [^{18}F]fluorodeprenyl-D2 (147). However, most of the DED fluorinated analogous radiotracers presented undesired characteristics such as brain permeable radiometabolites, poor brain uptake or complex radiosynthesis.

Other molecules with affinity to MAO-B, but not analogous to DED, have also been developed. The [^{11}C] SL25.1188 (148), a reversible MAO-B radiotracer, has already gone through its first-in-human clinical studies, and efforts to produce a fluorinated analogous have

already begun (149, 150). Another interesting reversible MAO-B radiotracer being developed is the [18F]SMBT-1 (151), which was originally designed to detect Tau tangles, but was found to bind MAO-B with high affinity (152). [18F]SMBT-1 was developed via lead optimization from [18F]THK-5351 and already showed promising results on its first human trials, which included a few MCI and AD patients (153). In a more recent work, [18F] SMBT-1-PET (154) was investigated in a higher number of patients across the AD continuum, showing an increase in cortical brain regions in the Aβ-positive groups, including Aβ-positive cognitively unimpaired (CU) individuals. Further studies, especially with a higher number of AD and MCI patients, are necessary to corroborate these encouraging findings.

Another PET target for reactive astrocytes is the I2-BS, mostly found in astrocytes' outer mitochondria membrane (155). Coincidently, it has been reported that I2-BS binding site is located in MAO-B (156). An autoradiography study with the radiotracer [11C]BU99008, which binds to I2-BS, showed that [11C]BU99008 and [11C] DED have similar regional distribution, but [11C]BU99008 has a higher specific binding in AD brains compared to CU individuals, (157). [11C]BU99008-PET studies in MCI and AD patients have identified a similar outcome to DED with an elevation on [11C]BU99008 uptake in Aβ-positive individuals; particularly, this elevation was superior in MCI than AD patients (158, 159).

A different approach for imaging astrocytes is to use a radiolabeled molecule that, in the brain, is mainly transported or metabolized by these cells, which seems to be the case of acetate (160). Therefore, the radiotracer [11C]acetate has been suggested as a marker of astrocyte metabolism (161). In Aβ-positive MCI patients, it was observed that [11C]acetate uptake was significantly elevated in the medial temporal lobe (162). An additional work, showed that [11C]acetate uptake was elevated in AD-vulnerable brain regions, such as the entorhinal cortex and the hippocampus, if compared to healthy controls (163).

Moreover, an alternative tracer developed for imaging astrocytes is Sulforhodamine 101 N-(3-[18F]Fluoropropyl) sulfonamide ([18F]2BSRF101) (164). [18F]2BSRF101 was developed based on the fluorescent dye Sulforhodamine 101 (SR101), which has been extensively used as an astrocyte marker. For a long time, the specific binding point or transporter of SR101 in astrocytes was unknown until the thyroid hormone transporter OATP1C1 (also known as SLCO1C1) was identified as the SR101-uptake transporter (165). It is important to note that SR101 is not specific to astrocytes, also labeling, to a minor degree, oligodendrocytes, although it is still dependent on SR101 uptake by astrocytes OATP1C1 transporters (166). [18F]2BSRF101 has not been tested in humans yet, but its binding was elevated in the cortex and hippocampus of the 3xTg, a mouse model that presents amyloid and tau pathologies. By contrast, no changes were found using [11C]DED in the same model, suggesting that [18F]2BSRF101-PET might bring additional information about the astrocyte heterogeneity in AD (167). The temporal changes in imaging biomarkers targeting astrocytes across AD continuum can be seen in Figure 2.

Other imaging biomarkers of neuroinflammation

Apart from the radiotracers primarily targeting glial cells, other available radiotracers targeting brain alterations may also be implicated in brain inflammatory changes such as glucose brain metabolism, cyclooxygenase (COX) isoenzymes, inducible nitric oxide synthase (iNOS) and ROS production.

