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Chapter 4

Cortical iron reflects severity of Alzheimer’s Disease

Sara van Duijn MSc 1, Marjolein Bulk, MSc 2,3,4, Sjoerd G. van Duinen MD PhD 1, Rob J.A. Nabuurs MD PhD 2,5*, Mark A. van Buchem MD PhD 2, Louise van der Weerd PhD 2,3, and Remco Natté MD PhD 1.

1Department of Pathology, Leiden University Medical Centre, Leiden, the Netherlands
2Department of Radiology, Leiden University Medical Centre, Leiden, the Netherlands
3Department of human genetics, Leiden University Medical Centre, the Netherlands
4Pecuros BV, Leiden, The Netherlands
5*Current address: Department of Neurology, VU-University Medical Centre, Amsterdam, the Netherlands

**Abstract**

Abnormal iron distribution in the isocortex is increasingly recognized as an in vivo marker for Alzheimer’s disease (AD). However, the contribution of iron accumulation to the AD pathology is still poorly understood. In this study, we investigated: 1) frontal cortical iron distribution in AD and normal aging and 2) the relation between iron distribution and degree of AD pathology. We used formalin-fixed paraffin-embedded frontal cortex from 10 AD patients, 10 elder, 10 middle aged, and 10 young controls and visualized iron with a modified Perl’s histochemical procedure. AD and elderly subjects were not different with respect to age and sex distribution. Iron distribution in the frontal cortex was not affected by normal aging but was clearly different between AD and controls. AD showed accumulation of iron in plaques, activated microglia, and, in the most severe cases, in the mid-cortical layers along myelinated fibres. The degree of altered iron accumulations was correlated to the amount of amyloid β plaques and tau pathology in the same block, as well as to Braak stage (p< 0.001).

AD and normal aging show different iron and myelin distribution in the frontal cortex. These changes appear to occur after the development of AD pathological hallmarks. These findings may help the interpretation of high resolution in vivo MRI and suggest the potential of using changes in iron-based MRI contrast to indirectly determine the degree of AD pathology in the frontal cortex.

Keywords: Alzheimer’s disease, iron, myelin, magnetic resonance imaging
**Introduction**

Future therapeutic interventions for Alzheimer’s disease (AD) require early and preferably non-invasive clinical tests. A promising approach is in vivo detection of amyloid β (Aβ) plaques, neuropil threads (NT) and neurofibrillary tangles (NFT), since these histological hallmarks of AD occur 10-20 years before cognitive decline [1, 2]. In vivo visualization of Aβ plaque burden is already possible using positron emission tomography (PET) [3-6], but this technique requires the use of a radioactive tracer and a highly-specialized infrastructure. Recent advances in PET tracers for tau are promising to study the spatial and temporal relation between amyloid and tau accumulation in vivo. Nevertheless, our understanding of AD is incomplete, and the role of other modulators of the disease such as microglia activation and iron accumulation is only partially understood. Recently, high field (7T) Magnetic Resonance Imaging (MRI) has been reported to discriminate AD from control neocortex by changes in phase shift, which is a reliable indication of iron content in the brain [7, 8]. Other MRI studies show susceptibility induced changes in vivo in AD transgenic mice [9-11] and in postmortem human AD brain tissue [12, 13] that correlate to Aβ plaques histologically. Aβ plaques show iron accumulation [14-20] and it has been suggested that this iron causes the high T2* susceptibility effects [11, 21-23]. However, there may be additional pathological substrates for iron-related MRI changes in AD. A subset of histological studies report iron accumulation in microglia in the cortex and hippocampus of AD patients [14, 16, 19]. In contrast, Smith et al shows iron accumulation in NFT but explicitly report absence of iron labelling in glial cells [20]. AD-related iron accumulation in other tissue compartments than Aβ plaques is also suggested by the presence of diffuse MRI hypointensities in postmortem AD cortex [19, 24], but the pathologic substrate of these diffuse MRI hypointensities is not known. Moreover, we found a diffusely increased iron labelling in layers III-V of the frontal cortex of AD patients in a study comparing different histological iron stains [25].

