Earliest accumulation of β-amyloid occurs within the default-mode network and concurrently affects brain connectivity

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It is not known exactly where amyloid-β (Aβ) fibrils begin to accumulate in individuals with Alzheimer’s disease (AD). Recently, we showed that abnormal levels of Aβ42 in cerebrospinal fluid (CSF) can be detected before abnormal amyloid can be detected using PET in individuals with preclinical AD. Using these approaches, here we identify the earliest preclinical AD stage in subjects from the ADNI and BioFINDER cohorts. We show that Aβ accumulation preferentially starts in the precuneus, medial orbitofrontal, and posterior cingulate cortices, i.e., several of the core regions of the default mode network (DMN). This early pattern of Aβ accumulation is already evident in individuals with normal Aβ42 in the CSF and normal amyloid PET who subsequently convert to having abnormal CSF Aβ42. The earliest Aβ accumulation is further associated with hypoconnectivity within the DMN and between the DMN and the frontoparietal network, but not with brain atrophy or glucose hypometabolism. Our results suggest that Aβ fibrils start to accumulate predominantly within certain parts of the DMN in preclinical AD and already then affect brain connectivity.
Accumulation of aggregated amyloid-β (Aβ) in the brain is believed to be the first pathological mechanism of Alzheimer’s disease (AD)\(^1\). Previous studies have shown that cerebral deposition of Aβ fibrils can occur for decades before an individual with AD reaches the dementia stage\(^2\). The widespread cerebral distribution of Aβ in later AD stages is well-established\(^3,4\), however it has been difficult to accurately identify the initial brain regions of Aβ accumulation due to the long time-lag between the start of the pathophysiology and onset of symptoms.

A neuropathological staging of different Aβ phases in AD has been proposed based on post-mortem examinations and it has been shown that the first phase is characterized by Aβ deposits throughout the frontal, parietal, temporal, and occipital neocortex\(^5\). Studies employing positron emission tomography (PET) to visualize fibrillar Aβ deposits have also attempted to identify early Aβ-accumulating regions. For example, when analyzing the earliest data from AD mutation-carriers, most cortical areas except for the sensorimotor cortex showed Aβ accumulation as early as 15 years before the expected onset of symptoms\(^6\). A shortcoming of such studies is the cross-sectional design, which precludes conclusions about longitudinal events. Another issue is that it has previously been difficult to identify the earliest preclinical AD cases; when mixing such cases with later asymptomatic AD cases that have been accumulating Aβ fibrils for years, the cortical sites of accumulation of Aβ identified are widespread.

In the present study, we applied a novel methodology of combining data obtained regarding Aβ levels in the brain via PET, and levels of the Aβ42 peptide in cerebrospinal fluid (CSF), to identify individuals who have just recently started to accumulate Aβ in the brain. Previous cross-sectional studies indicate that Aβ42 levels decrease in the CSF as Aβ starts to accumulate, before fibrillar plaques can be detected with PET\(^7-11\). Recently, this was confirmed longitudinally in a cohort of more than 400 non-demented individuals where we found that those with abnormally low CSF levels of Aβ42, but with still normal Aβ levels detected by PET (CSF+/PET−), accumulated Aβ fibrils longitudinally at a similar rate as those who had both abnormal CSF levels of Aβ42 and abnormal levels of Aβ detected by PET (CSF+/PET+) and four times higher than those with normal biomarker results on both parameters (CSF−/PET−)\(^12\). This indicates that individuals with AD may first be defined as being CSF+/PET−, and then progress to CSF+/PET+. It might therefore be possible to identify the earliest stages of preclinical AD by

### Table 1 Baseline characteristics of the ADNI cohort

|                          | Entire ADNI sample | A CSF−/PET− | B CSF+/PET− | C CSF+/PET+ | p-value |
|--------------------------|--------------------|------------|-------------|-------------|---------|
| N                        | 473                | 218        | 59          | 191         | A-B = 0.58 |
| Baseline CN/MCI          | 37%/63%            | 47%/53%    | 44%/56%     | 23%/77%     | A-B = 0.99 |
| Age (years)              | 72.0 (7.0)         | 71.0 (7.0) | 71.2 (8.1)  | 73.2 (6.3)  | A-B = 0.99 |
| Gender (women)           | 47%                | 47%        | 46%         | 48%         | A-B = 0.70 |
| Education (years)        | 16.5 (2.6)         | 16.7 (2.6) | 17.0 (2.2)  | 16.1 (2.6)  | A-B = 0.99 |
| Presence of the APOE ε4 allele | 40%            | 16%        | 46%         | 65%         | A-B = 0.47 |
| MMSE (0–30 points)       | 28.4 (1.7)         | 28.8 (1.4) | 28.8 (1.3)  | 27.9 (1.8)  | A-B = 0.99 |
| Baseline neocortical composite florbetapir SUVR\(^1\) | 0.90 (0.21) | 0.74 (0.05) | 0.78 (0.06) | 0.93 (0.09) | A-B = 0.99 |
| Years between florbetapir PET scans (range) | 2.0 (0.9–4.1) | 2.0 (0.9–4.1) | 2.0 (1.9–4.1) | 2.0 (1.7–3.0) | A-B = 0.92 |
| Years between FDG PET scans (range) | 2.0 (0.8–3.1) | 2.0 (0.8–2.7) | 2.1 (1.9–3.1) | 2.0 (1.8–3.0) | A-B = 0.92 |
| Years between MRI scans (range) | 2.0 (0.4–4.1) | 2.0 (0.4–4.1) | 2.0 (0.5–4.1) | 2.0 (0.6–3.1) | A-B = 0.92 |
| Hippocampus volume (cm\(^3\)) | 7.2 (1.1) | 7.5 (1.0) | 7.5 (0.9) | 6.9 (1.3) | A-B = 0.92 |
| CSF Aβ42 (ng/L)          | 186 (52)           | 234 (27)   | 165 (23)    | 136 (22)    | A-B = 0.92 |
| CSF T-tau (ng/L)         | 79 (46)            | 59 (26)    | 60 (31)     | 109 (53)    | A-B = 0.92 |
| CSF P-tau (ng/L)         | 39 (23)            | 28 (12)    | 30 (26)     | 54 (25)     | A-B = 0.92 |