The glucose brain metabolism has been widely investigated using the [18F]FDG tracer, a glucose analog molecule. In AD, a specific [18F]FDG-PET hypometabolism is seen in the later stages of the disease (for review, see Chételat, 2020 (168)). Interestingly, recent evidence identified a transient [18F]FDG-PET brain hypermetabolism in MCI patients (169). In parallel, correlations between glucose metabolism measured by [18F]FDG-PET and the astrocyte biomarkers, measured trough [11C]DED or plasma GFAP, have been found in ADAD patients (170) and sporadic AD patients (89). Indeed, studies have been consistently demonstrating that glial cells and inflammatory changes impact the brain [18F]FDG-PET signal. For instance, [18F]FDG-PET is sensitive to activation/deactivation of astrocytes and microglial cells (171-175). Thus, it is thought that [18F] FDG-PET may capture neuroinflammatory changes in AD.

Another target being used for radiotracers development are the COX enzymes. COX-1 and COX-2 are enzymes involved in forming prostaglandins and thromboxane (176). COX-1 is considered a housekeeping enzyme not induced by inflammation; however, a study observed an expression of COX-1 in microglia in association with Aβ plaques of AD patients. A pro-radiotracer of ketoprofen, [11C]ketoprofen-methyl-ester ([11C]KTP-Me), a non-selective inhibitor of COX, however, did not find statistical differences in MCI and AD individuals compared to healthy controls. Although predominantly neuronal, COX-2 is increased during neuroinflammation and is associated with the immunomodulation of the brain tissue (177). Indeed, [11C]MC1 binding, a COX-2 tracer, was higher in the brain lesion site of rhesus macaques injected with lipopolysaccharide in the right putamen (178). However, [11C]MC1 has not been used in AD patients or models.

The iNOS enzyme has also been a target for radiotracers development. In the brain, iNOS is expressed by microglial cells, astrocytes, neurons, and endothelial cells (179, 180). The expression of iNOS is low in healthy or non-inflammatory states but it is stimulated by inflammatory stimuli (181). PET radiotracers targeting

Table 1. Radiotracers targeting neuroinflammation

Target	Current Biological Interpretation	Cerebral Cellular source of the signal	Radiopharmaceuticals
TSPO	Microglial activation	Microglia, macrophages, astrocytes and endothelial cells.	[11C]PK11195 [18F]DPA-714 [11C]DPA-713 [18F]DPA [18F]FEBMP [11C]DAA1106 [18F]FEDAA1106 [18F]FEMPA [11C]AC-5216 [18F]FEPPA [11F]PBR06 [11C]PBR28 [18F]PBR111 [18F]GE-180 (S)-[18F]GE-387 [18F]GE-387 [11C]ER176 [11C]CB184 [11C]CB190 [11C]N′-MPB [18F]LW223
CB2R	Microglial activation	Microglia and Neurons	[11C]A-836339 [18F]2f [18F]RS-126 [18F]RoSMA-18-d6 [18F]JHU94620 [11C]NE40 [11C]MA2 [18F]MA3
P2X7R	Microglial activation	Microglia, astrocytes, oligodendrocytes, neurons and endotelial cells	[11C]A-740003 [11C]GSK1482160 [18F]JNJ-64413739 [11C]JNJ-54173717 [11C]SMW139 [11C]JNJ-47965567
P2Y12R	Microglial activation	Microglia and macrophages	[11C]JNJ-47965567 [11C]5 [11C]2
CSF1R	Microglial activation	Microglia and macrophages	[11C]CPPC [11C]GW2580
COX-1	Unspecific neuroinflammation	Ubiquitous	[11C]-KTP-Me [11C]PS13 [18F]PS2
COX-2	Unspecific neuroinflammation	Ubiquitous	[11C]MCI [18F]FMTP [18F]TMI
TREM1	Microglial activation	Microglia, monocytes and macrophages	[124I]TREM1-mAb
TREM2	Microglial activation	Microglia and macrophages	[124I]mAb1729
MAO-B	Reactive astrogliosis	Astrocytes and Monoaminergic neurons	[11C]DED [11C]SL25.1188 [18F]SBMT-1 [18F]fluorodeprenyl-D2 DL-4-[18F]fluorodeprenyl [11C]pargyline [18F]fluororasagiline [18F]fluororasagilineD2
I2BS	Reactive astrogliosis	Astrocytes and Monoaminergic neurons	[11C]BU99008 [11C]FTIMD [18F]FEBU (BU99018)
Acetate metabolism	Reactive astrogliosis	Astrocytes	[11C]acetate
OATP1C1	Reactive astrogliosis	Astrocytes	[18F]2B-SRF101
Glucose metabolism	Cell metabolism	Astrocytes, neurons and microglia	[18F]FDG
iNOS	Unspecific neuroinflammation	Ubiquitous	[18F]FBAT [18F]NOS
ROS	Oxidative stress	Ubiquitous	[18F]ROStrace [18F]oxROStrace [18F]dihydromethidine [11C]ascorbic acid [11C]dehydroascorbic acid

