The iron brain: Post-mortem and in vivo imaging of iron in brain diseases

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Summary and Discussion

8.1. Summary

This thesis aimed to gain more insight into the role of iron in neurodegenerative diseases using high-field MRI. I investigated the pathological correlates of susceptibility-based contrasts on MRI, and how iron accumulation is associated with disease progression both ex vivo and in vivo. Several MRI techniques sensitive to tissue iron concentration have been used in the last decades to investigate and quantify brain iron accumulation in neurodegenerative diseases. Increased R_2^* values, phase shifts and changes in susceptibility values have been reported not only in the cortex of patients with Alzheimer's disease (AD) and striatum of patients with Huntington's disease (HD) [1–6], diseases studied in this thesis, but also in other diseases such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). Even more promising, some studies showed the potential of iron-sensitive MRI as a marker for disease progression by identifying iron accumulation in early disease stages and correlating iron accumulation to clinical decline [3, 7]. However, there is still a large gap between the MRI observations and the interpretation of these observed changes in terms of the microscopic distribution and molecular nature of iron in the brain, as well as a lack of understanding of the mechanistic role of iron in the processes of ageing and disease. Validation and interpretation of in vivo MRI findings using MRI and histology on post-mortem brain tissue provide some answers to these questions as they provide unique information on the underlying pathological substrate of MRI contrast.

In *chapter 2* I first validated MRI-based detection of iron in post-mortem cortical brain

tissue of AD patients as well as non-demented controls using a unique pipeline that allows a direct comparison between full spatial maps of iron detected by T_2^*-weighted MRI, histochemistry, and a gold standard for iron detection, laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). Iron histochemistry as well as quantitative MRI methods (such as R_2^* mapping and QSM) provide reliable measures for iron content in the cortex. These results support the use of MRI studies of iron distribution in both the healthy and the diseased brain. One of the most surprising findings was that despite the obvious differences in iron distribution patterns within the cortex between AD patients and controls, no overall significant differences were found in absolute iron concentration as measured by LA-ICP-MS, nor in R_2^*, phase or susceptibility. Although QSM is the most reliable of the MRI methods for iron quantification, we found that the anatomical contrast within the cortex is often better appreciated on T_2^*-weighted images.

In *chapter 3* I further investigated the histopathological correlates of T_2^*-weighted MRI contrast changes to determine the exact pattern of iron accumulation and the colocalization with specific cells in the frontal cortex of AD patients. I applied ultra-high field MRI to post-mortem brain tissue and showed that AD patients have a different cortical appearance on MRI. Histology-MRI correlation analyses of pixel intensities showed that the MRI contrast is best explained by increased iron accumulation and changes in cortical myelin, whereas amyloid and tau showed less spatial correspondence with the MRI contrast changes. Subtypes of AD, namely early-onset and late-onset AD patients, showed different patterns of cortical iron accumulation and myelin changes that may be detected by high-field susceptibility-based MRI.

In *chapter 4* I extended the research in *chapter 3* to investigate the spatial distribution pattern of the observed MRI contrast changes over the entire cortex, using tissue blocks from different cortical regions selected based on the distribution pattern of tau pathology. Combining ex vivo high-resolution MRI and histopathology revealed that subtypes of AD, again early-onset and late-onset AD patients, had a different distribution pattern of AD pathological hallmarks and MRI contrast changes over the cortex. In general, early-onset AD showed more severe MRI contrast changes. Per lobe, the severity of AD pathological hallmarks correlated with iron accumulation and with MRI. Therefore, iron-sensitive MRI sequences allow the detection of the cortical distribution pattern of AD pathology ex vivo.

In *chapter 5* I studied the brain of patients with Huntington's disease (HD), another neurodegenerative disease in which mainly subcortical areas are involved. I again used post-mortem human brain tissue and assessed the histopathological correlates of the previously reported T_2^*-weighted contrast changes in the striatum in HD. Ultra-high field ex vivo MRI showed that the striatum of HD patients has a distinctive phenotype on T_2^*-weighted MRI compared to control subjects. On ex vivo MRI, these contrast changes are heavily biased by enlarged perivascular spaces; it is currently unknown

whether this is a fixation artefact or a disease-specific observation. Histopathology showed that besides iron within the vessel wall, reactive astrocytes are the predominant source of the general increase of iron within the striatum and hence the observed post-mortem MRI contrast changes.

