Title: Diagnosing Alzheimer's Disease from Circulating Blood Leukocytes Using a Fluorescent Amyloid Probe

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Abstract

Background: Toxic amyloid-β (Aβ) peptides aggregate into higher molecular weight assemblies and accumulate not only in the extracellular space, but also in the walls of blood vessels in the brain, increasing their permeability, and promoting immune cell migration and activation. Given the prominent role of the immune system, phagocytic blood cells may contact pathological brain materials.

Objective: To develop a novel method for early Alzheimer's disease (AD) detection, we used blood leukocytes, that could act as "sentinels" after trafficking through the brain microvasculature, to detect pathological amyloid by labelling with a conformationally-sensitive fluorescent amyloid probe and imaging with confocal spectral microscopy.

Methods: Formalin-fixed peripheral blood mononuclear cells (PBMCs) from cognitively healthy control (HC) subjects, mild cognitive impairment (MCI) and AD patients were stained with the fluorescent amyloid probe K114, and imaged. Results were validated against cerebrospinal fluid (CSF) biomarkers and clinical diagnosis.

Results: K114-labeled leukocytes exhibited distinctive fluorescent spectral signatures in MCI/AD subjects. Comparing subjects with single CSF biomarker-positive AD/MCI to negative controls, our technique yielded modest AUCs, which improved to the 0.90 range when only MCI subjects were included in order to measure performance in an early disease state. Combining CSF Aβ 42 and t-Tau metrics further improved the AUC to 0.93.

Conclusion: Our method holds promise for sensitive detection of AD-related protein misfolding in circulating leukocytes, particularly in the early stages of disease.

Alzheimer's disease (AD) is the most common cause of dementia throughout the world with nearly 60 million people affected (www.alzint.org). Despite much effort, there is neither a cure for AD nor a non-invasive clinical test to reliably establish the diagnosis with certainty. Given the failure of almost all therapeutic trials with anti-amyloid drug candidates (PMID: 29365294, 29914022), it is now generally accepted that any future therapy will only be potentially effective if started as early as possible in the course of the disease, possibly even in the clinically pre-symptomatic stage as a preventative measure. Therefore, it will be important to devise methods to detect disease early and predict cognitive decline based not on clinical status alone, but on the use of biomarkers in combination with clinical criteria, which is the most accurate predictor of future cognitive impairment due to AD (PMID: 24849862, 29653606). Thus, development of an inexpensive, non-invasive, rapid test for AD is of utmost importance as the population ages and AD prevalence is predicted to reach 82 million by 2030 (www.alzint.org).

This age-related progressive neurodegenerative disorder is pathologically characterized by β -amyloid (Aβ)-containing amyloid plaques, neurofibrillary tangles, and synapse loss in the brain (PMID: 11274343). Evidence indicates that pathological hallmarks of AD *i.e.*, misfolding and aggregation of cellular proteins, in particular Aβ peptides and tau, together with impaired clearance and accumulation, may contribute to the pathogenesis of this disease. Toxic Aβ peptides aggregate into higher molecular weight assemblies and accumulate not only in the extracellular space, but also in the walls of blood vessels in the brain (PMID: 11935076, 17430246), increasing their permeability (PMID: 19542618), and promoting immune cell migration and activation (PMID: 12831163). Macrophages/microglia ingest Aβ and are key players for Aβ clearance (PMID: 17222479, 18026096, 16476660). Monocytes clear Aβ accumulated around veins and are able to circulate back to the bloodstream (PMID: 24210819). Neutrophils also infiltrate into AD brain by virtue of blood-brain barrier (BBB) disruption (PMID: 15689955). Although lymphocytes are not phagocytic, intercellular protein transfer has been observed between these immune cells (termed trogocytosis) (reviewed in PMID: 17157208, 31481628). Interestingly, Aβ is transported bidirectionally across the BBB, which suggests a dynamic equilibrium between brain and circulating Aβ (PMID: 11438712, 9042383). Tau biomarkers have attracted attention because of this protein's prominent increase in preclinical AD patient cerebrospinal fluid (CSF), at a stage when only subtle changes of Aβ pathology are detected (PMID: 33169916). Very recently, novel plasma p-Tau181 and p-Tau217 biomarkers are significantly increased in the preclinical stage of AD (PMID: 33169916). Moreover, tau phosphorylation state changes are associated with neurodegenerative and clinical markers of disease, as early as the initial increases in aggregated Aβ begin (PMID: 32161412). Thus, all cellular elements in the blood could act as "sentinels", with a "memory" of their experience after trafficking through the brain and its microvasculature, where they encounter Aβ and/or fibrous tau assemblies in abnormal amounts and, more importantly, in pathological conformations.