CN cognitively normal, CSF cerebrospinal fluid, CSF−/− abnormal/normal CSF Aβ42 levels, MCI mild cognitive impairment, MMSE Mini Mental State Examination, N number of subjects, PET−/− abnormal/normal florbetapir SUVR, SD standard deviation, SUVR standardized uptake value ratio (using a composite reference region) corrected for partial volume errors. Comparison between non-accumulators (CSF−/PET−), early Aβ accumulators (CSF+/PET−), and late Aβ accumulators (CSF+/PET+). Values are given in mean (SD) if not otherwise specified. Groups were compared with Mann–Whitney U statistics if significant after the Kruskal–Wallis test. Significant p values are in bold. The CSF−/PET+ group was not included in this table as a separate group since they were not used in any analysis and they were also too few to be used in the group comparisons (N = 5).
studying individuals with abnormal CSF Aβ42 and normal Aβ PET (CSF+/PET−, also referred to as early Aβ accumulators to indicate their stage in the accumulation of Aβ).

The main hypothesis in the present study was that Aβ fibrils are more prone to start aggregating in certain regions of the brain before they can be found throughout the neocortex, and before neurodegeneration is present. Identifying these regions and examining their properties can provide important information about why Aβ aggregation starts in sporadic AD, which is a crucial step for developing disease-modifying drugs. The aim of the study was therefore to identify such regions by comparing the longitudinal fibrillar Aβ deposition rates in different brain regions of early Aβ accumulators (termed CSF+/PET−) with non-accumulators (termed CSF−/PET−). To avoid bias from non-optimal cut-offs for CSF Aβ42 levels, we also examined the correlation between continuous CSF Aβ42 levels and the regional Aβ PET signal in PET negative subjects. We performed the analyses in 68 predefined anatomical regions as well as employing voxelwise statistics. The analyses were performed both in a main cohort and an independent validation cohort. As the main cohort, we used the North American Alzheimer’s Disease Neuroimaging Initiative (ADNI) and included all non-demented participants with CSF Aβ42 and longitudinal Aβ PET data (473 individuals). The Swedish BioFINDER study was used as the validation cohort, from which we included all non-demented participants with baseline CSF Aβ42 and Aβ PET data (406 individuals).

We also examined early regional Aβ accumulation in the presumably earliest Aβ accumulators; those with normal CSF Aβ42 and normal Aβ PET at baseline who converted to abnormal CSF Aβ42 levels at follow-up but still had a normal Aβ levels identified by PET scan (CSF−/PET to CSF+/PET−). Consistently, using different cohorts and methods, we identified a specific set of early Aβ-accumulating regions, which largely corresponded to a functional brain network, the default mode network (DMN). The relationship between early stage Aβ accumulation and whole-brain functional connectivity was examined in detail and revealed that hypococonnectivity, especially within the DMN and between DMN and the frontoparietal network, was associated with low CSF Aβ42 levels in individuals that still had normal Aβ PET scans.

To test the hypothesis that early Aβ accumulation is an upstream mechanism that precedes overtly decreased energy metabolism and cell death, we compared the longitudinal changes in 18F-fluorodeoxyglucose (FDG) PET and volumetric magnetic resonance imaging (MRI) in the different groups. Finally, we examined the validity of using CSF/PET groups to stratify Aβ stages by comparing early (CSF+/PET−) and late (CSF+/PET+) accumulators to identify known late regions of Aβ accumulation, including the sensorimotor cortex.

**Results**

**Experimental outline and baseline characteristics.** We first analyzed the longitudinal results from the ADNI cohort, then replicated the result in the BioFINDER cohort and finally analyzed functional connectivity in BioFINDER.

Baseline characteristics of the ADNI sample and the different CSF/PET groups are provided in Table 1. Early stage Aβ accumulators (CSF+/PET−) had significantly higher prevalence of the APOE ε4 genotype and a slightly higher neocortical florbetapir standardized uptake value ratio (SUVR), but similar baseline cognition, hippocampal volume, and CSF tau levels as non-accumulators (CSF−/PET−) (Table 1). By contrast, the non-demented late stage Aβ accumulators (CSF+/PET+) showed, at baseline, signs of neurodegeneration with hippocampal atrophy, higher CSF tau levels and worse cognition, and had per definition significantly increased florbetapir SUVRs (Table 1).

**Identifying early Aβ-accumulating regions.** To identify early Aβ accumulation regions, longitudinal voxelwise comparisons of accumulation rates (SUVR/year) were performed between CSF+/PET− (early Aβ accumulators) and CSF−/PET− (non-accumulators). The results are shown in Fig. 1a (see Supplementary Fig. 1 for whole-brain axial images). The brain regions showing significant increases in Aβ PET signal over time in CSF+/PET− cases included the medial orbitofrontal cortex, anterior cingulate cortex, posterior, and isthmus cingulate cortex, precuneus and to a lesser extent temporal regions. Similar significant regions were identified by correlating CSF Aβ42 levels with the accumulation rates in PET− subjects to remove potential biases from the Aβ42 cut-off (Fig. 1b). In the region of interest (ROI)-based