The value of iron-based MRI changes for the diagnosis and staging of AD depends on an association between cortical iron accumulation and AD pathology. However, we are not aware of studies that have correlated the degree of cortical iron accumulation to Braak stage, local Aβ plaque load or local tau pathology. For the interpretation of iron-based cortical MRI changes as a biomarker for AD, it is also necessary to know the effect of
Aging on cortical iron accumulation. Aging itself leads to increased iron and ferritin concentrations in the cortex [26, 27], but the microscopically distribution of this age-related increase is unknown.

Our previous finding of increased cortical phase shift changes as a possible non-invasive clinical biomarker for AD [7] led us to investigate the following questions: 1) is the pathological substrate of iron based MRI changes in AD cortex limited to iron accumulation in Aβ plaques or is iron also accumulating in other tissue compartments? 2) Is cortical iron accumulation related to Braak stage, local Aβ plaques and local tau pathology? 3) How does iron accumulation in AD compare to normal aging?

Table 1: Demographics and pathology of subjects.

<table>
<thead>
<tr>
<th>No.</th>
<th>age/sex</th>
<th>AD/ control</th>
<th>Braak stage</th>
<th>Aβ plaque</th>
<th>Fe plaque/ mcg</th>
<th>band Fe/PLP</th>
<th>other brain pathology</th>
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</tr>
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<td>-</td>
<td>-</td>
<td>Old lacunar intact</td>
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<tr>
<td>7</td>
<td>33/M</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>Periventrically</td>
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</tr>
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<tr>
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<tr>
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<tr>
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<td>+</td>
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<tr>
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<td>AD</td>
<td>V</td>
<td>++++</td>
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Abbreviations: M = male; F = female; AD = Alzheimer Disease; FE = iron, mcg = microglia; PLP = proteolipid protein.
Symbols: - = absent, + = mild, ++ = moderate, +++ = severe. See Methods for definitions.
Materials and Methods

Patients
We included autopsy material of 10 AD patients (age 65-90 years old, 5 male and 5 female) with a clinical diagnosis of dementia and histological changes of at least Braak stage IV. Other neuropathological changes were absent, except for one patient with a 1 cm large meningioma in the parietal lobe which had not been noticed during life. We also included autopsy material of 10 young (17-38 years old, 8 male, 2 female), 10 middle aged (51-57, 8 male, 2 female) and 10 old (74-89, 4 male, 6 female) controls. No difference was found in sex distribution between the four groups (P>0.05). By definition, AD and old age controls were significantly older compared to the middle aged and young controls (P<0.05). The tissue was selected from the archive of the Department of Pathology, without knowledge of the degree of AD pathology in these blocks. All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for proper secondary use of human tissue, Dutch Federation of Medical Scientific Societies). Controls were included if the autopsy request file did not mention cognitive disorders, movement disorders, or extensive cerebrovascular disease. For all patients and controls, Braak stage was determined by two experienced neuropathologists (SGvD en RN) according to Alafuzoff [28] investigating the relevant brain areas. Patient characteristics, including Braak stage and additional neuropathological findings are presented in Table 1.

Tissue
Of each patient and control 1 block of formalin fixed paraffin embedded frontal cortex was taken. We choose the frontal cortex because increased diffuse iron in layers III-V was found earlier in a study designed to compare different histological iron stains [25]. Tissue was fixed in standard formalin for a minimum of 1 week and a maximum of 6, on average 1-2 weeks for both AD patients and controls. From each block, sequential 10-µm thick sections were cut and stained consecutively for myelin - iron and Aβ- iron-tau. In addition, 20-µm thick sections were stained for iron paralleled by a 10-µm section of Aβ immunohistochemistry. The iron stained 20-µm sections were used to count iron positive plaques (and in addition iron positive activated microglia) because at 20-µm the iron stain labelled much more plaques and labelled them more reproducibly.
Figure 1: A: Each row represents consecutive sections of frontal cortex stained for iron, myelin (PLP immunohistochemistry) and Aβ immunohistochemistry. Controls show a similar iron distribution, irrespective of age, with increased amounts in the myelin rich areas: white matter and the inner and outer line of Baillarger (arrows). AD cortex (bottom row) shows a diffuse band shaped iron and PLP increase in cortical layer IV/V, extending to the inner part of layer III (arrow, and indicated by lines).

B: higher magnification of A, Meguro iron stain. Controls show evenly distributed iron concentration in myelinated fibres and a good distinction between the lines of Baillarger. AD cortex shows diffuse iron increase in the middle cortical layers (arrows), as well as iron positive plaques (asterisk), also concentrated in the middle cortical layers.