Based on the results in *chapter 5*, I describe the rationale and design for an in vivo 7T MRI study in *chapter 6*. This study, which is currently ongoing, focusses on the correlation of brain iron levels obtained from 7T MRI of HD patients in different disease stages, to specific and well-known clinical cerebrospinal fluid markers for iron accumulation, neurodegeneration and neuroinflammation. Results from this study will provide a basis for the evaluation of brain iron levels as an imaging biomarker for disease state in HD and their relationship with the salient pathomechanisms of the disease on the one hand, and with clinical outcome on the other.

In *chapter 7* I used QSM to investigate iron accumulation in the basal ganglia of SLE patients with neuropsychiatric complaints. In contrast to AD and HD, SLE patients do not have significant brain atrophy, but many of them do have significant neuroinflammation, particularly those that belong to the inflammatory subgroup. Therefore, this study aimed to gain insight into the link between iron accumulation and neuroinflammation in the absence of neurodegeneration. Comparison of susceptibility values between age-matched controls and SLE patients showed that iron levels in the basal ganglia are not changed due to the disease. No subgroup of SLE showed higher susceptibility values and no correlation was found with disease activity or damage due to SLE. Histological examination of post-mortem brain tissue including the putamen and globus pallidus supported the in vivo findings.

In conclusion, the central findings of this thesis are that the T_2^*-weighted MRI contrast changes as observed in the cortex of AD patients and striatum of HD patients are indeed caused by iron accumulation. However, depending on the disease and brain area involved, the MRI contrast spatially correlates to different pathological phenomena, including local iron deposition which may be diffuse or associated with grey and white matter myelin organization, activated microglia and astrocytes. Lastly, although neuroinflammation is thought to play an important role in brain iron accumulation, this does not seem to be true for every neuroinflammatory disease; in SLE, iron accumulation within microglia does not seem to be an important contributor.

8.2. GENERAL DISCUSSION

To put the work presented in this thesis into perspective, we first go back to the earliest discoveries of iron accumulation within the human brain. These date back to the previous century, when researchers did pioneering studies on the presence of iron in brain tissue using the very limited laboratory techniques available at that time. Non-

haem iron was first demonstrated in the brain in the late 19th century, followed by the observation in 1915 that certain parts of the brain give stronger staining reactions than others [8, 9]. Based on these studies, Spatz divided the centers of the brain into four groups according their iron content [10]. This was the first, and at that time most extensive and systematic investigation reporting that the globus pallidus and substantia nigra show the most intense iron staining, followed by the red nucleus, putamen, caudate nucleus, dentate nucleus and subthalamic body. Diffuse iron was observed with the naked eye, but investigating the tissue at high magnification showed iron within neurons and oligodendrocytes. Also infiltration of iron within the walls of blood vessels of the globus pallidus was reported [11]. The form in which iron accumulated remained unclear, although it was suggested that at least part of it was present in ferritin [11].

At that time, apart from observations that less iron is present in the brains of children than in those of adults, the effect of aging on iron content was not systematically studied. In 1958, Hallgren and colleagues acknowledged that insight into the relationship between age and non-haem iron content is essential when investigating non-haem iron in pathological conditions. This idea was probably based on the hypothesis raised by Spatz [12] suggesting that pathological storage of iron within the globus pallidus and substantia nigra caused rigid akinetic syndrome with contractures (later known as Hallervorden-Spatz disease). Subsequently, Hallgren et al, nowadays still referred to as 'the hallmark study for brain iron concentrations', reported on what is now the well-known increase of iron content with advancing age in different brain regions [13]. A couple of years later, the same group reported that iron content was increased near senile plaques in AD patients [14]. This was followed by other groups reporting disrupted brain iron metabolism in several neurological disorders, including HD, PD, multiple sclerosis, and Pick's disease [15]. Already at this point and as also shown in this thesis, it was observed that regional iron accumulation depended on the disease involved: diseases affecting the basal ganglia such as PD and HD showed increased iron in those regions (*chapter 4*), whereas increased iron was found in cortical regions in AD patients (*chapter 3 & 4*). MRI studies in the 1990s also supported the accumulation of iron within the brains of PD and AD patients. However, the interpretation of observed regional hypointensities on T_2-weighted MRI was thought to be primarily caused by iron [15, 16], while other sources of negative contrast, such as calcium and myelin, were not considered as important contributors to these hypointensities. Also, apart from the brain areas being differently affected, differences in the cellular localization of iron among the different diseases were not reported. Although the exact role of iron in neurodegenerative diseases was unclear, the working hypotheses were quite general and heavily focused on oxidative stress as the main pathological mechanism. Neurodegeneration in PD and AD was thought to be associated with and even suggested to be caused by the increased presence of highly reactive oxygen species which may be generated by increased presence of redox-active iron [17].