**Fig. 1** Regions of Aβ accumulation from longitudinal voxelwise analyses in ADNI. **a** shows the regions where Aβ fibrils start to accumulate by comparing the annual florbetapir SUVR rate during 2 years between early stage Aβ accumulators (CSF+/PET−, n = 59) and non-accumulators (CSF−/PET−, n = 218). The lateral and medial projections in **a** show that the most significantly increased accumulation rate among the early Aβ accumulators was located in the posterior cingulate cortex, the precuneus, and the medial orbitofrontal cortex. A PET-data-derived ROI of these early-accumulating Aβ regions is available at http://biofinder.se. **b** confirms the regions in **a** without biases from a specific CSF Aβ42 cut-off. Here, we performed voxelwise correlations between annual florbetapir SUVR rates and CSF Aβ42 levels in Aβ PET negative individuals (n = 277). To contrast the early stage Aβ regions, **c** shows the regions with significantly increased annual SUVR rate in late stage Aβ accumulators (CSF+/PET+, n = 191) compared with non-accumulators (CSF−/PET−). A widespread pattern of Aβ accumulation is seen in these non-demented CSF+/PET+ subjects. Voxelwise two-sample t-tests were used and all analyses in **a-c** are adjusted for age and gender. The significant threshold was set at p < 0.001. The red and yellow colors illustrate significant t values according to the scale on the left
comparisons, 15 of 68 cortical regions had an increased rate in the CSF+/PET− subjects compared to the CSF−/PET− subjects (Table 2 and Supplementary Movie 1). The significant differences were in agreement with the voxelwise analyses and were mainly seen in the orbitofrontal cortex, precuneus, insula, and the posterior/isthmus cingulate cortex. On average the yearly Aβ accumulation rate of the CSF+/PET− group was ~4–5 times that of the CSF−/PET− group in the significant brain regions (Table 2). All results were corrected for multiple comparisons and adjusted for gender, age, and time between PET scans. To account for atrophy as a confounding factor, we also adjusted all significant models for the annual change in cortical thickness of each ROI. After this adjustment, all 15 regions still differed significantly between the two groups. The 15 early Aβ regions were also significant when adjusting for diagnosis (MCI or healthy control). Finally, we adjusted for APOE genotype to examine if different early Aβ patterns were observed depending on whether one carried an APOE ε4 allele, but the same regions as in Table 2 were found (details shown in Supplementary Table 1). The distinct pattern of early Aβ accumulation (Fig. 1a, b, Table 2) can be contrasted to the more global Aβ accumulation pattern seen in late, but still non-demented, Aβ accumulators (CSF+/PET+) compared with non-accumulators (CSF−/PET−) (Fig. 1c).

**Table 2 Annual Aβ accumulation rates in the significant early Aβ regions in ADNI**

| Region                              | CSF+/PET− | CSF−/PET− | p-value |
|-------------------------------------|-----------|-----------|---------|
| Posterior cingulate cortex, right   | 2.7% (1.8–3.6) | 0.9% (0.5–1.3) | 0.000051 |
| Medial orbitofrontal cortex, left   | 2.5% (1.4–3.6) | 0.4% (−0.2–1.0) | 0.0026 |
| Medial orbitofrontal cortex, right  | 2.5% (1.3–3.7) | 0.4% (−0.1–1.0) | 0.00038 |
| Precuneus, left                     | 2.3% (1.4–3.3) | 0.7% (0.2–1.2) | 0.00051 |
| Rostral anterior cingulate cortex, right | 2.0% (1.3–2.8) | 0.5% (0.0–1.0) | 0.00081 |
| Lateral orbitofrontal cortex, left  | 1.8% (0.9–2.7) | 0.3% (−0.1–0.7) | 0.0011 |
| Insula, right                       | 1.8% (0.8–2.8) | 0.2% (−0.3–0.7) | 0.0025 |
| Isthmus cingulate cortex, left      | 2.0% (1.1–2.8) | 0.5% (0.0–1.0) | 0.0027 |
| Rostral anterior cingulate cortex, left | 1.9% (1.1–2.6) | 0.6% (0.1–1.1) | 0.0033 |
| Isthmus cingulate cortex, right     | 2.1% (1.2–2.9) | 0.5% (0.0–1.0) | 0.0032 |
| Precuneus, right                    | 1.9% (1.1–2.8) | 0.6% (0.2–1.1) | 0.0041 |
| Superior frontal cortex, left       | 1.2% (0.5–2.0) | 0.2% (−0.2–0.5) | 0.0053 |
| Transverse temporal gyrus, right    | 0.8% (−0.3–1.9) | −0.8% (−1.5–0.2) | 0.0074 |
| Insula, left                        | 1.5% (0.7–2.3) | 0.2% (−0.3–0.7) | 0.0085 |
| Posterior cingulate cortex, left    | 2.1% (1.2–3.0) | 0.9% (0.5–1.4) | 0.01 |

Comparative Aβ accumulation rates are shown in Table 2 and Supplementary Movie 1. The signiﬁcant differences were observed depending on whether one carried an APOE ε4 allele, but the same regions as in Table 2 were found (details shown in Supplementary Table 1). The distinct pattern of early Aβ accumulation (Fig. 1a, b, Table 2) can be contrasted to the more global Aβ accumulation pattern seen in late, but still non-demented, Aβ accumulators (CSF+/PET+) compared with non-accumulators (CSF−/PET−) (Fig. 1c).

**Validation of our Aβ staging by identifying late Aβ regions.** We have used the CSF+/PET− subjects as a proxy for early stage Aβ accumulators and compared them with CSF−/PET− subjects (non-accumulators) to identify early Aβ regions. If our CSF/PET groups are valid for staging Aβ phases, we should also be able to compare early Aβ accumulators and CSF+/PET+ subjects (our proxy for late stage Aβ accumulators) to identify known regions of late Aβ accumulation. The results from the ROI-based comparisons are shown in Table 3 and Supplementary Movie 2. The largest differences were seen in the precentral, postcentral, paracentral, pericalcarine, and lingual regions. This means that our proxy group for late Aβ accumulation (CSF+/PET+) had a higher accumulation rate of the Aβ PET signal over time compared with early Aβ accumulators (CSF+/PET−) around the sensorimotor cortex and occipital lobe. Note that these significant, late Aβ regions did not overlap with the early Aβ regions (compare Tables 2 and 3). The corresponding voxelwise
comparisons between early and late accumulators are shown in Fig. 3 and highlight similar late regions (greatest significant difference in the sensorimotor cortex and parts of the occipital lobe).