The misfolding and aggregation of Aβ from its soluble physiological state into highly ordered fibrillar assemblies is associated with increased β-sheet content (PMID: 20190788). This pathological β-sheet-rich amyloid exhibits high-affinity binding to the classic amyloid probe Congo Red, leading to the term "congophilic" diseases. Conformationally-sensitive amyloid probes, such as luminescent conjugated

oligothiophenes and Congo Red derivatives, are effective for detecting pathological amyloids in tissue sections, such as Aβ fibrils, oligomers and tau tangles (refs: PMID: 12950445, Aslund2009, Nilsson2005, Hammarstrom2010). We reasoned that peripheral blood leukocytes that ingest pathological amyloid fragments as they traverse the brain vasculature (add refs to support this e.g. http://dx.doi.org/10.1016/j.celrep.2013.10.010) could be interrogated using spectral analysis of fluorescent amyloid probes, thereby reporting the degree of amyloid accumulation in the brain. To this end, we developed a blood cell-based technique for AD diagnosis using spectral microscopy of peripheral blood mononuclear cells (PBMCs) labeled with the Congo Red derivative K114 [(*trans*,*trans*)-1-bromo-2,5-bis-(4-hydroxy)styrylbenzene].

Methods

Human AD and control brains

Human brain samples from subjects that were given the diagnosis of AD with Braak stage 5 or 6, and samples from cognitively-normal subjects with Braak stage 0 or 1 were obtained from British AD bank.

Human subjects and PBMC sample collection

The study included a total of 90 subjects including 49 patients with AD, 19 patients with MCI, and 22 healthy controls (HC) diagnosed by standard clinical criteria?. All subjects underwent cognitive testing *(need details from the clinical folks below)*, lumbar puncture for the core AD CSF biomarkers and blood collection for staining and imaging of PBMCs. Blood samples were collected into EDTA-containing tubes and processed to isolate PBMCs by gradient purification using Ficoll-Paque (GE Healthcare). Isolated cells were fixed in 10% neutral-buffered formalin (VWR) and stored at 4°C. Formalin-fixed human PBMCs were smeared and dried on Superfrost Plus glass slides. Fixed PBMC smears were used for immunofluorescence and amyloid probe staining.

CSF analysis

CSF levels of Aβ42, total tau (tTau) and phospho-tau (pTau) were analyzed using the Innotest ELISA methods (Fujirebio, Ghent, Belgium), while CSF Aβ40/42 ratio [(Aβ42/Aβ40)x10] was determined using the V-PLEX Aβ Peptide Panel 1 (6E10) Kit (Meso Scale Discovery, Rockville, MD). The analyses were performed by board-certified laboratory technicians using established procedures for quality control and run acceptance. CSF ELISA-negative (CSF⁻) or -positive (CSF⁺) subjects (Table 1) were selected for validation.

Spectral detection of amyloid

Conformationally sensitive fluorescent probes that specifically bind amyloid have been widely used for the investigation of protein aggregation. Some of these probes have the additional property of detecting soluble oligomeric species and alter their emission spectra depending on the protein aggregate to which they bind, allowing them to identify minute deposits or subtle changes in conformation. K114 was selected as a best conformationally-sensitive fluorescent probe for our assay based on our preliminary studies with several candidates. PBMCs were stained with 300 µM K114 ((*trans,trans*)-1-bromo-2,5-bis(4-hydroxystyryl)benzene, Tocris Bioscience)) at pH 10.5 in 0.1 M $NaHCO_3$-NaOH buffer containing 20% DMSO and 50% Fluoromount/Plus mounting medium. Stained PBMC smears were imaged with a Nikon C2si 32 channel spectral confocal microscope using 405 nm excitation. Emission spectra were collected from 400 to 720 nm in 10 nm increments. Images were manually masked to select PBMCs for analysis; contaminating RBCs, platelets, large cellular aggregates and non-cellular debris were excluded. Spectral images were analyzed using custom software written by PKS (ImageTrak: https://www.ucalgary.ca/styslab/imagetrak).

