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Chapter 7

Summary and general discussion
Summary

The central theme of this thesis is to study the possibilities of an early diagnosis of Alzheimer’s disease (AD) using different MR techniques with pathological confirmation.

The current view on AD is that neuropathological changes start two decades before the occurrence of clinical symptoms (Jack, Jr. et al., 2010). The cognitive decline associated with AD represents a late stage of the disease when neurodegeneration is extensive and therapeutic interventions may be too late. In the coming years, the radiological changes associated with AD need to be validated and expanded to allow diagnosis in vivo, perhaps even at an early stage of the disease. The search for new imaging methods is driven by the promise that earlier diagnosis, preferably before obvious dementia is present, may help in the search for treatment and to identify patients early enough to benefit from future treatment.

In the study discussed in chapter 2 the findings of coarse hypointensities on postmortem brain MRI were examined, correlated with pathology and discussed. During experiments with ex vivo human brain material, large hypointensities were visible on MRI scans. These turned out to be tissue artifacts induced by prolonged formalin storage without refreshing the formalin. This chapter describes the usability and limitations of postmortem brain tissue for MRI. Postmortem brain tissue is often used for MRI and pathological research (Benveniste et al., 1999; Bobinski et al., 2000; Bronge et al., 2002; Englund et al., 2004; Fernando et al., 2004; Geurts et al., 2005; Gouw et al., 2008; House et al., 2007; Kangarlu et al., 2007; Larsson et al., 2004; Schmierer et al., 2010; van Rooden et al., 2009) but there are limitations. Fixed brain tissue stored in unrefreshed formalin for prolonged periods (longer than a year), shows structural tissue changes associated with hypointensities on MR images. The fixation protocol for prolonged storage of tissue without refreshment of the formalin may be common practice in brain banks. Researchers should be aware of this fixation artifact.

Iron

For many years, research has been focusing on Aβ accumulation in plaques and the intraneuronal accumulation of hyperfosphorylated of tau, as these are the pathological hallmarks of AD (Nelson et al., 2009). The last decade, increasing evidence suggests the importance of iron in the
The pathogenesis of AD (Bartzokis, 2011; Crichton et al., 2002; Haacke et al., 2005; Meadowcroft et al., 2009; Meadowcroft et al., 2015a; Nabuurs et al., 2011). Iron is present in plaques and microglia, which may serve as an early marker for AD. To demonstrate iron, various histological staining methods have been developed (LeVine, 1991; Meguro et al., 2007; Smith et al., 1997). In chapter 3 we describe the comparison of three histological iron staining methods and ferritin immunohistochemistry to visualize iron in paraffin-embedded human AD brain tissue. The specificity of the histochemical techniques was tested by staining sections after iron extraction. Iron was demonstrated in the white matter, in cortical layers IV and V of the frontal neocortex, in iron positive plaques and in microglia. In our hands, these structures were best visualized using the Meguro iron staining method (Meguro et al., 2007), a method that has not yet been described for iron staining in human brain or AD.

Chapter 4 discusses the iron changes in the frontal cortex of AD brains compared to control brains. The aim of this study was to assess the histological distribution of iron in aging and in AD. The frontal cortex with AD pathology showed iron accumulation in plaques and activated microglia. This is in line with earlier studies showing iron in human Aβ plaques (LeVine, 1997; Nabuurs et al., 2011; Nabuurs et al., 2013; Smith et al., 1997) and in activated microglia (Connor et al., 1992; LeVine, 1997; Nabuurs et al., 2011; Nabuurs et al., 2013). In addition to iron in plaques and microglia in AD cortex, we confirmed our preliminary observation (van Duijn et al., 2013) of an increased labelling of iron and myelin protein along myelinated fibres in and around cortical layers IV/V in a subset of AD patients with the most severe tau pathology. This is in line with the close relationship between iron and myelin synthesis extensively described in the literature. For example, activated microglia have been described to drive the recruitment and proliferation of oligodendrocyte proliferating cells (OPC) and their differentiation to oligodendrocytes, as well as the formation of myelin sheath around available axons (Crawford et al., 2013; Gudi et al., 2014; Miron and Franklin, 2014). Moreover, it is thought that microglia provide the high iron concentrations needed for myelinization and oligodendrocyte differentiation (Schonberg et al., 2012; Schonberg and McTigue, 2009; Todorich et al., 2009).

We observed that aging in the absence of Aβ plaques does not lead to changes in iron distribution. Finally, we showed a semi-quantitative association between iron accumulation and the stage of AD pathology. This
opens the possibility to grade the severity of AD pathology by MR in vivo. Figure 1 shows iron aggregations correlated with other histological changes during the development of AD. Plaque load does not increase much from Braak IV to VI stages but iron accumulation and tau pathology both progress, although we still do not understand how they are related.