Atrophy and glucose metabolism in relation to Aβ accumulation. To test the hypothesis that atrophy, as a marker of neuronal and synaptic degeneration, starts after the initiation of Aβ accumulation, we compared the longitudinal change in gray matter (GM) volumes between the CSF/PET groups using voxel-based morphometry (VBM) analysis (Fig. 2A). No clear atrophy pattern was seen in early Aβ accumulators (CSF+/PET−) compared with non-accumulators (CSF−/PET−) (Fig. 4a). In contrast, late Aβ accumulators (CSF+/PET+) exhibited the expected longitudinal atrophy pattern of over time involving mainly temporoparietal regions when comparing them to non-accumulators (Fig. 4b) and early Aβ accumulators (Supplementary Fig. 2A). Similar analyses were made with longitudinal FDG PET data to examine changes in glucose metabolism in relation to early Aβ accumulation. There were no differences in FDG uptake over time between non-accumulators (CSF−/PET−) and early Aβ accumulators (CSF+/PET−) (Fig. 4c), while a cortical metabolic reduction in the temporal lobe was seen in late Aβ accumulators (CSF+/PET+) compared with non-accumulators (CSF−/PET−) (Fig. 4d) and early Aβ accumulators (CSF+/PET−) (Supplementary Fig. 2B).

Replication of the early Aβ regions in the BioFINDER cohort. To test the generalizability of our results we replicated the early Aβ accumulation regions in the BioFINDER cohort. Only cross-sectional data was available from the BioFINDER study. Although longitudinal comparisons are preferred given our hypothesis, regional differences in Aβ deposition should still be noticeable when comparing CSF+/PET− and CSF−/PET− subjects cross-sectionally. A strength of this cohort is that another Aβ PET ligand (18F-flutemetamol) as well as another immunoassay for CSF Aβ (INNOTEST) were used, and the results would therefore be more generalizable if they were replicable in this cohort. The characteristics of BioFINDER is provided in Supplementary Table 2. Similar early Aβ accumulation regions were identified as in ADNI when comparing cross-sectional 18F-flutemetamol SUVs between CSF+/PET− and CSF−/PET− subjects using ROI-based analyses in BioFINDER (Supplementary Table 3). The most significant regions were found in the orbitofrontal cortex, the posterior cingulate cortex, and the precuneus (Supplementary Table 3, which can be compared with ADNI data in Table 2). Further, the cross-sectional voxelwise comparisons between CSF+/PET− and CSF−/PET− subjects in BioFINDER showed significant changes predominantly in the orbitofrontal cortex and posterior cingulate similar to the longitudinal analyses in ADNI (Fig. 5, compare with ADNI data in Fig. 1a, b).

Early Aβ accumulation and functional connectivity. Resting-state functional MRI was available in the BioFINDER study and this cohort was therefore used for this analysis. To examine the association between early Aβ accumulation and functional networks beyond the anatomical similarities (Fig. 2) we correlated
levels of CSF Aβ42 with whole-brain connectivity (Fig. 6). The groups of interest were the early Aβ accumulators (CSF+/PET−) and biomarker normal individuals with close to abnormal CSF Aβ42 (CSF−/PET−). In the CSF+/PET− group we found a significant network component where reduced connectivity was associated with decreasing levels of CSF Aβ42. The component predominantly consisted of intra-DMN links, but also links between the DMN and the frontoparietal network (Fig. 6a). The summed connectivity on the significant correlation component changed most drastically when CSF Aβ42 levels were closer to the cut-off (400–516 ng/L for the INNOTEST ELISA) and seemed to reach a floor effect as Aβ42 reached levels of around 350 ng/L.

Figure 6c depicts the intra-DMN links, which dominate the significant hypoconnectivity component correlating with lower CSF Aβ42 values in the early Aβ accumulators. Furthermore, a significant network component involving the DMN was associated with CSF Aβ42 in the CSF−/PET− group, but with fewer constituent links (Fig. 6b). Interestingly, the correlation was in the opposite direction showing an increased connectivity as CSF Aβ42 levels dropped from normal levels towards the abnormal cut-off. In summary, Fig. 6 indicates that when CSF Aβ42 levels drop within the near abnormal range they might be associated with an increased connectivity in connections involving the DMN. Then, when CSF Aβ42 levels become abnormal and decrease further as Aβ accumulates, they are instead clearly associated with a decreased connectivity within the DMN and mainly between the DMN and the frontoparietal network. The associations with hypoconnectivity and hyperconnectivity were similar when adjusting for CSF P-tau and T-tau, indicating the independent effect of Aβ on the connectivity (Supplementary Fig. 3).

Discussion

This study examined where Aβ fibrils are more prone to start accumulating, by comparing the longitudinal outcome of nondemented subjects with early signs of Aβ accumulation (CSF+/PET−) to those with no measurable signs of Aβ accumulation (CSF−/PET−), in a large cohort of 473 subjects. The results showed significantly increased rates of Aβ fibril accumulation predominantly in the precuneus, posterior cingulate cortex, and orbitofrontal cortex in this early Aβ stage. When examining subjects with even earlier signs of Aβ accumulation (CSF−/PET− subjects who converted to CSF+/PET− within 2 years), significantly increased Aβ fibril accumulation rate was again seen in the medial orbitofrontal and posterior cingulate cortex, compared with stable CSF−/PET− subjects. These regions were replicated in an independent cohort (n = 406). To our knowledge, this is the

