Iron-immune interactions in Alzheimer's disease

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Chapter 6

Summary & Discussion

Summary

The aim of this thesis was to further elucidate the importance of iron in the pathogenesis of Alzheimer's disease (AD). Iron has received increasing attention as promising biomarker due to its potential to be visualised *in vivo* by MRI and increasing evidence shows iron accumulation to be associated with cognitive decline. There are numerous hypotheses how iron accumulation could play a role in neuropathology/ cognitive decline, but the exact pathways involved in AD are unknown. The work presented in this thesis aimed to link MRI findings to a cellular substrate (**Chapter 2-3**), and develop or employ innovative post-mortem brain tissue microscopy tools (**Chapter 4-5**) and stem cell models (**Chapter 6**), with the intention of understanding how iron influences microglia function in AD (**Chapter 4-7**).

With ever increasing MRI field strengths, the possibility to study mesoscopic brain alterations beyond atrophy on structural MRI becomes reality. After a brief overview of the importance and value of comprehensive post-mortem MRI – immunohistochemistry studies in order to validate the substrate of the findings on MRI (**Chapter 2**), we addressed the feasibility of using T_2*-weighted 7T MRI to study changes in cortical lamination of the grey matter in AD (**Chapter 3**). Using post-mortem whole brain hemispheres, we could detect intracortical changes in structural appearance of the hippocampus and temporal lobe gyri. Further immunohistochemical validation found these to correlate not only with myelin alterations, but also iron accumulation and/or redistribution. This study suggests T_2*-weighted MRI to be suitable for studying iron-associated changes in the myelo- and cytoarchitecture of specific regions, to study regional disease involvement independent of atrophy.

To understand how iron potentially influences disease progression or cognitive function, we must also understand the effect on cellular function. Therefore, we investigated which cell types sequestered iron in post-mortem brain tissue of AD patients (**Chapter 4**). We found iron-positive cells to display characteristic microglia morphology and the iron to be stored in the main iron storage protein ferritin. To further characterize the microglia, we developed a new multispectral immunofluorescence panel with an automated cell segmentation and phenotyping pipeline. This revealed that the ferritin light chain (FTL)-positive iron-loaded microglia appeared activated, exhibited as a loss of expression of several homeostatic markers and dystrophic morphological appearance. Additionally, of all amyloid β (Aβ) plaque-infiltrating microglia, iron-loading microglia were the predominant subtype.

Microglia are increasingly recognized as key players in AD pathogenesis, with a complex phenotype requiring characterization with multiple microglia markers. Using our previously developed microglia multispectral immunofluorescence panel, we studied the expression patterns of three widely used microglia markers

(**Chapter 5**). We found all markers to show distinct, complementary, expression patterns, reflecting the microglia's activation status, and depending on the (micro-) environment. This chapter serves as a reference study aiding the interpretation of a wide body of literature using only one of these markers.

Even though we identified iron inside a subset of microglia in AD, which appeared to have a prominent dysfunctional phenotype, this data was all correlative. It is still unclear whether iron uptake is the cause or consequence of microglia activation and how iron uptake affects microglia functioning. Therefore, in **chapter 6**, we investigated the primary effect of high iron concentrations on microglia. We differentiated human induced pluripotent stem cells into microglia and exposed them to an increasing concentration of iron, in combination with different inflammatory stimuli, to try and replicate the ferritin^{+} microglia phenotype which we identified in post-mortem brain tissue, *in vitro*. Firstly, we showed that increased ferritin expression and iron uptake is the result of exposure to an increase of iron and not merely pro-inflammatory activation. Secondly, *in* vitro iron-loaded ferritin^{+} microglia appeared to be activated, but in contrast to previous literature using murine or peripheral macrophage models, proinflammatory pathways were actually dampened by the iron loading. Instead, microglia showed transcriptomic evidence of induced cellular toxicity and oxidative stress. In line with this, iron-loaded microglia appeared to be metabolically stressed and showed slower phagocytosis.