Table 2. Ongoing clinical trials of neuroinflammation in AD (https://clinicaltrials.gov last accessed 07/11/2022)

Study	Drug	Sample	Mechanism	Phase	Completation date	Primary outcome
NCT04838301	Allopregnanolone	Probable AD	Maintenance of structural integrity	2	June 2025	Hippocampal volume
NCT05318976	XPro1595	Mild dementia and Aβ-positive	Neutralization of soluble TNF	2	June 2023	Change in Early and Mild Alzheimer's Cognitive Composite (EMACC)
NCT05321498	XPro1595	MCI due to AD and Aβ-positive	Neutralization of soluble TNF	2	January 2023	Change in Early and Mild Alzheimer's Cognitive Composite (EMACC)
NCT05522387	XPro1595	Patients who completed another XPro1595 trial	Neutralization of soluble TNF	2	December 2025	Number of participants who experience adverse events and serious adverse events
NCT04795466	Canakinumab	MCI due to AD or mild AD	Immunomodulation with an anti IL- 1β monoclonal antibody	2	February 2026	Change from baseline in cognition as measured by the Neuropsychological Test Battery (NTB) total score
NCT05521477	Dietary Supplement: SLAB51 (probiotic)	MCI due to AD, Aβ-positive and APOEe4 carrier	Regulation of gut microbiota	NA	September 2023	Concentration of plasma AD biomarkers: Amyloid, Tau and NfL
NCT03435861	Neflamapimod	Prodromal AD and Aβ-positive	Inhibition of the alpha isoform of the mitogen-activated serine/threonine protein kinase p38 MAPK	2	June 2021 (delayed)	Brain inflammation assessed by [18F]-DPA714, Standard Uptake Value (SUV)
NCT05564169	Masitinib	Dementia due to AD	Inhibition of tyrosine kinase that targets activated cells of the neuroimmune system	3	December 2025	Absolute change from baseline in ADAS-Cog-11 score
NCT05468073	Proleukin(IL-2)	Progressive amnestic syndrome and positive for AD biomarkers	Non-specific immunomodulation	2	September 2026	Change from baseline CDR score at 18 months
NCT04740580	GlyNAC	Amnestic syndrome and tau positive	Correction of glutathione deficiency by supplementation of its precursors (glycine and cysteine)	1	May 2025	Change in ADAS-Cog, FDG-PET scan, TSPO-PET scan
NCT05004688	Bacillus Calmette-Guerin (BCG) vaccine	MCI due to AD or mild AD	Non-specific immunomodulation	2	October 2023	Changes in blood and CSF biomarkers related to AD pathology and inflammation and in cognitive scores
NCT05551741	IBC-Ab002	Early AD	Immunomodulation with an anti-PD-L1 monoclonal antibody	1	December 2024	Incidence of subjects with adverse events, serious adverse events
NCT04777409	Semaglutide	MCI or mild dementia due to AD and Aβ-positive	Non-specific immunomodulation	3	August 2024	Change in the Clinical Dementia Rating

NA= not applicable

iNOS, such as [^{18}F]NOS (182) and [^{18}F]FBAT (183), are promising to track neuroinflammation, but have not yet been tested in AD patients.