| Region               | CSF+/PET− | CSF+/PET+ | p-value |
|----------------------|-----------|-----------|---------|
| Precentral, right    | 0.1% (−0.5–0.7) | 2.1% (1.7–2.6) | 0.000002 |
| Postcentral, right   | 0.1% (−0.7–0.9) | 2.3% (1.8–2.7) | 0.000005 |
| Pericalcarine, left   | 0.2% (−0.8–1.3) | 2.8% (2.2–3.6) | 0.000006 |
| Postcentral, left     | 0.2% (−0.5–1.2) | 2.4% (1.9–2.8) | 0.000008 |
| Precentral, left      | 0.3% (−0.3–1.0) | 2.3% (1.9–2.9) | 0.000024 |
| Paracentral, left     | 0.6% (−0.2–1.4) | 2.4% (1.9–2.8) | 0.000057 |
| Lingual, left         | 0.5% (−0.7–1.9) | 2.8% (2.2–3.4) | 0.00019 |
| Lateral occipital, left | 0.4% (−0.4–1.3) | 2.3% (1.8–2.9) | 0.00026 |
| Pericalcarine, right  | 0.4% (−0.6–1.5) | 2.9% (2.3–3.6) | 0.00033 |
| Paracentral, right    | 0.6% (−0.2–2.1) | 2.2% (1.7–2.7) | 0.00038 |
| Fusiform, left        | 1.1% (0.3–2.0) | 2.4% (2.0–2.8) | 0.00049 |
| Lingual, right        | 0.6% (−0.4–1.8) | 2.8% (2.2–3.5) | 0.0006 |
| Cuneus, left          | −0.3% (−1.2–0.8) | 2.0% (1.4–2.8) | 0.00062 |
| Middle temporal, left | 1.1% (0.3–2.0) | 2.2% (1.9–2.6) | 0.0007 |
| Lateral occipital, right | 0.6% (−0.1–1.4) | 2.4% (1.9–3.1) | 0.00088 |
| Fusiform, right       | 0.9% (0.0–1.8) | 2.4% (1.9–2.9) | 0.0017 |
| Inferior temporal, right | 1.3% (0.5–2.2) | 2.4% (1.9–2.8) | 0.0022 |
| Inferior temporal, left | 1.5% (0.7–2.5) | 2.6% (2.2–3.1) | 0.003 |
| Superior temporal sulcus, left | 1.4% (0.6–2.3) | 2.4% (1.9–2.8) | 0.0047 |
| Caudal middle frontal, left | 1.3% (0.5–2.1) | 2.5% (2.0–3.1) | 0.0054 |
| Superior parietal, left | 1.0% (0.2–1.9) | 1.9% (1.5–2.3) | 0.0074 |
| Superior frontal, left | 1.2% (0.5–2.0) | 1.9% (1.5–2.3) | 0.013 |

CSF+ cerebrospinal fluid Aβ42 ≥192 ng/L, PET+ >0.872 SUVR, ROI region of interest, SUVR standardized uptake value ratio (using a composite reference region). Comparisons in ADNI between early Aβ accumulators (CSF+/PET−) and late Aβ accumulators (CSF+/PET+) to identify late stage Aβ accumulation regions. The analysis was performed using general linear models with the Aβ PET SUVR change/year in each ROI as the dependent variable and CSF/PET groups, sex, age, and time between PET scans as covariates. Data are given in mean values (95% CI of the mean) in order of significance and Aβ accumulation is shown in % yearly SUVR change. Only significant regions after the Benjamini & Hochberg correction are shown.
Voxelwise two-sample data and fewer subjects compared to ADNI, which results in less statistical power. Voxelwise two-sample $t$-tests were used and all results in a-d were adjusted for age and gender. The VBM analysis was also adjusted for total intracranial volume. Only voxels with a $p < 0.001$ are shown. The colors illustrate significant $t$ values according to the scales.

**Fig. 4** Group comparisons of annual change in brain volume and glucose metabolism in ADNI. a Voxel-based morphometry (VBM) comparison of annual MRI change over 2 years in early $\beta$ accumulators (CSF+/PET$, n = 59$) compared with non-accumulators (CSF−/PET$, n = 218$). The results are adjusted for age, gender, and intracranial volume. The contrast is reduction in cortical thickness in early compared with non-accumulators and shows no clear pattern of longitudinal atrophy in the early $\beta$ accumulators (CSF+/PET−). b The same VBM analysis as in a, but with a comparison between non-accumulators and late $\beta$ accumulators (CSF+/PET+, $n = 191$). The contrast is reduction in late compared with non-accumulators and shows a typical AD atrophy pattern in the non-demented late $\beta$ accumulators (CSF+/PET+). c Voxelwise analysis of annual FDG PET change over 2 years in non-accumulators ($n = 153$) compared with early $\beta$ accumulators ($n = 41$). No significant difference is seen. d The same FDG PET analysis as above, but with a comparison between non-accumulators and late $\beta$ accumulators ($n = 124$). The contrast is reduction in glucose metabolism in late compared with early $\beta$ accumulators and shows a temporal and to a lesser extent parietal reduction in metabolism in the non-demented late $\beta$ accumulators. Voxelwise two-sample $t$-tests were used and all results in a-d were adjusted for age and gender. The VBM analysis was also adjusted for total intracranial volume. Only voxels with a $p < 0.001$ are shown. The colors illustrate significant $t$ values according to the scales.

**Fig. 5** Replication of the early $\beta$ regions in BioFINDER. Comparison of $^{18}$F-flutemetamol SUVR between early accumulators (CSF+/PET−, $n = 30$) and non-accumulators (CSF−/PET−, $n = 219$) in BioFINDER to identify early $\beta$ regions. The highest significance was seen around the posterior regions of the cingulate cortex and the orbitofrontal cortex similar to the results in ADNI (Fig. 1a, b). Note that the BioFINDER analysis included cross-sectional data and fewer subjects compared to ADNI, which results in less statistical power. Voxelwise two-sample $t$-test was used. The significance threshold was set at $p < 0.001$ and the comparison was adjusted for age and gender. The red/yellow colors illustrate significant $t$ values according to the scale on the left.
and frequent fluctuations in activation and deactivation) and not the neuronal activation per se. Although the core DMN was the most prominent network that we found that had anatomical overlap with the early Aβ regions, we want to point out that involvement of other networks was also evident in the early Aβ accumulation, especially the frontoparietal network (Fig. 2). This indicates that early stage accumulation is not related to a unique feature of the DMN, but in a more general sense to hubs in the brain with high connectivity. This interpretation is supported by previous studies showing an association between Aβ distribution and several networks and network hubs.