All in all, in this thesis we confirmed iron homeostasis to be dysregulated in AD, and showed that specifically the inhomogeneous accumulation pattern in the cortex could serve as potential *in vivo* biomarker. On a more cellular level, we found that microglia play a prominent role in sequestering excessive iron and an association with activation and Aβ plaque infiltration is evident. Results obtained with induced pluripotent derived microglia showed microglial iron sequestration to be the consequence of iron accumulation rather than inflammatory activation, and to cause oxidative stress and dampen the immune response.

Discussion

Iron has been studied in Alzheimer's disease (AD) and other neurodegenerative diseases for several decades. However, its involvement in many biochemical and cellular processes has made it challenging to understand exactly via which pathways iron contributes to disease pathogenesis, and secondly, how it could be leveraged as biomarker. Here, I will discuss how this thesis has aided to both better understanding the pathogenesis of AD and biomarker development, while also highlighting the remaining questions in the field.

1. Iron as biomarker in AD

1.1 Iron-associated laminar changes in the cortex of AD patients

As discussed in the introduction, in 2011 the clinical guidelines for diagnosis of AD were updated, striving for a more accurate diagnosis and estimation of clinical course based on evidence from biomarkers[1]. While CSF and imaging biomarkers for Aβ and p-tau have greatly improved both diagnostics and research alike, significant disadvantages are the invasiveness of obtaining CSF via a lumbar puncture and the necessity of radioactive tracers for PET, although encouraging data is emerging on plasma assays for Aβ and p-tau[2–6]. Moreover, complementary biomarkers that could be predictive of disease progression and rate of cognitive decline would be incredibly valuable. One recently identified and promising candidate is neurofilament light, a marker for neuroaxonal degeneration and injury. Changes in neurofilament light levels are identified approximately ten years prior to the expected clinical onset[7,8]. Additionally, surrogate markers of iron accumulation, as we explored in **chapter 3,** could be interesting candidate biomarkers. Iron originally gained attraction due to its potential to be studied in vivo using susceptibility-weighted MRI, and because accumulation was found to be closely associated with both Aβ and tau pathology[9–13]. It has long been debated whether iron accumulation merely follows these two pathologies, or is a pathological mechanisms itself. Interestingly, recent studies found higher iron levels of specific cortical regions, independent of Aβ and tau load, , to be associated with accelerated cognitive decline[14–17], which is indicative of an independent pathological mechanism.

Nevertheless, finding appropriate iron-associated biomarkers has not been straight forward. A meta-analysis revealed great heterogeneity among regions and studies looking at iron accumulation[18]. This could potentially be explained by the fact that it is still unresolved whether there is actually a net increase of iron in the brain, or iron is merely redistributed, resulting in local increases of iron, as will be discussed in section 1.2. Moreover, it is even suggested that within a single region iron is inhomogenously deposited, responsible for a laminar pattern within the cortex[19,20]. The intracortical distribution of iron has been relatively unexplored compared to

quantitative measurement of a net increase of total iron in the cortex or iron-rich regions such as the basal ganglia, even though there are several reasons making it worthwhile exploring. Firstly, if iron is primarily redistributed instead of net increased, an average measurement of iron would not be able to appreciate this. For example, recently Bulk *et al* showed that differences in the redistributive pattern of iron were best appreciated as local contrast changes on T_2*-weighted MRI, whereas overall no significant differences in absolute iron concentrations were found, as measured by LA-ICP-MS or quantitative MRI[21]. Secondly, by looking at changes in the intracortical distribution pattern also allows for evaluation of an association with myelo- and cytoarchitecture, which could be a potential source of iron. For these reasons, in **chapter 3** we set out to explore the feasibility of studying intracortical alterations on T_2*-weighted MRI. Even though there have been tremendous advances in the field of MRI, and impressive resolutions can be achieved with 7T MRI, compared to preclinical systems, there is still a big gap in resolution for *in vivo* MRI. Thus, as bridging step between post-mortem tissue blocks and *in vivo* scans, we scanned whole brain hemispheres in a human 7T scanner, to explore whether we could detect the previously observed iron-associated laminar alterations on tissue blocks[20], in these hemispheres.