Results

Fluorescence spectroscopy of K114 in human AD brain

Pathologically confirmed human brain samples were used to define the spectral characteristics of K114-labeled amyloid as a typical K114-positive AD plaque spectrum. Our optimized fluorescent amyloid probe staining method with K114 showed morphologically very clear senile plaques with red-shifted emission spectra in the human brains (Fig. 1A). "Truecolour" images, calculated from the spectral confocal micrographs to approximate the actual colour that the naked eye would perceive if viewing the sample through the microscope oculars, showed typical human senile plaques brightly stained with K114 (Fig. 1A, left panels). Each pixel in the spectral image contains 32 channels of spectrally resolved data ranging from 400 to 720 nm with 10 nm bins. Two extreme fluorescence spectra, from healthy control (HC) brain background (very blue-shifted non-pathological background (violet trace)) and AD plaque (very red-shifted plaque core (red trace)) spectra are extracted from spectral images of human subjects (Figure 1B). The spectral pseudocolour image shows an AD brain plaque with a large spectral shift between core and rim (Fig. 1A, right panels). The deposition of β-sheet-rich amyloid in the plaques caused an intensity increase and a marked red-shift of emitted fluorescence. This shift exhibited notable spectral variation even within a single plaque, which is indicative of intraplaque heterogeneity of amyloid structure with various degrees of amyloid aggregation varying continuously from plaque periphery to plaque core.

Spectral scatter analysis (SSA)

In order to analyze and quantitate these information-rich spectral micrographs, a novel method of analyzing spectral image data was developed, which we termed Spectral Scatter Analysis (SSA), allowing for high sensitivity and specificity. Briefly, spectral image data were divided into square kernels (0.86 μm^2), from which an overall fluorescence intensity and averaged spectrum are calculated, together with a continuous index based on spectral shape, representing the extent of amyloid pathology in each kernel (index 0 is normal, index 1 is mature pathological amyloid). Pixel intensities were plotted against spectral shape (computed as a single scalar index, with higher values indicating a more red-shifted spectrum) in a 2D scatterplot, from which an overall score is calculated representing an "amyloid load" based on a specific subpopulation of kernels as shown in the green ROI of Fig. 1C. The main cluster in both HC and AD graphs represents the majority of background pixels in the brain parenchyma. Notably, the AD samples exhibited a unique additional collection of points extending upwards (higher intensity) and to the right (redder spectra, higher index), representing the plaques seen only in the AD brains. Selecting the AD-specific pixels (green rectangle in Fig. 1C), and applying the same ROI for all samples, allowed us to calculate objective statistics representing the "amyloid load" as brain plaque amyloid (brain plaque amyloid = index × intensity) in each sample. These results are plotted in Fig. 1D, indicating a highly significant difference between HC and AD as expected, mainly driven by plaque deposition in the latter.

Fluorescence spectroscopy of K114 in human PBMCs

The previous examples from brain illustrate our approach for quantitating amyloid load in biological samples. We hypothesized that circulating leukocytes could act as "sentinels", reflecting the amyloid status of diseased brain. To test this idea, we examined K114-labeled PBMCs from HC and MCI/AD patient blood to see if amyloid-like spectral shifts could be detected. In contrast to brain images, where bright plaques are easily seen, K114-labeled human PBMC truecolour images from HC vs AD patients are not distinguishable visually (Fig. 2A, left panels). *Fig. 2B illustrates* that in PBMCs, the spectral differences between HC and AD samples are less pronounced compared to brain, accounting for the similarity in the truecolour image appearance. Importantly, however, although spectral differences were more subtle, PBMC spectra from AD subjects also exhibited a red-shift, as in brain, which is a characteristic of this probe when binding to Aβ (Levine 2005). Pseudocolour images, designed to emphasize subtle spectral shifts, revealed clear differences between HC and AD, quantitatively shown by SSA analysis in Fig. 2C. Although much more subtle, similarly to brain, PBMCs from AD subjects tended to exhibit red-shifted spectra (right-shifted distributions along the horizontal index axis) whose intensities were higher than corresponding pixels from control subjects (green ROI in Fig. 2C). However, although the patterns were similar to those in brain, the extent of spectral red-shift and intensity increase was less pronounced in PBMCs from AD

compared to HC subjects. This likely mirrors differences in quantity and state of amyloid from the two types of sample.

