The irony of iron

Hagemeier, J.

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Chapter 5

Discussion, summary and future perspectives

The aim of the present thesis was to use an iron sensitive MRI imaging technique (SWI), in the context of the inflammatory, demyelinating and (neuro)degenerative disease multiple sclerosis (MS). For several decades, MRI studies using techniques sensitive to iron, such as SWI but also T2, T2*, R2*, FDRI and post-mortem studies have been conducted in neurodegenerative disorders such as Alzheimer's disease. However, MS has received much less attention, most likely because many have regarded this disease as a strict white matter (WM) disorder, whereas increased iron levels are usually observed in the gray matter (GM). More recent developments in MS research have led to a shift in the focus of research to also include GM pathology. Throughout the present work, the SWI-filtered phase technique, which is sensitive to (para-) magnetic substances, most notably iron, was used as a surrogate *in vivo* marker of iron deposition. In Chapter 2, we first established a baseline for the behavior of SWI phase in healthy aging. From there on, in Chapter 3, we investigated SWI-derived phase measures in deep GM structures in different stages of MS, starting in early MS cases (pediatric and clinically isolated syndrome) and later stages (relapsing-remitting and secondary progressive MS). In Chapter 4, the hallmark pathological finding in MS, white matter signal abnormalities/lesions, are investigated for appearance, prevalence, and diagnostic value using SWI.

Deep gray matter

Hallgren and Sourander published a hallmark histological study in 1958 where they observed that non-heme iron was elevated in older people; occurring mostly in the putamen, caudate nucleus, globus pallidus, substantia nigra, dentate nucleus, and thalamus, as well as the prefrontal, sensory, cerebellar, and motor cortices.[1] Iron levels of the globus pallidus, thalamus, red nucleus, substantia nigra, and sensory and cerebellar cortices increase rapidly during the first decades of life, but remain relatively constant starting at 30 years, with the exception of the thalamus where iron levels slowly decreases after young adulthood (Chapter 2). In contrast, iron contents of the putamen, caudate nucleus, and motor cortex increase somewhat slower, with maximum levels being reached in older age. Many recent studies have used MRI to investigate the link between healthy aging and brain iron levels. Among the MRI techniques used are SWI, magnetic field correlation, quantitative susceptibility mapping, and T2 and R2 relaxometry.[2-7] Collectively, their main findings imply that MRI changes suggestive of increased iron increase with age. In order to have a reference framework, it is important to also understand SWI phase behavior in healthy individuals. In Chapter 2, we extend previous findings in the normal population to include not only mean phase measures as obtained by SWI, but also mean phase measures of low phase voxels (MP-LPV) as a measure of highly affected tissue, as well as volumetric analysis of deep GM structures. Our findings are in line with the literature with respect to the association of increased age and lower mean phase and MP-LPV measures, indicative of higher iron levels. However, a novel finding was that –unlike in previous studies– the association of SWI mean phase measures and age was not strictly linear but are plateauing or even reversing in middle age. In several prominent brain structures, including the caudate nucleus and thalamus, the association of age with mean phase was better explained by a quadratic fit, as opposed to a linear fit. This suggests that deep GM iron levels peak between 40 and 60 years of age, after which iron levels tend to slowly decrease again. However, when considering only MP-LPV, i.e. highly affected voxels, the associations with age were strictly linear. This finding sheds light on brain phase behavior

in healthy individuals; the total iron concentration peaks in middle-age, after which it rebounds, whereas the iron content of tissues with already high levels of iron increases steadily with age. In a recent SWI study by Haacke et al.,[8] it was shown that not only did measures of such high iron content areas increase with time, they appeared to even accelerate with age. This increase of high iron tissues can potentially have deleterious effects in the form of atrophy. Indeed, we observed that mean phase and MP-LPV measures from deep GM structures, especially the thalamus, were strongly associated with neocortical and lateral ventricle volumes. One proposed hypothesis postulates that the excessive iron levels cause free radical damage to cells through chemical (Fenton and Haber-Weiss) reactions.