The association between functional networks and earliest stages of Aβ accumulation was further examined using functional connectivity analyses in BioFINDER. When we correlated whole-brain functional connectivity and CSF Aβ42 levels in the early stage Aβ accumulators (CSF+/PET−), we found a decreased connectivity in intra-DMN connections and between DMN and the frontoparietal network as CSF Aβ42 decreased (Fig. 6a). The disruption of these connections in BioFINDER is especially interesting given the distribution of early Aβ fibrils in ADNI in the DMN and frontoparietal networks (Fig. 2). This correlation between the disruption of functional connectivity and Aβ pathology has recently been described in AD, but to our knowledge the present study is the first to show that this association is present already at the very earliest preclinical stages of AD when Aβ fibrils are just starting to accumulate. In our second connectivity analysis (Fig. 6b) we examined the correlation between functional connectivity and CSF Aβ42 in those with indications of very early Aβ accumulation (CSF low/PET−). Aβ accumulation is instead associated with hyperconnectivity in similar neuronal connections. Correlation coefficients (r) refer to Spearman correlation between summed connectivity and CSF Aβ42 levels. Age, gender, and APOE e4 status was controlled for by partial correlation. Network components correlating with CSF Aβ42 were calculated using a method similar to the NBS algorithm (see “Methods for the BioFINDER study” for statistical details). Acronyms: BG basal ganglia, CSFlow normal CSF Aβ42 levels close to the abnormal cut-off (517–750 ng/L), DA dorsal attention, DM default mode, FP frontoparietal, FT frontotemporal, HI hippocampus, QC quality control, SM sensorimotor, VA ventral attention, VI visual network.
transgenic AD models suggesting that neurons become hyperactive very early, independently of the deposition of Aβ into plaques, and that the silencing of neurons emerges only later in the disease course\textsuperscript{2}. 

Although altered functional connectivity was found already in the early Aβ accumulators (CSF+/PET−), our longitudinal analyses (Fig. 4a, c) showed that no changes in glucose metabolism or atrophy were present at this stage. However, decreased temporoparietal glucose metabolism and atrophy was observed in the late Aβ accumulators (CSF+/PET++; Fig. 4b, d and Supplementary Fig. 2A, B). This finding can have practical implications for the enrollment in anti-Aβ clinical trials since anti-Aβ agents should be introduced after Aβ accumulation has started but before neurodegeneration is present. This should make CSF+/PET− subjects suitable for inclusion in clinical trials, especially if the drug is intended to target Aβ accumulation over long time periods using AD biomarkers as outcomes of its efficacy in this preclinical disease stage.

The temporal sequence of Aβ accumulation preceding neurodegeneration confirms the most acknowledged hypothetical model on the development of AD\textsuperscript{1}. It also strengthens the rationale for using CSF/PET groups as proxies for Aβ stages in AD. To further validate the use of CSF/PET groups, we compared Aβ accumulation in our proxy group for early Aβ accumulation (CSF+/PET−) with the proxy group for late Aβ accumulation (CSF+/PET+). As hypothesized, we identified known regions of late stage Aβ accumulation, including the sensorimotor cortex and occipital lobe (Fig. 3, Supplementary Movie 2 and Table 3)\textsuperscript{3, 19}. In a recent study, we also showed that CSF+/PET− subjects accumulated Aβ at a similar rate as CSF+/PET+ subjects and about four times higher than CSF−/PET− subjects\textsuperscript{12}. The high accumulation rate in CSF+/PET− subjects was not accompanied by cognitive decline, but we found a subtle decline in memory over 5 years in CSF+/PET+ subjects that further validates the use of CSF/PET groups for stratifying Aβ stages. Nonetheless, we acknowledge that CSF+/PET− subjects are not all early Aβ accumulators since this state also can be caused by CSF analytical factors, failed PET scans, and medical conditions other than AD\textsuperscript{36–38}.

As part of our methodology we chose to primarily compare longitudinal changes between the CSF/PET groups instead of comparing cross-sectional values or just within-group changes to the identify the early Aβ regions. This had the advantage of accounting for physiological differences in Aβ burden, age-related non-specific Aβ accumulation rates, and the fact that the individuals within each CSF/PET stage had reached slightly different time points in the disease progression. In addition, the early Aβ-accumulating brain regions remained significant when adjusting for confounding factors such as longitudinal changes in cortical thickness, APOE genotype and clinical status. These adjustments show that the results were not confounded by differences in atrophy between the groups or differences between healthy controls and MCI subjects (discussed further in the Methods section). It should be noted that the early Aβ regions are derived from group analyses, which show where Aβ is prone to start accumulating but they do not exclude the possibility that the accumulation might start in other regions in some individuals.

A limitation of our early Aβ regions is that they have been identified using PET ligands that bind to primarily dense-core Aβ aggregates and to a lesser extent or not at all to diffuse plaques\textsuperscript{39, 40}. We can therefore not pinpoint the exact Aβ forms within the early Aβ regions, neither can we say if Aβ aggregates not detected by PET are present in other areas of the brain at this early stage. Future PET ligands that binds to different forms, e.g., Aβ protofibrils, might reveal a different early Aβ pattern\textsuperscript{41}. The binding affinity of current PET ligands could explain some of the differences with neuropathological studies that either use silver techniques\textsuperscript{39}, which stain not only Aβ but also tau, or anti-Aβ antibodies\textsuperscript{5} that bind to specific Aβ epitopes and stain different Aβ forms. Nonetheless, a strength of the methodology is that we could detect similar early Aβ regions in two different cohorts using two different types of PET ligands; \textsuperscript{18}F-florbetapir (a stilbene compound) and \textsuperscript{18}F-flutemetamol (an \textsuperscript{18}F-labeled analog of \textsuperscript{11}C-Pittsburgh Compound B (PiB) derived from thioflavin T)\textsuperscript{40}. The cut-offs for the two PET ligands were established separately in each cohort, and the data were not pooled in the analyses, nor were the groups compared to each other. We therefore chose to not transform SUVr into a standardized scale such as centiloid units\textsuperscript{42}.