Additionally, ROS has also been a focus of PET radiotracers. ROS are a byproduct of physiological cellular functioning and are also signaling molecules. Abnormal ROS production or clearing and resulting oxidative stress are linked to aging and degenerative disease in general (184). Even though this is not a specific neuroinflammatory process, it can cause DNA damage and mitochondrial dysfunction (i) in neurons, promoting a proinflammatory environment through the production of damage-associated molecular patterns; and (ii) in glial cells, resulting in glial changes and cell death (185). The brain is especially prompt to oxidative stress-related damage because it is the most energetically active organ in the human body, meaning intense cellular metabolism and, consequently, intense ROS production. In the specific context of AD, along with mitochondrial production of ROS, there is evidence that Aβ plaques may also be a source of ROS production (186, 187). Some effort has been made to develop PET tracers that target ROS, such as [^{11}C]Ascorbic acid, [^{11}C]dehydroascorbic acid and [^{18}F]dihydromethidine, but these tracers were not yet investigated in the context of AD (188, 189). On the other hand, [^{18}F]ROStrace was associated with amyloid burden in a mouse model, and [64Cu]ATSM was shown to be related to Aβ accumulation in a preliminary study comparing individuals with early biomarker-evidenced of AD (190, 191). A list of proposed PET radiotracers targeting neuroinflammation is presented in Table 1.

Neuroinflammatory biomarkers as prognostic markers

Converging evidence suggests that the pathogenesis of neurodegenerative disorders begins years before symptom onset. Theoretically, a perfect marker of disease progression should track different stages since the earliest pathological alterations. In AD, prognostic biomarkers have promising clinical applications because drug therapies are supposed to start before symptoms (192). Indeed, AD biomarkers positivity has been consistently demonstrated in preclinical phases, which is thought to be the most suitable timing for disease-modifying treatments and secondary prevention strategies. Together with target engagement biomarkers, prognostic markers may also be a method to enrich participants' inclusion in clinical trials with early AD.

Prognostic markers diverge from diagnostic markers in many aspects. Biomarkers of disease progression should be sensitive to cognitive deterioration and the pathological burden of the disease (193). Currently available evidence has pointed out a myriad of markers of disease progression, including neuroimaging, cognitive testing, and fluid markers. Peripheral biomarkers of neuroinflammation have been increasingly mentioned as promising prognostic markers due to their early identification in peripheral tissue in different conditions (194). However, current evidence of the validity of these markers is still under debate. In the following session we in-depth analyze the prognostic value and test accuracy of neuroinflammation biomarkers of AD.

Therapeutic targets of neuroinflammation

Recent advances in the biomarker field have allowed for classifying individuals in the AD spectrum before the onset of symptoms. The importance of using biomarkersto develop treatments that prevent the development of dementia was brought to light (195). Therapies targeting amyloid plaques, such as aducanumab (FDA-approved) and lecanemab (FDA-approved), can remove amyloid aggregates (196). Still, it is unclear whether a single target will halt the progression of a complex disease like AD. Thus, there is a need to explore other treatment possibilities, including drugs targeting inflammatory changes. A list of ongoing clinical trials targeting neuroinflammatory pathways is presented in Table 2.

Some attention has been paid to this in the past. The ADAPT trial (197) was released in 2008, with negative results regarding preventing dementia onset using non-steroidal anti-inflammatories celecoxib and naproxen. Not only no evidence for efficacy was achieved in delaying cognitive decline or preventing dementia onset, but the trial also held the possibility of naproxen use being associated with increased cognitive decline. The study was terminated because the intervention group showed increased cardiovascular risk.

Another attempt to halt disease progression in cognitively unimpaired individuals using NSAID naproxen was the INTREPAD trial. For this trial, a composite primary endpoint comprising cognitive, neurologic, and biomarker data was developed and used. As occurred in the ADAPT study, naproxen did not show good outcomes and was associated with important side effects (198). It is important to note that, even with limited statistical power, these studies were marked by a tendency for adverse results.