After establishing that SWI-filtered phase measures are altered in older age, and that mean phase and MP-LPV behave differently (quadratic vs. linear), it was important to highlight differences of these measures among different MS disease types and stages. First, we scanned adolescent MS patients (Chapter 3.1). Twenty patients with a mean age of 15 were recruited and phase measures of deep GM structures were compared to 21 age- and sex-matched healthy individuals and eight adolescent patients with other neurological disorders. In this study, multiple measures of abnormally high iron content were utilized: (1) as considered previously, MP-LPV which takes into account the mean phase values only of voxels 2 standard deviations below the mean of a reference healthy control group, (2) LPV volume which quantifies the volumetric size (in milliliters) of these low phase voxels, and (3) the inverse; normal phase tissue volume (NPTV) which is defined as the volume of tissue that is not severely affected (voxels within the >2 standard deviations normal range). Consistently the pulvinar nucleus of the thalamus had the lowest MP-LPV, increase in LPV volume, and biggest decreases in normalized volume and NPTV, all of which are suggestive of increased atrophy and iron content. In addition, the putamen and caudate nucleus had prominent healthy tissue (NPTV) reductions. Although not always significantly so, in other structures among adolescent MS patients (caudate, putamen, globus pallidus, thalamus, amygdala, nucleus accumbens, substantia nigra), the MP-LPV and NPTV was consistently the lowest, whereas the LPV volume was consistently the

highest. Showing both measures of mean phase and volumes serves to create a link between SWI phase measurements suggestive of iron increases and volume reduction. Clearly, longitudinal studies can shed more light on the causality of this relationship. However, an interaction between iron and cell destruction appears evident. This study showed that excessive iron is present at the earliest stages of MS, a finding also observed in the caudate nucleus by Ceccarelli et al.[9] using T2 hypointensity, lending credence to the notion that its detrimental effects could potentially not only cause localized damage, but may also lead to eventual disease progression.

Investigating clinically isolated syndrome (CIS), as done in Chapter 3.2, is another crucial step in understanding the phase behavior of early MS disease course. Similar to adolescent MS patients, patients diagnosed with CIS have significantly lower MP-LPV values and increased LPV volumes in especially the pulvinar nucleus of the thalamus. In addition, they also show signs of iron deposition in the caudate and putamen compared to age- and sex-matched healthy individuals. Interestingly, no global or regional volumetric differences were found between the study groups. It stands to reason that pathology measured by SWI-filtered phase images is not reflected in atrophy yet. This study supports the concept that iron deposition is present in early MS disease stages, even in patients with a single clinical attack, which may contribute to disease development and brain damage. In these early stages, volume loss is minimal yet pathology is visible on SWI phase. It would be expected that in later stages of MS the atrophy of deep GM structures is concomitantly present along with increased iron content, a finding which has been observed previously.[10]

To test whether SWI-filtered phase metrics have any clinical relevance, we recruited an adult sample of relapsing-remitting and secondary-progressive MS patients in order to assess the association of SWI mean phase and Kurtzke Expanded Disability Status Scale (EDSS)[11] and disease duration. In Chapter 3.3 it is shown that deep GM MP-LPV is independently related to increases in EDSS, even when the statistical model is corrected for age and gender as well as conventional MRI measures (T2 and T1 lesion volume, and normalized cortical and WM volume). Specifically, caudate and red nucleus

MP-LPV were associated with EDSS increases. Interestingly, in stepwise models, the deep GM MP-LPV measure was retained, whereas conventional MRI metrics commonly found to be associated with MS were not. These results suggest that decreased mean phase, indicative of increased iron content, in the deep GM is clinically relevant.[12] In addition, loss of thalamus volume was associated with longer disease duration.