**Histochemistry**

Iron was detected using a modified diaminobenzidine (DAB)enhanced Perl’s protocol as described previously [22, 25]. Sections of 10 µm were incubated for 40 min in 1% potassium ferrocyanide (pH 1) and washed, followed by 75 minutes incubation with methanol containing 0.01 M sodium azide (NaN3) and 0.3% H2O2. Then the sections were washed in 0.1 M phosphate buffered saline (PBS), before visualization by 0.025% 3,3’-diaminobenzidine (Sigma, St Louis, MO, USA) and 0.005% H2O2 in
0.1 M PBS for 40 min. The reaction was stopped by washing. For the 20 μm sections incubation times were modified to: potassium ferrocyanide 80 minutes, methanol-NaN3-H2O2 100 minutes and DAB 80 minutes. A slightly adapted protocol was used to compare the potential impact of using fixed versus fresh-frozen post-mortem tissue on the detection of iron. Frozen sections (20 μm) of the contralateral hemisphere of two AD patients were post fixed for 1 minute to prevent floating of the frozen sections from the slide during the staining procedure. The rest of the protocol was identical as described above apart from the 75 minutes incubation in methanol containing 0.01 M NaN3 and 0.3% H2O2: PBS was used instead of methanol.

**Immunohistochemistry**

Immunohistochemistry (IHC) was used to detect Aβ protein, hyperphosphorylated tau, proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and HLA-DR. Specific information regarding the antibodies and the dilutions used can be found in Table 2.

After de-paraffination, all 10 μm sections were blocked with methanol and H2O2 (0.3%) for 20 minutes at room temperature. Slides for Aβ IHC were pre-treated by 85% formic acid at room temperature for 1 hour, followed by 30 minutes in trypsin at 37°C. Pre-treatment for MBP and MOG was citrate buffer, pH 6, at boiling temperature for 20 minutes and cooling down for an hour. No pre-treatment was performed for tau and PLP IHC. All incubations with primary antibodies were overnight at room temperature. For Aβ, tau, PLP, MBP the second step was performed with rabbit anti mouse biotin (DAKO, dilution 1:200) for 1 hour at room temperature. For MOG swine anti-rabbit biotin was used (DAKO, dilution 1:600) for 1 hour at room temperature. The third step was performed by avidin biotin complex (Vectastain, Vector, Burlingame CA, USA), according to the manufacturer's instructions (incubation for 30 minutes at room temperature). DAB (0.05%) with H2O2 (0.005%) was used as a chromogen with an incubation time of 10 minutes at room temperature. Double labelling for ferritin and HLA-DR was performed by pre-treatment with boiled citrate buffer pH 6.0 for 20 minutes and cooling down for one hour. Then, tissue was incubated overnight with a 1:1 cocktail of the antibodies for ferritin and HLA-DR (Table 2). The second step was performed by 2 hours incubation with a 1:1 cocktail of goat anti rabbit biotin (resulting dilution of 1:800) and goat-anti mouse alkaline phosphatase (resulting dilution 1:60), followed
Figure 2: A: iron accumulation in plaques (left) and microglia (middle) (both Meguro stain). On the right double labelling of iron positive cells by ferritin (brown) and MHCII (red).

B: band shaped increase of iron and PLP labelling in AD (right column) compared to an old control (left column). The right column illustrates that the normal distinction of the lines of Baillarger (arrow) is blurred by a band shaped iron/PLP increase in cortical layer IV/V extending to layer III (arrow heads). This is due to darker and thicker iron/PLP labelling of more crowded myelinated fibres (bottom panel).
by 30 minutes incubation with avidin-biotin complex (Vectastain, Vector, Burlingame CA, USA), according to the manufacturer’s instructions. First, ferritin was visualized by 10 minutes incubation with DAB (0.05%) with H2O2 (0.005%) and 1% cobalt-chloride, followed by visualization of HLA-DR by 25 minutes incubation with liquid permanent red (DAKO, 1:60), both at room temperature.