Since then the hypotheses on the role of iron in neurodegenerative diseases evolved and became more disease-specific. Importantly, the first studies were published reporting data that point to a causal role of iron in neurodegeneration rather than just a correlative bystander effect. Whereas in AD iron was initially found to be only spatially related to $A\beta$ plaques and neurofibrillary tangles, evidence from animal and in vitro studies showed that the presence of iron actually promotes and increases the toxicity of $A\beta$ aggregation through the furin pathway [18]. Additionally, iron also promotes hyperphosphorylation of tau, thus proving a causal link between iron and two key components of AD pathology. Following the discovery that mutations in the HFE gene are responsible for most cases with hereditary hemochromatosis [19], several studies attempted to identify a potential link between HFE-associated hereditary hemochromatosis and AD [20–23]. Although still controversial, some studies showed significant associations between HFE mutations and AD and increased frequency in AD [23]. Especially the combination of H63D HFE mutant allele together with the ApoEϵ4 allele was shown to significantly reduce age of onset of AD compared to ApoEϵ4 carriers alone [24].

Concurrently, the MRI field also made significant advances, and the increasing availability of clinical scanners operating at higher field strength (3T) allowed for more sensitive detection of iron in the brain [25]. The number of studies investigating iron accumulation using MRI in several neurological diseases significantly increased and the potential of MRI as a potential biomarker for the presence and progression of important neurological disorders was increasingly recognized.

During the course of this thesis accumulating evidence hinted to iron as a potential contributor to disease progression, with most of the evidence coming from studies in AD. Starting from correlative studies showing that iron accumulation is negatively correlated with cognitive performance in AD [2, 26, 27], recent studies showed that increased CSF levels of ferritin do not only predict poorer cognition [28], but also increase the risk of conversion from mild cognitive impairment to AD [29]. Subjects with mild cognitive impairment with high CSF ferritin levels demonstrated an earlier age of diagnosis compared to patients with low CSF ferritin levels [28]. This was further supported by a post-mortem study from the same group investigating the association between brain iron and the rate of cognitive decline assessed during the 12 years prior to death [30]. A central finding of that study was that iron burden contributes to cognitive decline upon the underlying AD pathology, further supporting the hypothesis that in the presence of AD pathology, brain iron affects the symptomatic progression of the disease. Complementary findings are reported in *chapter 3 and 4* from this thesis, reporting that patients with early-onset AD show more severe changes on both MRI and iron histochemistry compared to patients who develop the disease after the age of 65 (late-onset AD).

Research over the last years has also shown that iron plays an important role in ad-

ditional mechanisms beyond oxidative stress. Interestingly, as also described in the introduction of this thesis, a mechanism that has been shown to be present in several neurodegenerative diseases is neuroinflammation. In both AD and HD, neuroinflammation has received much attention and studies including patients without or only mild symptoms showed an early and substantial involvement of inflammation in the disease pathogenesis [31, 32]. Based on the observation that within the brain iron colocalizes with activated microglia, and that a pro-inflammatory environment induces iron accumulation within microglia, the possibility was raised that imaging of iron using MRI would potentially reflect neuroinflammation [33]. On a cellular level, we indeed found iron-positive microglia in the AD cortex, particularly in the vicinity of $A\beta$ plaques (*chapter 3*). These findings put earlier observations in numerous MRI studies on the detection of $A\beta$ plaques in a new light; much of the colocalization of iron in $A\beta$ plaques, visible as hypointense foci on iron-sensitive MRI, may actually reflect neuroinflammation rather than the $A\beta$ plaque itself [4, 34, 35].

As shown in *chapter 5*, iron accumulation within microglia was less prominent in the striatum of HD patients. A possible explanation may be that brain tissue with relatively high disease stages was used. I hypothesize that initially in HD, microglia likely play an important role in sequestering iron, but due to prolonged activation, microglia become dystrophic and degenerate. Finally, after microglial degeneration in end stage HD, activated astrocytes take over the role of microglia as the predominant iron-sequestering glia cells. Nevertheless, it remains unknown whether microglia accumulate iron as a consequence of their pro-inflammatory activation status or is it the other way around: do microglia become activated as a consequence of increased iron?