PBMC spectral analysis and CSF biomarkers

To determine how well the spectral amyloid assay could detect disease, we analyzed the PBMC score by using the enhanced quantitative method based on our preliminary study and compared with CSF biomarkers. Subject characteristics, PBMC scores and CSF biomarkers are reported in Table 1. Mean ages did not differ between the groups. Established AD CSF biomarkers showed characteristic profiles of reduced Aβ42 and increased t-Tau and p-Tau in the clinical MCI and AD subjects, whereas the HC group had the expected higher levels of Aβ42 and low levels of t-Tau and p-Tau as previously reported (PMID: 27068280).

As an initial gauge of the performance of our fluorescent method, we plotted PBMC scores against single CSF biomarker status based on previously published cut off values (Table 1), independent of clinical diagnosis (all 90 subjects included). Although PBMC scores were significantly different between control and disease cohorts for each of the CSF Aβ42 (Fig. 3A), t-Tau (Fig. 3B) and p-Tau (Fig. 3C) single biomarker grouping, the receiver operating curves resulted in modest AUCs (0.71 - 0.74 range; Fig. 3D). Plots of continuous sensitivities, specificities and Youden indexes as a function of various cutoffs were shown in Fig. 3E (Aβ42) and F (t-Tau). All three comparisons grouped by CSF Aβ42, t-Tau and p-Tau showed very similar diagnostic accuracy (dashed line, used as a baseline in later analyses). Interestingly, near-optimal Youden indexes are found for a narrow cutoff in the Aβ42-based group (Fig. 3E) compared to a relatively wider range of peaks in both t-Tau- (Fig. 3F) and p-Tau- (data not shown) based groups. The limitation of this analysis includes the potential uncertainty of clinical diagnosis of AD-related cognitive impairment particularly in the earlier stages of MCI, nor do we know *a priori* whether our assay mirrors clinical dementia, brain amyloid status independent of functional cognitive decline, or a combination of both. To shed further light on this question, we next analyzed the PBMC scores from subjects who were CSF biomarker-negative, clinically HC and those who were biomarker-positive, clinically MCI, as MCI subjects are considered pre-dementia and later often progress to AD (ref)-(Fig. 4). Comparing CSF biomarker-negative to CSF biomarker-positive subjects within this restricted cohort (AD subjects excluded) yielded statistically significant differences in the PBMC score once again (Fig. 4A-C), and with AUCs improving markedly to the high 0.8 range; notably, classification by clinical HC vs. MCI and by CSF p-Tau biomarkers yielded a PBMC score AUC approaching 0.9 (Fig. 4D compared to 3D) as well as a Youden index improving from 0.45 (Fig. 3 E and F) to 0.72 (Fig. 4E and F).

Combining CSF markers often improves diagnostic accuracy (PMID: 22078172), therefore, we next evaluated the PBMC score grouped based on combination of CSF Aβ and tau. A recent study showed ratios of Aβ42/Aβ40 and Aβ42/t-Tau are very reliable and have high diagnostic accuracy (PMID: 31010420). Comparing either Aβ42/Aβ40- or Aβ42/t-Tau-negative to Aβ42/Aβ40- or Aβ42/t-Tau -positive PBMC score groups with all

HC, MCI and AD subjects yielded statistically significant differences with much higher statistical significance for the Aβ42/t-Tau comparison (Fig. 5B). However, when we analyzed the diagnostic accuracies for these two combined CSF biomarkers, the AUCs did not differ from the analyses with single CSF biomarkers (Aβ42, t-Tau or p-Tau alone) (Fig. 5D compared to 3D). The prevailing view of AD pathogenesis has been that initial Aβ precipitation in the disease process triggers a detrimental cascade involving tau pathology and neurodegeneration for many years. However, accumulating evidence now suggests that both Aβ and Tau pathologies have synergistic effects (PMID: 32778792), and thus combining these two biomarkers could improve diagnostic reliability. Therefore, we calculated combined Aβ42 and t-Tau metrics based on the hypothesis that Aβ and Tau synergistically represent a patient's disease severity (Fig. 5C; 550/Aβ42 + t-Tau/400). Interestingly, the PBMC score grouped by this method alone, without considering clinical status showed markedly higher AUC (Fig. 5D) and Youden index (Fig. 5F) compared to Aβ42/Aβ40- or Aβ42/t-Tau-grouped comparisons (Fig. 5E). When we further analyzed the PBMC score using combined CSF biomarker-negative and -positive groups (using the above CSF metrics) only in clinically established HC and MCI subjects (Fig. 6), comparing combined Aβ42&t-Tau -negative to -positive within HC and MCI subjects (AD subjects excluded) showed the highest group separation (Fig. 6D and F).