**Semi-quantitative scoring of AD pathology and iron accumulation**

Severity of the different pathological features in the frontal cortex was assessed semi-quantitatively by an experienced neuropathologist (RN). Tau pathology was scored as: absent (-): no AT8 immunoreactivity; mild (+): any AT8 immunoreactive structures up to occasional AT8 positive NT/NFT; moderate (++): microscopic diffuse AT8 positive NT up to visible cortical layer V; severe (+++): macroscopically visible layer V and other cortical layers, especially layers II/III.

Aβ pathology was scored as: absent (-): no Aβ plaques; mild (+): 1 Aβ plaque up to 10 fields with >50 plaques/100X field; moderate (++): >10 fields of >50 plaques/100X field; severe (+++): all cortical areas show >100 plaques/100X field.

The degree of abnormal iron accumulation was scored by counting the amount of iron positive plaques and iron positive microglia with activated morphology outside plaques in the cortical 100X field with the highest density and by assessing the presence or absence of a band like increase of PLP and myelin associated iron labelling in cortical layers III-V. Scoring categories were defined as: absent (-): no plaque/microglial iron accumulations; mild (+): 1-30 plaque/microglial iron accumulations; moderate (++): 31-100 plaque/microglial iron accumulations; severe (+++): >100 plaque/microglial iron accumulations. Abnormal iron accumulation in the cortex was scored as ++++ when the tissue showed a band of diffusely increased PLP and iron in layer III–V in addition to >100 plaque/microglial iron accumulations.

**Statistical methods**

Age was compared between groups using a one-way ANOVA followed by a Bonferonni post hoc test. A chi-square test was used to compare sex distribution between groups. Semiquantitative iron score was correlated to Braak stage, and semiquantitative scores for Aβ plaques and tau pathology by a linear by linear association test for ordinal variables [29]. All statistical analyses were performed using Statistical Package of Social
Sciences (SPSS, version 23; SPSS, Chicago, USA). A significant level of 0.05 was used.

**Results**

**Iron distribution in aging and AD**

For histochemical visualization of iron, we used a modified Perl’s procedure described by Meguro [22]. With this method, only non-heme iron is stained, mostly Fe3+ but also some Fe2+ [22]. Thus, if we refer to iron in this study, this is DAB enhanced Perl’s detectable iron meaning non-heme iron, mostly Fe3+ and some Fe2+. In the absence of Aβ plaques and tau pathology (20 out of 30 controls), iron distribution in the frontal cortex was identical in young (9), middle aged (7), and old (4) controls. These samples showed a finely diffuse, regular iron distribution with evenly increased density in myelin-rich areas: the white matter, the lines of Baillarger and the radially projecting thickly myelinated fibres from the white matter into the cortex (Fig. 1). 5 Controls (1 young, 2 middle aged and 2 old) had minimal Aβ plaques with or without minimal tau pathology and showed the same iron distribution as controls without these AD lesions. The last 5 controls (1 middle aged, 4 old) showed variable iron accumulation in plaques and cells, directly correlating to the amount of Aβ plaques (Table 1). All 10 AD patients showed iron accumulation in plaques and cells (Table 1, Fig. 2A), most frequent in cortical layers III-V (Fig 1, bottom row). Intracellular iron was mainly localized in rounded microglia

![Figure 3: Semi-quantitative relation between iron accumulation and Braak stage (A), Aβ plaques (B) and tau pathology (C). Iron accumulation: 0: no iron accumulation, 1: low, 2: intermediate and 3: high iron accumulation in plaques and microglia, 4: high iron accumulation in plaques and microglia and a band shaped iron/PLP increase in the middle cortical layers. For grading definitions see Methods.](image-url)
with dilated cell-branches (Fig 2A), morphologically identified as activated microglia [30, 31]. Iron laden microglia were often seen in or directly around Aβ plaques but also apart from Aβ plaques. Iron accumulation was not observed in neurofibrillary tangles. In 3 out of 10 AD patients, we observed a diffuse band-shaped increase of both iron and PLP labelling in cortical layers IV/ V, and part of layer III, blurring the normal distinction between the lines of Baillarger (Fig. 1, 2B). At higher magnification, we observed darker and thicker labelling of neuropil fibres by iron and myelin proteins (PLP, MOG, and MBP) in a more crowded network (Fig. 2B). The band of increased mid-cortical iron and myelin labelling is not reflected by Aβ plaques, which are most dense in cortical layers I-IV.