To conclude, we believe that the identified early Aβ regions can have several important implications. These brain regions give a pathophysiological insight into where Aβ fibril accumulation is prone to start in AD and its relationship to functional networks and their connectivity. Our data indicated that Aβ starts accumulating before overt metabolic changes or atrophy, and that hypometabolism within the early Aβ-accumulating regions has already occurred when Aβ fibrils just starts to accumulate. The most prominent early Aβ-accumulating regions were the pre-cuneus, posterior cingulate cortex, and the medial orbitofrontal cortex, possibly with an overlap to the anterior cingulate. Based on our results, future studies should be able to examine early unique pathophysiological events and triggering mechanisms of Aβ accumulation by comparing molecular and physiological properties of the early-accumulating regions to later-accumulating regions. These early-accumulating Aβ regions should also be advantageous to use in an Aβ PET composite ROI to better assess early fibrillar Aβ deposition (available at http://biofinder.se), which can have practical implications for early AD diagnostics, Aβ staging and enrollment in clinical trials that target Aβ accumulation.

**Methods**

**Study data.** Longitudinal data from the ADNI were used for all the statistical analyses in the study, except for replicating the finding of early Aβ accumulating regions and for the functional connectivity analyses for which data from the Swedish BioFINDER study were used. The methodology described in the next sections refers only to analyses performed in the ADNI cohort. Methodology referring to analyses in the BioFINDER cohort is described separately under Methods for the BioFINDER study.

**Participants.** Only non-demented individuals were included in this study (characterized as either cognitively normal or diagnosed with mild cognitive impairment, MCI). Demented subjects were excluded since the study aim was to identify early AD pathology. We chose to include MCI subjects in addition to healthy controls since the cognitive impairment did not necessarily have to be caused by an underlying AD process and because about a third of MCI subjects have shown to be incorrectly diagnosed with cognitive impairment\textsuperscript{43–45}. We also attempted to include a range of different pre-dementia AD phases for a better staging of late Aβ accumulators, early Aβ accumulators and non-accumulators. To account for this inclusion the models were adjusted for clinical status (MCI/healthy control) in subanalyses. The specific inclusion/exclusion criteria for the ADNI cohort can be found at http://www.adni-info.org. Briefly, all subjects were enrolled from the ADNI-2 study, were between the ages of 55 and 90 years, were fluent in Spanish or English, had completed at least six years of education, had a Mini-Mental State Examination score (MMSE) of ≥24\textsuperscript{46}, and were free of any significant neurologic disease other than AD. Subjects classified as cognitively normal (n = 176) had a Clinical Dementia Rating scale (CDR) score of 0\textsuperscript{47}. MCI (n = 297) was defined as having preserved activities of daily living, absence of dementia, and an objective cognitive impairment as shown on the delayed recall test of the Wechsler Memory Scale - Logical Memory II as well as a CDR score of 0.5. Only subjects with a complete set of baseline and follow-up Aβ PET scans and baseline CSF Aβ42 data were included. Our study baseline was defined as the first visit where both CSF and PET data were available. Study subjects gave written informed consent and the study was approved by each participating site’s Institutional Review Board.

**Image acquisition.** For MRI, 3 Tesla scanners were used. High-resolution 3D T1-weighted images were acquired for volumetric measures, anatomical segmentation,
and template normalization using an MPRAGE sequence (for details, see ref. 48).

Methods for the BioFINDER study. The Swedish BioFINDER study was used for replicating the main results from ADNI and for the functional connectivity analyses. BioFINDER is a prospective study that focuses on identifying key mechanisms and improvement of diagnostics in AD and other neurodegenerative disorders. For details about study design, methods, and specific inclusion/exclusion criteria, see http://biofinder.se. The study was approved by the ethical review board in Lund, Sweden, and all participants gave their written informed consent. All non-demented individuals with CSF and Aβ PET data were selected for this study. This resulted in a cohort consisting of cognitively healthy elderly subjects (n = 138) and consecutively recruited patients who had been referred to memory clinics due to cognitive complaints (n = 268). The PET ligand 18F-flutemetamol was used for Aβ PET, and images were acquired 90–110 min post-injection. The PET scanning procedures have been described previously46. CSF Aβ42 and P-tau were analyzed with INNOTEST ELISAhs (Fujirebio Europe, Ghent, Belgium) and T-tau with EUROIMMUN ELISAhs (EUROIMMUN AG, Lubeck, Germany) as previously described46, 62. Mutation modeling was performed in the sample to determine the cut-offs for abnormal CSF Aβ42 (<517 ng/L) and abnormal Aβ PET (<0.759 SUVR). Only baseline data was available for analysis in the BioFINDER cohort. Imaging was performed on a 3 Tesla Siemens Tim Trio scanner (Siemens Medical Solutions, Erlangen, Germany). The high-resolution 3D T1-weighted volume used for segmentation and normalization was acquired using an MPRAGE sequence (in-plane resolution = 1 × 1 mm2, slice thickness = 1.2 mm, TR = 1950 ms, TE = 3.37 ms, flip-angle = 9°). Spontaneous BOLD oscillations in the absence of external stimuli were imaged with a gradient-echo planar sequence (eyes closed). In-plane resolution = 3 × 3 × 3 mm, TR = 2000 ms, TE = 30 ms, flip-angle = 90°, 180 dynamic scans, 6 min).

Resting-state data preprocessing was performed with a pipeline composed of FSL45, AFNI50, and ANTS55. Analytical processing involved skull stripping, segmentation of white matter (WM)/GM/CSF and normalization to MNI152 space. Data were preprocessed in anticipation of a potential transformational data was bulk motion, and slice timing corrected, furthermore nuisance regressed using the WM/CSF average signal, 6 components of physiological noise24, 26 motion parameters42, and linear/quadratic trends. Finally, the functional data was transformed to MNI space. Frames causing outliers in total frame-to-frame signal intensity >15% and mean drift during the first 50 and last 50 frames were discarded. The band-pass signal was calculated and summed. Outliers in this measure likely originate in a motion-induced global signal confound capable of eluding conventional motion estimation95 and were removed. The processed fMRI data was resampled using trilinear interpolation to 6 × 6 × 6 mm3 resolution and masked with GM decay from the Harvard-Oxford subcortical network atlas61 and Harvard-Oxford subcortical atlas52. Fisher-z transformed Pearson correlation between the resulting 5071 GM voxel time series then yielded a measure of functional connectivity (FC), corresponding to a weighted graph with nodes (voxels) and links (voxel BOLD time series correlations).