In chapters 2, 3.1 and 3.2 the thalamus, especially the pulvinar nuclei of the thalamus are consistently shown to have lower phase values and thereby elevated iron content, in addition to volume loss. This begs the question, are the thalamic nuclei, specifically the pulvinar, more heavily involved than previously thought in MS? Several recent studies have found that extensive volume loss (1) occurs in the thalamus[13] (2) is related to cognitive decline[14] and (3) is associated with conversion from CIS to clinically definite MS.[15] The pulvinar nucleus of the thalamus has not been researched as extensively, although researchers have found atrophy of this structure among relapsing-remitting MS patients.[14] Because of the extensive cortical connections of the thalamus and pulvinar nucleus,[16] research efforts will have to be made to investigate their involvement in MS. In recent years, the focus of research has already shifted somewhat away from WM, toward GM.[17, 18] Several studies,[19-21] including the present work, have shown that elevated levels of iron, as assessed using different MRI techniques, are associated with GM atrophy. Therefore, increased levels of iron could potentially be a piece of the puzzle of, or biomarker for, GM pathology and the associated clinical signs.

White matter phase signal abnormalities (lesions) in multiple sclerosis

White matter signal abnormalities (WM-SAs) are a hallmark feature of MS, yet the clinical importance of the occurrence of such lesions as observed on T2-weighted imaging are disappointing.[22-25] T2- and T1-weighted WM-SAs are thought to represent focal pathology, and to be caused by inflammation, edema, demyelination and/or gliosis.[23] Even though T2 WM-SAs are present at the first demyelinating episode, the poor specificity of conventional

MRI[22,26] limits their predictive value. Previously, the differential diagnosis of MS vs. other central nervous system disorders was considered using brain and spinal cord MRI, and incorporating number, localization and morphology of T2 WM-SAs in the diagnostic criteria of MS,[27] or by using non-conventional MRI techniques.[26,28-30] We sought to add SWI-filtered phase to the mix of non-conventional techniques in order to examine focal brain pathology in CIS and MS, and presence, prevalence, localization, and clinical relevance of observed white matter phase lesions.

A substantial subset of WM-SAs have negative phase shifts,[27] and have morphological differences.[10,27-30] Recent studies have confirmed histologically, that WM-SAs visible on MRI phase and $R2^*$ correspond to focal iron deposits, although other factors are likely to influence MRI phase as well (e.g. demyelination, deoxyhemoglobin, tissue microstructure and fiber orientation).[31-33] Phase changes of WM-SAs have been proposed to specifically signal early lesion development.[33, 34] WM-SAs visible on SWI-filtered phase images may appear initially, but signal intensity will be lost when the pathology advances, possibly due to lesion microstructural changes.[33] Even though phase WM-SAs may disappear over time, it has to be noted that they are not synonymous to active lesions (ie. they are not contrast enhancing). In fact, in a recent pilot study by Bian et al.[35] some phase visible WM-SAs persisted over an extended period (>2 years), far longer than one would expect a contrast-enhancing lesion to be active. Also, in one case, the phase WM-SAs even preceded hyperintensity on magnitude images.[35] In a combined MRI and histopathological study of WM lesions, Bagnato et al.[36] found that activated microglia (CD68) co-localize with ferritin and iron (Perl's/Turnbull Blue). Since oligodendrocytes possess the highest concentrations of iron in the healthy brain, the breakdown of myelin in the context of WM lesions would cause the extracellular release of iron, followed by macrophages ingesting copious amounts of iron as a means of detoxification (by transforming the more toxic $Fe^{2}+$ to $Fe^{3}+$, and binding it to ferritin).[36] Furthermore, leakage of the blood brain barrier may lead to an increase in iron concentration by allowing iron to seep into perivascular regions.[37] This mechanism may have further importance especially in ring-

like phase WM-SAs, which tend to be situated around central penetrating veins and where macrophages may remain for an extended period of time in an anti-inflammatory, protective state. The previously mentioned distinct pathologies, including increased iron levels, are most likely strongly associated with each other, have the potential to influence phase shift in WM-SAs, and are observed in MS and related disorders.[10, 38-42] Because of this, investigating both the occurrence and relevance of phase WM-SAs remains important.