**Correlation between iron accumulation, myelin changes, plaques and tau pathology.**

Iron accumulations were scored from 0-3 depending on the amount of iron positive plaques and microglia together (see “Materials and Methods”). In the presence of an increased iron band in the mid-cortical layers the degree of iron change was scored as 4. This histological iron score correlated with Braak stage (P<0.001) and with the semi-quantitatively scored amount of Aβ plaques (P<0.001) and degree of tau pathology (P<0.001). This level of statistical significance was the same for stratification by age as well as comparison of AD with the old age controls only (Fig. 3). Note that absence or low (score 0-1) iron accumulation in plaques and microglia excluded advanced Braak stage, significant Aβ plaques or significant tau pathology. A band-shaped increase of diffuse iron and PLP/MBP/MOG labelling was only present in AD frontal cortex with the severest tau pathology (Fig. 3 and 4), despite comparable amounts of Aβ plaques for all AD patients (Fig. 4, Table 1).

**Discussion**

**Technical Considerations**

In the present study, we show non-heme iron accumulation in Aβ plaques and activated microglia in the frontal cortex of AD patients. This is in line with earlier studies showing iron in human Aβ plaques [17, 19, 20], and in activated microglia [14, 17, 30-33]. We did not identify iron accumulation in NFT or NT and this is in line with most [14, 17, 19], but not all [20], previous reports. The modified Perl’s protocol we employed was also used in other studies [17, 20] and principally stains Fe 3+ but also some Fe 2+.
Figure 4: Iron accumulation in AD, related to tau pathology and plaques. A: Both patients (first and second row) show similar amounts of plaques but only the tissue with the most severe tau pathology shows a diffuse band shaped increase of iron/PLP labelling in the middle cortical layers (arrows). B: 20µm sections of the same blocks. Cortex with the most severe tau pathology shows more iron accumulation in plaques and microglia.

[22, 34]. In addition to iron, other metals like copper, zinc, magnesium and aluminium have been shown in plaques and have also been implicated in AD and plaque pathogenesis [18, 35-37]. Although the ferrocyanide that is applied in the Perl’s staining can also bind to other metals apart from iron, the precipitation of ferrocyanide with these metals does not appear to catalyze polymerization of DAB [22]. Indeed, ferrocyanide in combination with DAB, detects iron with much higher sensitivity than copper, magnesium, zinc, or calcium [34]. This excludes a significant contribution of other metals to our visualization of iron in the tissue.

Fixation and storage of tissue in formalin could lead to leakage of iron [38]. We took care not to include tissue that had been stored for prolonged
periods of time. Nevertheless, we investigated the potential impact using fixed versus fresh-frozen postmortem tissue of the same patients on the histochemical detection of iron. Briefly-fixed frozen sections qualitatively showed a similar band of increased iron staining as in the paraffin sections. Also, iron-laden plaques and microglia could be observed, but visual comparison showed that this was not more prominent than in the paraffin sections. Therefore, we conclude that a qualitative assessment of iron accumulation in different cell types is reliable in our selected samples.

Iron and Myelin
In addition to iron in Aβ plaques and microglia of AD cortex, we confirmed our preliminary observation [25] of a band-shaped increase of iron and PLP labelling in cortical layers III-V in a subset of AD patients with the most severe tau pathology, a finding we have not seen reported before.