Results from this thesis also show that in AD, HD and SLE the MRI contrast is not necessarily explained solely by iron accumulation within microglia. In *chapter 3*, I showed that on a pixel-by-pixel basis iron accumulation in the AD cortex is mainly related to changes in cortical myelination. Rather than demyelination as generally observed in the white matter in neurodegenerative diseases, an increased myelin staining covering layer II-IV of the cortex was found in AD patients. First, the observation of increased myelin staining does not necessarily reflect an absolute increase of myelin content. It might be caused by tissue compaction due to the loss of neurons and supporting cells. However, myelin alterations and specifically the association between $A\beta$ pathology and focal myelin disruption in the cortex of AD patients have been previously reported [36, 37]. Whereas focal myelin loss implies that myelin alterations are a consequence of AD pathology, recent studies suggest that myelination might has a key role in AD pathogenesis [38]. Using single-cell transcriptomic analysis, it was shown that myelination-related processes were perturbed not only in oligodendrocytes and oligodendrocyte precursor cells, but also in neuronal and other glial cells suggesting a regulatory response to maintain myelin integrity [38]. Interestingly, changes in genes related to myelination, such as upregulation of LINGO1, were already found early dur-

ing pathological progression [38].

In addition to changes in myelin which are present in both AD and HD, reactive astrogliosis is also a pathological hallmark in both diseases and common to nearly all neurodegenerative diseases. However, as shown in *chapter 5* of this thesis, only in the striatum of HD patients we found that iron within astrocytes significantly contributed to the observed MRI contrast changes, suggesting that the glial response is different in the striatum compared to the cortex. Indeed, several studies have shown clear morphological, molecular and functional differences between astrocytes present in the gray and white matter and even among the different cortical layers [39, 40]. Aging studies showed that during aging significantly more gene expression changes related to astrocyte reactivity were found in the mouse hippocampus and striatum, compared to the cortex [41]. Results from studies focusing on neurons in these brain regions suggest that astrocytes within the hippocampus and striatum are more vulnerable to oxidative stress and the dysregulation of ion homeostasis [42]. This corresponds to a study showing that striatal astrocytes have a decreased calcium mitochondrial buffering capacity compared to cortical astrocytes [43, 44], suggesting again increased vulnerability of the striatum.

Conversely, although the same brain regions were investigated as in HD, the amount of iron within glial cells in SLE patients as a consequence of neuroinflammation in the absence of neurodegeneration seems to be unchanged compared to controls (*chapter 7*). This raises the possibility that although brain diseases as AD, HD and SLE share many mechanisms, the relative contribution of each of these to the pathology might differ between the diseases.

Taken together, it seems that depending on the disease involved, iron accumulates within specific cell types and brain regions. Why certain cell types and brain regions are more susceptible to increased iron levels needs further investigation. Single-cell profiling techniques such as single-cell RNA sequencing, can be powerful tools to give more insight into the underlying mechanisms of selective cellular iron accumulation. Recently, gene expression profiles which classify the different cell types within the human cortex, individual cortical layers and brain regions have become available, which may help to further understand the selective accumulation of iron within the brain[45, 46].

Although the field has clearly advanced over the last years and provided new insights into the role and relevance of iron accumulation in neurodegenerative diseases, it is currently unknown whether the observed increase of iron is a net increase or a redistribution of iron as a consequence of neuroinflammation or atrophy. Apart from an influx of iron due to, for example, increased blood-brain barrier permeability, vascular events and increased expression of iron importers [47, 48], it might be more plausible that iron redistribution explains the observed changes. As observed in AD and HD, but also in diseases as multiple sclerosis, demyelination and an attempt to remyelinate increases

the local availability of iron [49]. In addition, increased intracellular iron upon activation within microglia and astrocytes can be a consequence of increased extracellular iron originating from degenerating neurons or myelin debris [50–52]. This might also explain why we did not observe increased iron within microglia in SLE, as this disease is characterized by significant neuroinflammation, but not significant neurodegeneration. Alternatively, the observed increase of iron within the AD cortex and HD striatum can also be a result of tissue compaction due to atrophy and loss of neurons and supporting cells. In that case, the total amount of iron in the cortex would not change, which could explain why absolute iron concentrations were not changed in chapter 2. However, measuring atrophy is not possible in small post-mortem tissue samples such as we used in our studies as deformation due to formalin fixation might be present. Therefore, future in vivo or in situ post-mortem scans should link iron-based MRI scans to local atrophy. In addition, although high-resolution MRI within clinical time frames is becoming more realistic with increased magnetic field strength, MRI will be limited by its inability to measure absolute iron concentrations and intracellular localization of iron. Also, in vitro experiments with cell-type-specific human iPSC derived neurons and microglia in combination with iron challenges will give more insight into the underlying mechanisms of iron accumulation within these cells.