First, in Chapter 4.1, we investigated the number, volume, and mean phase of SWI-filtered phase visible WM-SAs in a sample of 135 MS patients. As expected, MS patients had more, and higher volumes of phase WM-SAs compared with healthy individuals. However, phase WM-SAs were much less prevalent than T2 lesions. Of T2- and T1-weighted imaging WM-SAs, only 23.6% and 37.3% respectively overlapped with phase WM-SAs, indicating that the majority of T2 and T1 lesions are independent of phase lesions. This suggests that some of the phase lesions are unique to SWI-filtered phase images. Phase WM-SAs were also observed to consist of several morphological subtypes: the most prevalent of which were nodular and scattered, while ring lesions were more rarely observed. The presence of multiple (>5) phase WM-SAs could readily distinguish MS patients from healthy control subjects with a sensitivity of 75.6% and specificity of 89.9%. Indeed, presence of phase WM-SAs appears to be a good differentiator of patients from healthy individuals, and may be a valuable tool in differential diagnosis, in addition to the classic, hallmark, T2 WM-SAs. However, the lack of significant correlations between phase WM-SA volume, number, or mean phase with clinical outcomes mirror the limited clinical relevance that T2 WM-SAs have.[24]

Even though the presence of WM-SAs lacks strong clinical and phenotypical associations, they do seem to constitute genuine brain pathology. In order to be of value as a diagnostic measure, disease stages before relapsing remitting MS, such as CIS, will have to be assessed for phase WM-SAs. As is described in Chapter 4.2, phase WM-SAs possess diagnostic value in patients with a single demyelinating episode. In addition to corroborating findings from the

previous study that phase WM-SAs are more prevalent in patients, it was also determined that the mere presence of phase WM-SAs could not only distinguish between CIS and healthy subjects, but also between CIS vs. patients with other neurological disorders, and CIS vs. neurological autoimmune disorders, with a high sensitivity and specificity. An interesting finding relates to the commonness of T2 WM-SAs. Because it is not unusual to have at least one T2 WM-SA, the presence of such abnormality is not highly specific in classifying CIS patients. In order to still have valuable diagnostic properties, additional lesions and localization data are necessary, such as with the McDonald 2005[43] and 2010[27] criteria. However, differentiating CIS patients from patients with other neurological disorders using 1 or more phase WM-SA could be done with an accuracy of 73.1%. Compared to an accuracy of 64.1% for the presence of any T2 WM-SA, 62.8% for satisfying the McDonald 2005, and 67.9% for satisfying the McDonald 2010 (based on the dissemination in space of T2 WM-SAs; longitudinal analyses were not conducted). The mere presence of phase WM-SAs could classify the CIS patients. Furthermore, presence of multiple phase WM-SAs was associated with progression to clinically definite MS from CIS and approximately half of phase WM-SAs were not detected by T2 and may represent unique pathology. Phase lesions may be less prevalent, but when they occur they can classify CIS patients with relatively high specificity, on par with, or even exceeding, McDonald criteria for dissemination in space, rendering it a potential tool for differential diagnosis.

Brain iron as a biomarker; genetics and underlying biology

Throughout this work it has been argued that the detection of brain iron using MRI techniques provides valuable information in healthy brain aging and disease states. However, as opposed to for example demyelination and inflammation, iron deposition is not regarded a classic hallmark pathology of MS. It is therefore imperative to describe what biological mechanisms may underlie these changes seen on MRI, and what subsequent pathologies those may cause, in order to fully understand the implications of the presented MRI findings.

It has long been known that iron is present in the brain, a finding first published by Zaleski in 1886 using the Perl's stain on a single human brain.[44] Guizetti and Spatz first described that staining for iron was most evident in deep GM structures of the extrapyramidal system,[44] and Hallgren and Sourander published a hallmark study in 1958 where they observed that deposition of non-heme iron in the brain was correlated with age. Basal ganglia structures tend to have the highest level of iron, with oligodendrocytes being the most prominent cell-type to stain for iron,[45] while ferritin is the most common iron-storage protein. In the substantia nigra, neuromelanin is the location of the most prominent iron storage, with levels also increasing with age.[46]