Discussion

Whereas analyzing post-mortem brain is the *de facto* standard for AD diagnosis, there is no non-invasive clinical test to reliably establish the diagnosis with certainty. Biological fluid testing, including CSF and other developing blood-based analyses, rely on quantification of the monomeric units of amyloid, either Aβ or Tau, and on their phosphorylation state. While monomer status is strongly correlated with amyloid state, it is not a direct measurement of amyloid status. In this study, we introduce a novel blood AD biomarker based on fluorescence microspectroscopy of the amyloid-binding molecular probe K114 after staining of circulating blood mononuclear cells (PBMCs). These circulating phagocytic cells are known to ingest amyloid while traversing the brain microvasculature. The AD brain parenchyma accumulates two main amyloid species: misfolded/aggregated Aβ and Tau. Interestingly, when we measured total levels of these proteins in PBMCs using conventional immunocytochemical methods that generally do not distinguish between normally folded vs. misfolded/aggregated proteins, we did not detect quantitative differences between controls and MCI/AD subjects (Fig. X, upcoming IHC). We reasoned that it may not be the total amounts of Aβ and Tau proteins contained in these cells, but rather, amounts of misfolded, aggregated β-sheet-rich species that could detect AD brain pathology. A number of fluorescent probes have been developed that bind to and are relatively specific for β-sheet amyloids. Congo Red and Thioflavin-T are the best known, but a variety of other probes with very dissimilar structures have also been reported. We chose K114 because it is a bright, high-affinity probe for a variety of amyloids, including aggregated Aβ and Tau. Importantly, K114 undergoes a shift in its emission spectrum when binding amyloids, which allows detection by spectral shape, rather than absolute fluorescence intensity alone, which is very difficult to control. Staining PBMCs with this spectrally variable probe allowed us to compute a score, based on relative intensity and spectral shape. The initial analysis using this PBMC fluorescence

score to identify MCI and/or AD compared to HC was very encouraging and showed similar diagnostic accuracy of CSF t-Tau and p-Tau, but lower than Aβ42 and combined CSF biomarkers (Table 1), which suggests our novel assay may correlate better with CSF Tau markers than Aβ markers.

The trend of better correlation between our novel blood AD test and CSF Tau markers was observed throughout this study, even when subjects were classified based on single CSF biomarker positivity, independent of clinical diagnosis. Interestingly, this trend was more prominent when we compared only the clinically normal subjects (HC) and subjects with MCI, including possibly pre-clinical AD but not symptomatic AD subjects (Table 2). This suggests our PBMC method may be better at detecting early stages of AD (in the MCI, MBI (or even pre-clinical) rather than already established late-stage AD. This confers an important advantage since recent data suggest that disease-modifying therapies are likely to be most effective when started early in the disease (refs).

The highest diagnostic accuracy with single CSF biomarkers was shown when the subjects were classified by p-Tau within the restricted (HC and MCI) cohorts. Our results are consistent with recent studies that reported increased plasma p-Tau181 levels in the preclinical stage of AD (PMID: 33169916), and strong association of tau phosphorylation state changes with neurodegenerative and clinical markers of disease, occurring as early as the initial increases in aggregated Aβ (PMID: 32161412).

Grouping the PBMC score based on the combination of both Aβ and Tau CSF biomarkers improved the diagnostic accuracy overall compared to the grouping by any single CSF biomarker (Fig. 6). We attempted three different combinations: Aβ42/Aβ40, Aβ42/t-Tau and Aβ42&t-Tau. The highest diagnostic accuracy was found when subjects were classified by combined Aβ42 & t-Tau metrics based on the hypothesis that Aβ and Tau synergistically reflect disease severity. This may represent unique characteristics of our novel spectroscopy blood AD detection method that are able to report overall amyloid load, including both Aβ and Tau, which is suggested by the best results from a combined Aβ42 & t-Tau metric (Fig. 6C, D and F).

Together, these results indicate that our method is capable of sensitive and potentially AD-specific detection of protein misfolding/aggregation in circulating PBMCs. These data also support our hypothesis that circulating leukocytes act as "sentinels" with the potential ability to mirror brain amyloid status in a semi-quantitative manner.

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