Iron-sensitive MRI is nowadays commonly used as a non-invasive proxy for brain iron and several studies showed the improved quantification of iron using QSM over phase and R_2^* measurements [53–56]. Especially with the advent of high-field MRI, QSM at 7T is particularly advantageous, since MRI at higher magnetic field is more sensitive to tissue-related magnetic field disturbances caused by presence of iron [57]. Also, scanning at higher resolution due to increased signal to noise at high-field allows better visualization of iron distribution within structures as for example the cortex [57]. High-resolution MRI will lead to more accurate parcellation of the cortex and even individual cortical layers. This is especially of interest for research on AD as, as shown in *chapter 2*, sometimes a simple T_2^*-weighted image is still preferred over an average regional susceptibility or R_2^* value as this contains information on the spatial distribution patterns over cortical layers. Multi-modal approaches will remain extremely valuable as the combination of MRI and histological/molecular tools yields information on the cellular localization and for example changes in the expression of iron regulation proteins.

8.3. CONCLUSION AND FINAL REMARKS

The combination of MRI with other techniques has increased our understanding on what iron-sensitive MRI reflects in healthy controls and patients enormously. Besides iron, myelin is proven to be an important source of T_2^*-weighted MRI contrast [58–61]. Due to observation by several studies that iron is present within $A\beta$ plaques and

induces hypointense foci on MRI, numerous studies focused on the detection of $A\beta$ plaques in vivo using iron-sensitive MRI [4, 34, 35]. Studies combining QSM as a proxy for iron and a PET-ligand specific for fibrillary $A\beta$ plaques showed that the 11C-PIB ligand is indeed significantly associated with cortical iron as measured with MRI in $A\beta$-positive cognitively normal, MCI as well as AD patients [7, 29]. As shown by van Duijn et al [62], and in *chapter 4* of this thesis, the amount of $A\beta$ plaques is indeed positively correlated with iron, however, on a pixel-by-pixel basis changes in cortical myelin and associated iron are the predominant cause of MRI contrast changes in AD (*chapter 3*). Also, iron within microglia surrounding $A\beta$ plaques and extracellular diffuse iron should not be neglected as contributors to the observed MRI contrast changes. As such, T_2^*-weighted MRI is not a simple proxy for just iron nor amyloid. It is the sum of several components, like iron associated with myelin, $A\beta$, tau and microglia, that together form the final image.

Maybe one of the most important outstanding questions is whether iron is a cause or a consequence of neurodegeneration. Several studies, as also described above, have shown that the presence of iron can lead to oxidative stress, promotes protein aggregation and neuroinflammation. In addition, oxidative stress itself also potentiates the neurotoxic oligomerization of proteins like $A\beta$ and tau, but also promotes neuroinflammation by the activation of microglia and astrocytes [63, 64]. Recently, a newly described iron-dependent form of cell death termed 'ferroptosis' has been described which involves iron dysregulation, lipid peroxidation and inflammation as major hallmarks [**?**]. Taken together, all these mechanisms are known to be promoted by increased levels of iron, but also the other way around: these mechanisms can lead to increased levels or redistribution of iron within the brain. Therefore, with the currently available evidence, regardless of the of the primary event, a flywheel concept is the most probable hypothesis.

In vivo evidence on how iron-sensitive MRI is related to brain iron levels, neuroinflammation and disease progression in neurodegenerative diseases is needed and can, as suggested in *chapter 6*, be accomplished by correlating MRI with specific cerebrospinal fluid markers for iron accumulation, neurodegeneration and neuroinflammation. These conjectures deserve further consideration, for example by using the wealth of data available from larger population studies such as the UK Biobank, Alzheimer's Disease Neuroimaging Initiative (ADNI) and the Religious Order Study (ROS).

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