Figure 5: Schematic representation of the observed combinations of AD pathology and iron accumulation in frontal cortex. A: aging without plaques or tau pathology. B: controls with little diffuse plaques without iron accumulation. C and D: controls with increasing iron accumulation in plaques and microglia depending on the amount of plaques. E: AD with high Aβ plaque load and diffuse limited increase of tau pathology with increased iron accumulation. F-H: Similar high plaque load with increasing tau pathology and iron accumulation.
This band reflected a more crowded network of neuropil fibres with thicker and darker staining for iron and several different myelin proteins. This finding is in line with a meta-analysis by Tao et al. [39] and a recent laser-ablation inductively coupled plasma-mass spectrometry imaging study [40], both showing increased iron levels in the cortex of AD patients compared to controls. Interestingly, the investigators also showed a trend of increased iron along white matter fibres, including myelinated fibres in the lines of Baillarger, in AD patients [40]. This is in line with our observations of increased iron along neuropil fibres in the mid cortical layers. This qualitative observation of increased myelin staining in a late stage of AD, a neurodegenerative disease, is counter-intuitive. This study was not designed to prove this observation quantitatively, and therefore we cannot exclude the possibility that atrophy or other disease-associated tissue changes may result in better antigen retrieval or compaction of myelinated fibres. Furthermore, increased labelling of myelin proteins does not prove an increased amount of functional myelin or myelinated fibres. However, we found a consistent increase of several myelin proteins by different antibodies, making a true local myelin increase more likely. The mechanism by which severe AD may lead to increased cortical myelin proteins is open for speculation. Re-myelination is an almost default reaction of the brain to demyelination caused by myelin damage and/or death of oligodendrocytes [41-44]. Thus, maybe the continuous severe tissue and cell damage in late stage AD leads to unbalanced de- and re-myelination. Indeed, myelin damage and restoration with increased oligodendrocyte proliferating cells (OPC) has been shown in an AD mouse model, although in the same study a decrease of OPC was found in the brains of human AD patients [45]. We observed a band shaped increase of iron/myelin proteins in cortical layers III-V in a context of increased iron laden microglia. This is in line with the close relationship between iron and myelin synthesis extensively described in the literature [46, 47]. For example, activated microglia have been described to drive the recruitment and proliferation of OPC and their differentiation to oligodendrocytes, as well as the formation of a myelin sheath around available axons [41, 42, 48]. Moreover, it is thought that microglia provide the high iron concentrations needed for myelinization and oligodendrocyte differentiation [47, 49, 50]. We observed increased labelling of myelin proteins as a late phenomenon, occurring only in cortices severely affected by both Aβ plaques and tau pathology. This does not support the hypothesis of an early role of myelin dyshomeostasis in the pathogenesis of AD [1]. However, disruption of
cortical myelin may contribute to the ongoing neurological deterioration in late stage AD.

Iron and Microglia
In our cases, there was a clear presence of activated and iron laden microglial cells around Aβ plaques. The polarization of microglia, as in macrophages, plays an important role in the neuroinflammatory response [51]. Classically activated, or M1, microglia drive the pro-inflammatory response, whereas the alternative activation state, or M2, is apparently an anti-inflammatory state, mediating tissue maintenance and repair. In macrophages, iron accumulation can directly activate the M1 phenotype. Conversely, M2-polarized cells are thought to be involved in iron recycling by expressing molecules involved in iron uptake and export [52]. However, recent evidence shows that increased intracellular iron can alter microglia polarization to a pro-inflammatory M1 phenotype [53].

Iron and Neurodegeneration
Iron accumulation is not merely a marker of neurodegeneration, but has been shown to play a crucial role in the progression of neurodegeneration through several mechanisms [54-56]. Production of reactive oxygen species (ROS) leading to DNA damage and cell death is a well-known consequence of iron overload and a common feature in many neurodegenerative diseases [56]. The mechanisms behind iron dyshomeostasis are still not fully understood, but several key processes in AD have been found to be of importance. Aβ processing and regulation of intracellular iron are e.g. both modulated by furin, thus mutually activating pro-amylogetic and intracellular iron accumulation pathways [57].
In addition, apart from driving the amyloid cascade, the intracellular complexation of a certain iron species, magnetite, with Aβ appears to have an adverse effect on neuronal network function to a much larger extent than Aβ alone [54]. Also iron has been implicated in lipid metabolism through an interaction with Apolipoprotein E (ApoE); with the APOE-ε4 allele inducing high levels of cerebrospinal fluid ferritin [58] and brain iron levels on MRI [55], possibly due to reduced clearance of iron because of the low affinity of APOE-ε4 to high-density lipoprotein [58]. As such, locally high iron concentrations in AD are likely the result of an intricate disbalance of iron uptake, mediated by furin and iron-regulating elements and iron clearance through microglial lysosomal degradation and ApoE-mediated transport [36, 59], see also further reviews on this topic. The fact that
transferrin and HFE mutations have a higher incidence in AD patients stresses the self-perpetuating loop caused by iron and amyloid deposition. In this context, a recent paper by Maher et al. raised the intriguing concept that iron deposition as an initiator of AD pathogenesis may not only be the result of iron-regulation gone haywire, but may also be caused by environmental factors such as airborne iron-bearing nanoparticles entering the brain through the olfactory bulb, [60]. In conclusion, at this moment it appears that iron toxicity in the brain is a complex phenomenon, which may be both the cause and consequence of neurodegenerative processes. Further research should focus on the cellular localization of iron and its interaction with other mediators of AD, such as Aβ, as well as on the characterization of different iron species in the brain [61].