Figure 1: Schematic representation of the relation between AD pathology and pathological Fe accumulation in frontal cortex. a: normal aging showing diffuse myelin associated iron in the lines of Baillarger; b: controls with few diffuse plaques without pathological Fe accumulation; c: controls with little diffuse plaques and minimal pathological iron accumulation; d: controls with increasing plaques, including classical plaques and minimal AT8+ neuropil threads (AT8+NT) with increasing Fe accumulation in plaques and activated microglia; e: AD with maximum Aβ plaques and diffuse increase of AT8+NT with increased iron in plaques and activated microglia; f: AD with similar Aβ plaques and increased AT8+NT, macroscopically visible on the slide in cortical layer V and increased iron in plaques and activated microglia; g: AD with similar Aβ plaques and increased AT8+NT, macroscopically visible in cortical layer V and other layers; h: Same severe AD pathology as in g but with diffuse myelin associated increase of Fe in and around cortical layer IV/V.
**Magnetic Resonance Imaging**

The clinical diagnosis of AD is based on neuropsychological testing combined with hippocampal atrophy assessed by magnetic resonance imaging (MRI) and Aβ and tau levels in CSF (Fox et al., 1996; Hyman et al., 2012; Jack, Jr. et al., 2010; Li and Wahlund, 2011; Nasrallah and Wolk, 2014).

Over the past few years, the feasibility to detect the histological hallmarks of AD using MRI has been explored; in general these efforts have focused on detecting individual amyloid plaques (Chamberlain et al., 2011; Wadghiri et al., 2013). Increased iron accumulation in amyloid plaques induces a magnetic susceptibility effect, visible as hypointense foci on T2*-weighted or susceptibility-weighted (SW) MRI in the cerebral cortex of transgenic AD mouse models and in human post-mortem brain slices (Chamberlain et al., 2011; Meadowcroft et al., 2009; van Rooden et al., 2009). These findings have not yet been convincingly replicated in vivo in patients. It is doubtful whether detecting individual amyloid plaques with MRI will be possible in a clinical setting given the required high resolution, limited scanning time and physiological movement of the patient. However, recent advances in human MRI systems operating at an ultra-high magnetic field strength (7 Tesla and higher) show that the increased sensitivity to susceptibility effects generates iron based contrasts in the human brain that have not been observed before (Fukunaga et al., 2010; Meadowcroft et al., 2015b).

Previous studies showed band like changes (van Rooden et al., 2009) and single plaques (Chamberlain et al., 2011; Meadowcroft et al., 2009) in the frontal cortex of AD patients and not in non-demented controls. These studies suggested effects of the amyloid plaques on the MR images. In chapter 5 we examined changes in cortical appearance of AD patients using susceptibility weighted 7T MRI and correlated these findings with ex vivo scans and pathology. The aim of this study was to establish whether 7T MRI allows in vivo detection of differences in the cerebral cortex between probable AD patients and healthy controls. Having observed a difference, we then determined the histological substrate of the changes by comparing MRI, light- and electron microscopic analyses on human post-mortem material of AD patients and controls. On MRI, diffuse hypointens bands were frequently found in the cortex of the frontal lobe of AD patients (57%), which were not observed in healthy age matched control subjects. Further histologic correlation revealed that the pattern of the susceptibility-weighted contrast in the cortex of AD patients did not primarily co-localize with amyloid plaques or neurofibrillary tangles, but
with myelin-associated iron accumulation and with an altered myelin cytoarchitecture. This band-like pattern of iron/myelin in the neuropil fibres of cortical layers III-V appears to be accentuated by the iron accumulation in Aβ plaques found more frequently in layer III of the cortex, confirming the findings in chapter 4. Iron is, due to its magnetic characteristics, easy to see on an MRI (Fjell and Walhovd, 2012; Karran et al., 2011). The current findings show great promise for the in vivo detection of the underlying pathological changes in AD.

To verify our results in the frontal cortex, described in chapter 5, a larger set of ex vivo brain material of different brain locations should be studied. Another study should include comparison of AD patients to other neurodegenerative diseases and control subjects. The study described in chapter 5, suggests that MRI changes can be observed only in a late stage in AD. This has potential use to follow treatment and to improve the clinical diagnosis at a late stage, however is not useful for early diagnosis.

**Magnetic Resonance Spectroscopy**

One way to examine the metabolic pathways non-invasively during the early phases of AD is by focusing on the metabolic changes in a transgenic (tg) mouse model, by means of magnetic resonance spectroscopy (MRS). MRS is a non-invasive tool which can be used to measure the concentration of various brain metabolites in vivo (Rupsingh et al., 2011), making it possible to follow family members of AD patients with the possibility to identify AD at an early stage, prior to the onset of cognitive symptoms (Jack 2010).