Mutations of several iron metabolism genes can have severe implications in age related diseases, such as cardiovascular disorders, but also more importantly for the present work, in disorders of the central nervous system. Homozygous carriers of the relatively common Cys282Tyr mutation of the hemochromatosis (HFE) gene, which causes systemic iron overload, have a significantly increased risk for acute myocardial infarction.[47, 48] Male carriers with hereditary hemochromatosis seem to be especially vulnerable to develop further iron overload related problems, such as cirrhosis and hepatocellular carcinoma.[49] It has also been observed that healthy male carriers of the Cys282Asp and/or transferrin C2 mutations have significantly higher brain ferritin levels than non-carriers, which could possibly be a contributing factor for gender differences observed in neurodegenerative disorders.[50] Furthermore, these iron metabolism genes have been located within the brain, indicating that they not only affect systemic iron levels. Studies have linked increased incidence of several neurodegenerative disorders such as amyotrophic lateral sclerosis and Alzheimer's disease, to the presence of HFE gene mutations such as Cys282Tyr and His63Asp. However, the role of these genetic mutations remains uncertain in other disorders such as MS, Parkinson's disease, and ischemic stroke,[51-53] and would thus require further research.

Even though brain iron deposits have been found in healthy individuals, it may still be related to functional impairment. For example, Penke et al.[54]

showed that elevated levels of brain iron are inversely related to cognitive ability and successful cognitive aging in a cohort of healthy elderly individuals. Furthermore, in a group of 10 healthy elderly individuals, caudate and putamen iron estimates corresponded to lower scores on the Dementia Rating Scale.[55]

On the molecular level, excessive levels of iron, as well as other redox active metals, have the ability to promote the generation of reactive oxygen species. These subsequently overwhelm antioxidant protection mechanisms and cause harm to membranes and DNA, and can promote or exacerbate protein misfolding and aggregation.[56] Specifically, free (labile) iron, has the ability to catalyze the generation of highly reactive hydroxyl radicals ($^{\cdot}OH$). In the Fenton reaction, ferrous iron (Fe^{2+}) and hydrogen peroxide (H_2O_2) react to generate ferric iron (Fe^{3+}), hydroxyl anion (OH-), and the reactive hydroxyl radical ($^{\cdot}OH$), potentially resulting in molecular damage. The Haber-Weiss reaction, wherein superoxide ($^{\cdot}O_2^-$) participates in the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), is followed by the Fenton reaction to also produce hydroxyl radicals (see Chapter 1).[57, 58] Hydroxyl radicals can cause DNA strand breakage and chemical alterations of the deoxyribose and purine and pyrimidine bases, as well as damage to membranes through lipid peroxidation, causing mitochondrial dysfunction.[57] Oxidative damage has been known for some time to occur in and around MS lesions.[59, 60] For example, lesions contain significant amounts of mRNA encoding for human inducible nitric oxide synthase.[61] Other mechanisms leading to nitric oxide radical increases in WM lesions include the widespread expression of NADPH oxidase, which induces oxidative damage and may act in targeting oligodendrocytes for microglia destruction.[60] Other factors may exacerbate or amplify oxidative damage. For example, anterograde or retrograde axonal degeneration can lead to microglia activation and lesion formation in areas which are connected to previously injured sites.[62] Clearly, excessive iron is not the sole cause for oxidative injury in the MS brain. However, iron may hold an important key in that iron in the healthy brain is mostly stored in oligodendrocytes and myelin sheets.[63] Therefore, widespread destruction of specifically these cells in MS could mean that there is a particular

susceptibility to a vicious cycle of iron-induced oxidative injury.[64] This would suggest that demyelination and neurodegeneration in MS is at the least partly driven by free radical induced damage. At this stage it is impossible to implicate excessive iron levels as a causative factor in MS disease initiation. However, it seems likely that in early disease stages both inflammation and oxidative stress together with excess iron, play an important role. Even though increased iron levels may play a role in the early stages by causing oxidative injury, it stands to reason that the more severe the demyelination, the more labile iron is released, resulting in a cascade of oxidative damage and microglia activation, leading to even more severe pathology. Because excessive iron has the potential to cause harm in the brain, *in vivo* monitoring of patients with CNS disorders may prove valuable in MS.