**Iron and ageing**
Some [27, 62] but not all [26] quantitative studies show increased frontal cortical iron concentrations with aging. The present study was not designed to quantify iron load, but we found no changes in frontal cortical iron distribution from young to old controls without Aβ plaques. This suggests that, although there may be increased iron load in aging cortex, the distribution of frontal cortical iron remains the same. This finding is helpful if iron based MRI changes may be used in the differentiation between an aging related disease like AD and aging.

**Iron and APOE**
APOE-ε4 is a well-known risk factor for AD and has recently been shown to correlate with ferritin levels in cerebrospinal fluid of APOE-ε4 carriers [58]. Van Bergen et al. (2016) demonstrated that magnetic susceptibility, as measured with MRI, was significantly higher in APOE-ε4 carriers with mild cognitive impairment (MCI) compared to MCI patients with other APOE variants [55]. These results confirm the hypothesis that the APOE-ε4 allele may increase the risk of developing AD via brain iron accumulation. In our study, we unfortunately only had DNA available for 8/10 of our AD patients, of which 2 were ε4/ε4, 5 were ε4/ε3 and only 1 ε3/ε3. As expected, APOE-ε4 was highly prevalent in these patients and the APOE-ε3/ε3 patient showed frontal cortical iron accumulations similar to the others. However, our sample size was too small and thus our data do not allow any conclusion to be drawn about a relation between APOE status and frontal cortical iron accumulations. Lastly, besides APOE status, mutations in some iron-regulating genes, such as HFE (hemochromato-
sis), are also known to associate with age of onset in AD [63]. Future studies correlating iron-related genetic variants to brain iron accumulation would be of interest.

Iron and AD pathology
Finally, we showed a statistically significant correlation between iron accumulations (in Aβ plaques/microglia and myelinated fibres in the mid cortical layers) and the amount of Aβ plaques and tau pathology, as well as between these iron accumulations and Braak stage. To our knowledge this is the first report that correlates frontal cortical iron accumulations with Braak stage and local tau pathology. This opens the possibility to grade the severity of AD pathology by MR in vivo. In Fig 5 we schematically represent the different combinations of AD pathology and pathological iron accumulations we encountered. These combinations suggest that histochemically detectable iron accumulation is secondary to Aβ plaques and tau pathology and follows its progression. In controls, iron accumulation only occurs in the presence of Aβ plaques and the amount of iron accumulation is correlated to the amount of Aβ plaques. For AD, Aβ plaque load does not increase much from Braak IV to VI, but iron accumulation and tau pathology both progress. The band shaped increase of diffuse iron-/myelin protein labelling in cortical layers III-V especially appears to be a late phenomenon, occurring when several cortical layers, especially layer V and II/outer III are heavily involved by tau pathology.

Conclusion
The presented data show a correlation between frontal cortical iron accumulation and AD pathology independent of age. Iron is not only accumulated in Aβ plaques but also in activated microglia and in myelinated fibres in the middle cortical layers with increased labelling of myelin proteins. Ex vivo MRI has shown iron related hypointensities in iron rich cortical myelin tracts and iron accumulation in microglia and Aβ plaques [13, 64, 65], suggesting the potential to detect these iron rich cortical areas with in vivo MRI. Complementary work from our lab showed that changes in iron and in myelin organization co-localize on a pixel-by-pixel basis to MRI contrast changes, demonstrating that iron is a major source of cortical MRI contrast in AD (data submitted for publication). The direct clinical relevance of these findings is supported by an earlier study in which high field susceptibility-weighted MR already demonstrated that iron-related MR contrast was detectable in human subjects, and shows different con-
trast between controls, patients with cognitive impairment and patients with clinically diagnosed AD [7]. Thus, iron imaging with MR reflects multiple aspects of AD pathology beyond Aβ plaques and therefore offers complementary in vivo imaging possibilities beyond PiB-PET amyloid and tau-PET imaging.

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Conflict of Interest
The authors have no conflict of interest to report.
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