Numerous MRS studies have been performed using tg AD mice. Until now N-acetylaspartate (NAA) and glutamate (glu) have been found to decrease in tg mice compared to control mice (Braakman et al., 2008; Choi et al., 2010; Dedeoglu et al., 2004; Marjanska et al., 2005; Oberg et al., 2008; von Kienlin M. et al., 2005). NAA is found in neuronal cell bodies and is a source of myelin synthesis in oligodendrocytes, the glial cells that myelinate neuronal axons. A decrease in NAA indicates neuronal damage. Glu is a neurotransmitter involved in memory formation and learning; decrease of glu indicates less neuronal activity. Myo-inositol (mIns) and taurine (tau) are involved in osmoregulation and are found in astrocytes, indicating an immune response; both increase during the development of AD in tg mouse compared to wt mice (Chen et al., 2009; Choi et al., 2010; Dedeoglu et al., 2004; Marjanska et al., 2005; Westman et al., 2009).

Chapter 6 describes the findings in the first longitudinal MRS study on a
transgenic mouse model. This study showed decreased glu and NAA in tg mice compared to wt mice at all time points. There was a decrease in mIns found in tg mice compared to wt mice at 3 months. Over time this decrease became less and at 18 months there was no difference between tg and wt mice in the level of mIns. At 3 months the level of tau was increased in tg mice compared to wt mice, this difference disappeared over time and at 18 months there was no difference in tau levels between tg and wt mice. Our findings confirm earlier findings in studies on transgenic mice (Braakman et al., 2008; Chen et al., 2009; Dedeoglu et al., 2004; Li and Wahlund, 2011; Marjanska et al., 2005; Oberg et al., 2008; Rupsingh et al., 2011; von Kienlin M. et al., 2005; Westman et al., 2009; Xu et al., 2010). These differences were not significant, probably due to the small size of the study group.

Currently, the long acquisition times limit the use of MRS in AD patients. If technical improvements are successful, the obvious next step would be to study large test groups of AD patients over time and compare those to non-demented subjects and, in a later stage, to patients with other neurodegenerative diseases.

Another finding, described in chapter 6 is a sex difference in AD mice and wt mice using MRS. It is known that females are at higher risk for developing AD than men (Andersen et al., 1999; Kim et al., 2015; Musicco, 2009). In this study female mice started with increased levels of NAA, compared to male mice, in both tg and wt mice. Female mice have a larger axonal density compared to male, which might explain the higher levels of NAA in both female control and tg mice at 3 months of age. The decline of NAA in tg female mice was more precipitous compared to control mice. The difference in glu levels between male and female wt mice at 18 months was a significant one. Female wt mice showed higher levels of glu compared to male wt mice. The level of taurine was higher in tg female mice compared to tg male mice at 3 months and showed a faster decline over time.

The effect of sex on the pathophysiology of AD has not yet been carefully examined. Hormonal influences have been suggested, however more research is necessary (Janicki and Schupf, 2010). It will be of great interest to study sex differences between AD and control groups in animals or in a non-invasive way in human subjects.

Our study is the first to show that metabolic changes during AD development are influenced by sex differences. This study is done in a small test group and should be considered a pilot, giving information for further
research.

**Conclusions**

This thesis aimed to improve the clinical diagnosis of AD by using native contrast MRI and MRS. MRI was correlated to histology with a special focus on iron because iron has high magnetic susceptibility effects on MRI and some reports show iron accumulation in Aβ plaques (LeVine, 1997; Nabuurs et al., 2011; Nabuurs et al., 2013; Smith et al., 1997) as well as in activated microglia (Connor et al., 1992; LeVine, 1997; Nabuurs et al., 2011; Nabuurs et al., 2013) and myelin (van Duijn et al., 2013).

We found changes in neocortical layers III-V, seen as band like hypointensities, on MRI scans which were correlated to a histological band-like pattern of iron/myelin in the neuropil fibres of cortical layers III-V. This band reflects a more crowded network of neuropil fibres with thicker and darker staining for iron. Iron was found in plaques, microglia and myelin in the neuropil. We also found a correlation between iron and neurofibrillary degeneration and plaques.

These MRI changes were observed only in a later stage of AD but may improve clinical diagnosis and enable monitoring effect of future experimental treatments.

The MRS studies showed early changes in metabolic levels between control mice and AD mice and between male and female mice.

Our results on MRI and MRS have potential to improve the clinical diagnosis of AD, however, more research is necessary. Follow up studies should be done in human test groups using MRI and MRS to confirm our findings that a hypointense band on MRI and metabolic changes predict AD. These studies should focus on a large group of direct family members of early AD patients, who are at risk for AD and compare these to a control group. These groups should be followed over time, doing MRI scans to check for the myelin/iron band and MRS scans to check for metabolic changes with caution for sex differences. Another study should focus on the specificity of the band like changes found on MRI scans. These band like hypointensities might not be specific for AD, which makes it necessary to compare AD patients also to other neurodegenerative diseases.
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