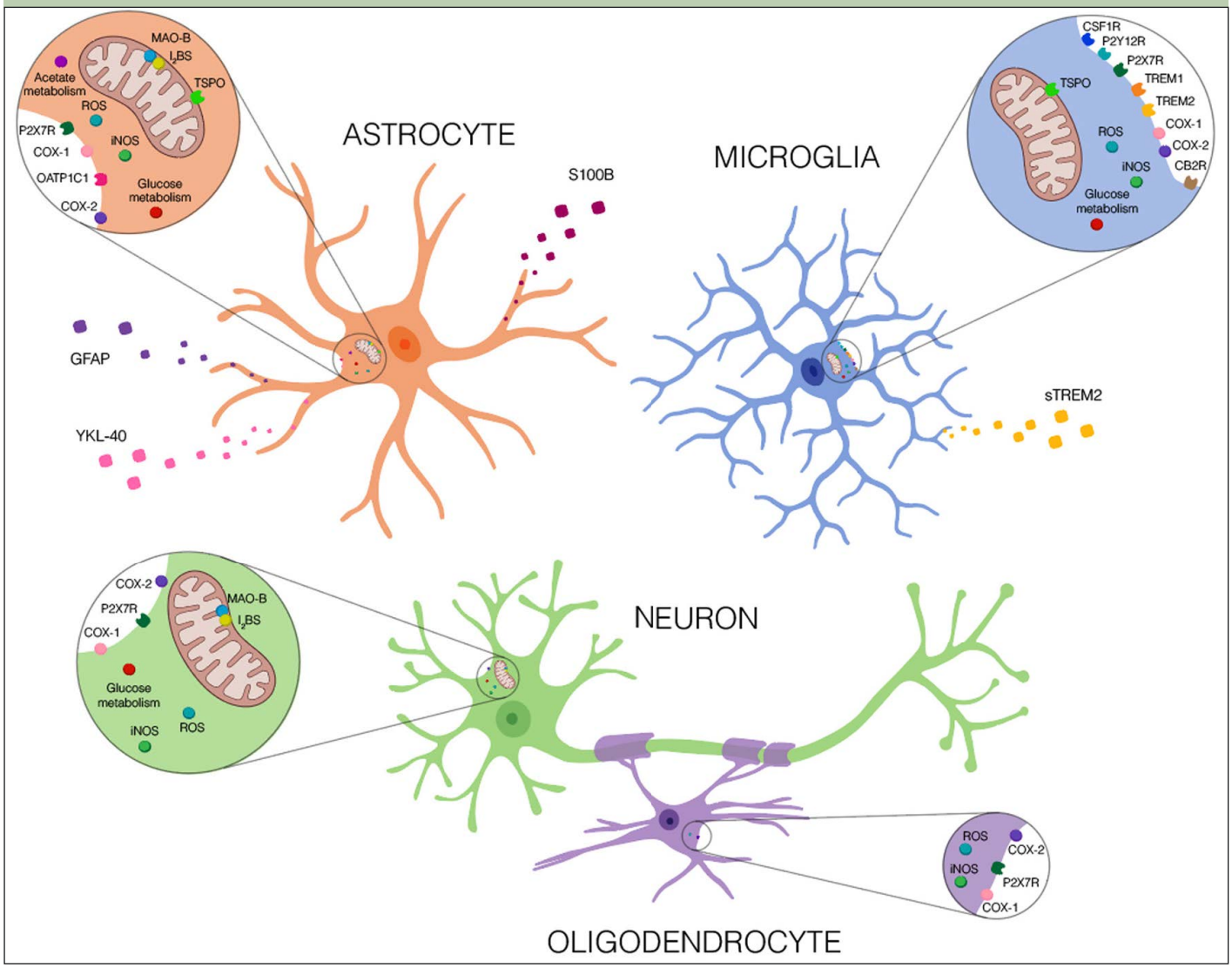
Other attempts to treat AD targeting inflammatory processes were made. For instance, minocycline was found to have an anti-neuroinflammatory effect, inhibiting microglia in animal studies, but clinical trials did not show clinical impact (199). Rosiglitazone, a PPAR inhibitor developed as an anti-diabetic, was also considered a good candidate to prevent AD due to its modulating neuroinflammatory response (200, 201). But, once again, results in clinical trials were not able to achieve its primary clinical outcome.