Group classifications. All subjects were categorized into different groups according to their CSF Aβ42 and Aβ PET status. The following groups were derived according to the diagnostic criteria: 1) normal CSF Aβ42 and normal Aβ PET (CSF+/PET−) referred to as no-accumulators (n = 218), 2) abnormal CSF Aβ42 and normal Aβ PET (CSF−/PET−) referred to as Aβ accumulators (n = 59), and 3) abnormal CSF Aβ42 and Aβ PET (CSF−/PET+) referred to as late Aβ accumulators (n = 191). Those with normal CSF Aβ42 and abnormal Aβ PET were not used in any analysis (n = 3). Abnormal CSF Aβ42 values were defined using the previously established cut-off of <192 ng/L57. The cut-off for abnormal Aβ PET SUVR was determined using a mixture modeling analysis since no previous cut-off had been established for PVE corrected florbetapir data in ADNI (using the GTM method). Mixture modeling statistics provide an unbiased threshold for normality (i.e., the distribution of sizes, thus controlling for the family-wise error rate in the weak sense at α = 0.05 The result of the algorithm is a network component on which sum of z-scores correlates significantly higher than for randomized sets of subject FC-CSF pairs. Age, gender and APOE e4 status was controlled for by partial correlation with CSF Aβ42 and Aβ PET data. In order to simplify the analysis of network components, we grouped nodes using a resting-state network atlas51 containing: default mode, dorsal and ventral
attention, sensory motor, visual, fronto-parietal, and fronto-temporal (medial temporal lobe/orbitofrontal cortex). To this set of labels we added two anatomically defined subcortical structures from the Harvard-Oxford atlas: the Basal Ganglia (BG: thalamus, caudate, putamen and pallidum) and hippocampus/amygdala (HI). Note that the permutation-based approach generates $p$ values for the network component as a whole, but since these are too large and complex to visualize, a network-based break up is needed in some cases.

Only Aβ PET negative subjects were used in the connectivity analysis. In addition to the previously described group of early Aβ accumulators (CSF+/PET−, $n = 23$ after MRI quality control) we also defined a group of biomarker negative subjects with indications of very early Aβ accumulation ($n = 80$). Those with low levels of CSF Aβ42, but still within the normal range, have a high risk of becoming abnormal within the next couple of years, which suggests that sub-threshold CSF Aβ42 levels indicate very early Aβ accumulation. This group was characterized as Aβ PET negative with CSF Aβ42 between 517–750 μg/mL (CSF−/PET−).

Statistical analysis. Group comparisons of baseline characteristics (Table 1 and Supplementary Table 2) was performed with Mann–Whitney U statistics if significant after the Kruskal–Wallis test. For ROI analyses, full factorial general linear models were used to analyze group differences and correlations of the 68 available cortical Freesurfer ROIs from the Desikan-Killiany Atlas. The dependent variable was Aβ accumulation rate ([SUVR at follow-up – SUVR at baseline]/years between baseline and follow-up) in ADNI and baseline Aβ PET SUVR in BioFINDER. The variance of the SUVRs was similar in each group. All results were adjusted for gender and age and, in the longitudinal analyses, time between Aβ PET examinations. We also performed additional analyses adjusting for changes in cortical thickness over 2 years in each ROI and clinical status (healthy control/MCI) in addition to the above covariates. Longitudinal changes within a specific group of subjects were analyzed with the Paired T-test. Because of the limited number of subjects in the subanalysis of “CSF converters” ($n = 11$), we primarily used non-parametric statistics for this subgroup (the Mann–Whitney U and Wilcoxon signed rank test). To control for multiple testing in the ROI-based analyses we used the Benjamini–Hochberg procedure with a conservative false discovery rate (FDR) of 0.05 (not to be confused with a $p$ value of 0.05)42. Original $p$ values are presented for the ROI-based analyses but are only reported as significant if they are significant after the FDR correction. The effect size of the significant results when comparing the CSF/PET groups are given in % annual increase of SUVR (yearly determined using actual SUVR or SUVR/year data. SPM12 was used for all voxelwise analyses, thresholded to present results with a voxel size uncorrected for family-wise error, and a $k = 0.001$ threshold was assigned a $p$ value as a whole based on the component size relative a permutation-generated null distribution, thus correcting for family-wise error (see “Methods for the BioFINDER study” for details). Finite mixture models for establishing biomarker cut-offs were performed with the package “mixtools” in R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria, 2013). FDR correction of multiple comparisons was performed using the Benjamini–Hochberg formula in an Excel sheet (Microsoft Excel for Mac, 2011, version 14.4). All other statistical analyses were performed with SPSS for Mac, version 22.0 (SPSS Inc., Chicago, IL).

Data availability. MRI and PET images were downloaded online at https://ida.loni.ucla.edu/ and further processed locally (see Image Processing above). All other ADNI data were also downloaded from the same site. BioFINDER data and processed ADNI data are not publicly available for download, but might be retrieved from the principal investigator Oskar Hansson. A PET-data-derived ROI of the cingulate sulci from the Harvard-Oxford atlas was available in MN152 space and Nifti format at http://biofinder.se.

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Author contributions

S.P. contributed to the study design and concept, interpreted data, performed the ROI-based statistical analyses, and drafted the manuscript. M.S. contributed to the study design and concept, interpreted data, and performed the voxel-based analyses. O.S.
processed all image data, interpreted data, and performed the connectivity analyses. N.M. contributed to the study design, interpreted data, and provided data on CSF converters. E.S. provided data on cognitively healthy elderly in BioFINDER. H.Z. and K.B. performed CSF analyses in BioFINDER and interpreted data. S.L. and W.J. contributed with ADNI florbetapir data and interpreted data. O.H. was P.I. for BioFINDER, was responsible for the study design and concept, and interpreted data. All co-authors have read and critically revised the manuscript.

Additional information
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