Conclusions and future perspectives

Even though several factors can influence MRI phase (inflammation, microstructure, myelin, fiber orientation), it remains likely that a substantial contribution of the observed signal comes from differences in iron concentration, as it is the most abundant paramagnetic substance. Some effort has already been put into histopathological studies;[65] however, future experimental studies should extensively expand on the investigation of excessive iron levels using a combination of histopathology and MRI, to further validate what the observed MRI signals represents in both human studies and animal models of MS. In either case, regardless of the source of signal change, if it can distinguish between disease and health states, as well as disease type, and provide clinically relevant information, it should be considered a promising measure. Combined, the results from the described studies demonstrate that imaging MS patients using SWI or other iron sensitive imaging techniques may enhance the understanding we have about some of the underlying pathologies, and can potentially aid in the detection and diagnosis in early disease development.

The described studies are all cross-sectional in nature. We attempted to overcome the issue of causality by investigating patients at different disease stages and different ages, and concluded that in patients with a single

demyelinating event there are MRI phase changes present in the deep GM structures, though no structural volume reductions are observed. However, what the exact temporal relationship of phase pathology with hallmark MRI abnormalities (such as atrophy and lesion formation) observed in MS is, can only be addressed by longitudinal follow-up studies, preferably among patients recruited at or before first onset. Several studies[66] have proposed that excessive iron levels can cause tissue destruction through its free radical properties, whereas others[67] have argued that excessive iron levels may be an after-the-fact epiphenomenon. There is currently no definitive proof which of these hypotheses, and to what extent, is true.

Further research would also need to address whether phase measures and WM-SAs are associated with clinical and cognitive measures. Results from Chapter 3.3 showed correlations with EDSS. However, it will be crucial to utilize extensive neuropsychological and memory test batteries to investigate whether phase WM-SA presence or deep GM phase measures can predict alterations in cognition. Among MS patients, such studies would have to be carefully designed, due to confounding factors such as fatigue and fluctuating disease severity in the form of relapses.

In the future, SWI and other iron sensitive MRI methods could potentially be used in clinical trial designs to monitor the brain iron levels of MS patients. Although mostly hypothetical at this stage, longitudinally following patients with MS using such MRI measures could hold potential in studies of iron-chelating agents and anti-oxidant therapies targeting reactive oxygen species catalyzed by excessive iron. For example, a recently (2013) FDA approved therapy (BG-12/Tecfidera, Biogen-Idec) has been shown to have beneficial effects by reducing oxidative stress mostly by mediating the nuclear 1 factor (erythroid-derived 2)–like 2 (Nrf2) antioxidant response pathway.[68, 69] Although studies on iron chelators are in their infancy, and are mostly conducted in animal models, selective chelators have the potential to remove excessive levels of brain iron, and studies are currently being carried out in several other neurodegenerative disorders.[70-72]

In conclusion, the main findings of this thesis are:

- SWI-filtered phase allows indirect imaging of deep GM iron content, although other physical properties are also likely to influence phase changes.
- In the deep GM, mean phase, an overall measure of phase visible pathology where more negative values represent more severe pathology, decreases with age until middle-age, after which mean phase increases again (a quadratic effect).
- The mean phase of low phase voxels of the deep GM, a measure of the severity of only highly affected tissues, likely due to excessive iron content, continuously decreases (ie. more putative iron) with age.
- Both adolescent MS patients and CIS patients have lower deep GM SWI phase measures indicative of higher iron levels, even though structural atrophy is minimal or not present yet.
- In MS patients, disability (EDSS) can be better explained by the deep GM MP-LPV of some structures than with conventional MRI measures.
- Phase WM-SAs partially overlap with T2 and T1 WM-SAs. However, the remaining (approximately 50% among CIS patients) may represent unique pathology.
- Phase WM-SAs are much more common in CIS patients than in healthy individuals and individuals with other neurological disorders, and may be useful as part of diagnostic criteria because of high sensitivity and specificity.
- Overall, these (indirect) MRI measures suggest that increased iron levels play a role in MS disease pathology, although no conclusions about causality can be drawn. Reduction of both paramagnetic substances and oxidative stress may prove to be viable therapeutic targets.

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