A possible explanation for previous failures is that anti-inflammatory drugs impact both protective and harmful inflammatory states of glial cells (20). As we develop knowledge toward a more specific understanding of the inflammatory states of glial cells, more selective therapies may be developed to inhibit processes related to harmful phenotypes selectively. An example is the Etanercept, a TNF-α blocker that has been considered a plausible approach for reducing detrimental inflammation in AD 202).

Another approach that has been recently explored is the use of drugs with pleiotropic effects that may be repurposed for AD prevention by acting on neuroinflammation. Pioglitazone, another PPAR inhibitor, is being studied despite the initial unsatisfactory results of similar drugs (203). Candesartan, an ACE inhibitor, is a potential candidate, with preclinical studies indicating immune modulation towards a more protective state in animal models of AD (204). Also, the anti-inflammatory effects of statins, a lipid-reducing class of drugs, has been gaining attention in the context of AD (205). Semaglutide, a GLP-1 receptor agonist, is entering phase 3 evaluation for treating early AD (206). It is proposed that pleiotropic anti-inflammatory effects are behind its anti-dementia potential (207). Multiple phytotherapeutic and antioxidant drugs, such as resveratrol, omega-3 fatty acids, and folic acid, have been suggested as supplements to treating AD due to their anti-inflammatory potential (208-210). Even though these drugs tend to have little, unspecific effects, they are well-tolerated and safe interventions that may be further explored as adjuvant therapies.

In conclusion, no therapy targeting inflammation has effectively prevented or halted the cognitive decline in AD. Still, with an improved understanding of biological processes and biomarkers, a more specific approach may warrant a better response. With the recent advances in

Figure 3. Imaging and fluid biomarker targets for tracking neuroinflammation in Alzheimer's disease



Schematic representation of cell-derived origins of imaging and fluid biomarkers of neuroinflammation in AD. Abbreviations: AD – Alzheimer's disease; TSPO – 18 kDa translocator protein; I2-BS – imidazoline2 binding sites; MAO-B – monoamine oxidase B; sTREM2 – soluble triggering receptor expressed in myeloid cells 2; YKL-40 – chitinase-3-like protein 1; GFAP – glial fibrillary acidic protein; S100B – calcium-binding protein B; iNOS – inducible nitric oxidase; P2X7R – P2X7 receptor; ROS – reactive oxygen species; COX-1 – cyclooxygenase 1; COX-2 – cyclooxygenase 2; OATP1C1 – organic anion transporter 1C1; P2Y12R – P2Y12 receptor; CB2R – cannabinoid receptor type 2.

Aβ-targeted therapies for AD, neuroinflammation may be a secondary target aiming for effective disease-modifying treatments.

Neuroinflammation in the AT(N) framework

The AT(N) system is proposed to be dynamic and open for including novel biomarkers indicating additional pathophysiological processes in AD. The question is straightforward, are we ready to include neuroinflammation as an "Im" (microglial positivity), "Ia" (astrocytic positivity), "G" (glial positivity) or simply "I" (neuroinflammation)? First, they add little information as diagnostic tools compared to AD core biomarkers – Aβ and tau. Second, the biological interpretation of neuroinflammation imaging biomarkers is far from conclusive, relying on the overexpression of proteins in immune-associated cells. Also, these proteins are rarely specific to the cell type of interest. Third, as happens to fluid biomarkers, proteins that leak or are secreted by immune-associated brain cells are used as surrogate markers of neuroinflammation. Forth, similarly to systemic inflammatory biomarkers such as the C-reactive protein, biomarkers of neuroinflammation present low specificity for AD. In clinical research, however, neuroinflammation biomarkers have been useful in elucidating additional pathophysiological mechanisms and proposing therapeutic targets in AD. A summary of neuroinflammatory-related imaging targets and fluid proteins measured in AD can be seen in Figure 3.

Concluding remarks

In summary, neuroinflammatory markers of AD have potential clinical relevance. However, their role in clinical practice remains elusive. Large longitudinal, multicentric studies in diverse populations with AD core biomarkers associated with neuroinflammation biomarkers in the AD continuum are needed. In addition, it is necessary to advance these biomarkers' biological interpretation. Thus, although promising, more evidence is needed to propose a new biomarker group representing neuroinflammation in the AT(N) framework.

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