We found that we could detect earlier identified laminar changes in specific gyri of the temporal cortex with our human 7T scanner. There were significant differences, even between adjacent regions, confirming the previously noted heterogeneity between different cortical regions. Secondly, we verified the histological substrate of the observed changes; myelin was the main source of contrast in controls, and the myelinated pattern appeared disturbed in AD. Alongside this, in AD changes in the distribution of intracortical iron were observed, which followed the observed myelin changes and were responsible for the observed changes in cortical lamination, corresponding with previous literature[10,20]. The finding that the observed changes in iron distribution was associated with a disturbed myelin pattern is an important finding, which will be further discussed in the following section discussing the source of the observed iron accumulation/iron redistribution. We did not have the clinical information nor power to directly assess whether brains that showed the most considerable contrast changes reflecting iron, also showed a more progressive clinical history. Important to note, is that these findings were found in regions that showed extensive atrophy and end-stage disease, and are therefore of limited value for predicting future rate of cognitive decline. Therefore, even though we have shown that gross intracortical cytoarchitectonic alterations can be detected using a human 7T MRI scanner, it would be worthwhile in future studies to investigate whether more subtle changes can be detected at an earlier stage. Moreover, to validate the relevance of this potential biomarker for clinical use, we must also assess whether an association with rate of cognitive decline exists for cortical lamination changes, as exists for certain net quantitative measures. Following, although two *in vivo*

studies already achieved sufficient resolution to study intracortical iron distribution changes[11,22], it should be studied whether these specific AD-associated cortical lamination alterations can be detected *in vivo*.

1.2 Origin of iron accumulation/redistribution

With biomarker data suggesting iron to be a correlate of AD disease or clinical cognitive decline, it is important to uncover the origin of the observed iron accumulation, in order to find a specific therapeutic target. However, this remains one of the biggest questions in the field. In the introduction I explained how iron enters the brain via the blood brain barrier (BBB) and how it is subsequently transferred and stored (Chapter 1, Fig. 2). Taking this into consideration, I will discuss two possibilities of how iron can accumulate in the brains of AD patients. Firstly, it is most commonly proposed that excessive iron could enter the brain from the blood, where erythrocyte heme is a major iron-storage pool, due to an imbalance between iron influx and efflux. Considering iron's indispensable role during neurodevelopment and physiological processing [23], it is important for the brain to prioritize receiving sufficient iron. While the entering of iron into the brain has been clearly described, it is unclear how iron can leave the brain, other than via a bleeding[24]. Such an imbalance would also explain the general increase of iron observed in aging. In light of clinical evidence of the role of iron in AD[15–17], a hypothesis could be that specifically subjects with an increased iron influx, are susceptible to cognitive decline with age. Another explanation for increasing quantities of iron to enter the brain could be chronic BBB breakdown. Both in aging and AD, BBB breakdown is observed and found to be associated with cognitive decline[25–27]. It was shown using both murine transgenic models[28] and human tissue[29,30] that iron can enter the brain due to BBB breakdown, either as free iron or in hemosiderin.

Alternative to an increased influx of systemic iron, a second possibility for increased brain iron accumulation in certain regions, is a redistribution of brain iron. Oligodendrocytes have the highest iron content of all central nervous system (CNS) cell types and the majority of iron is associated with myelin[31,32]. As was indicated in the previous section discussing the results from **chapter 3**, we found iron alterations to colocalize with structural myelin alterations in the cortex of AD patients, which has previously also been noted by others[20,33,34]. Additionally, cortical iron reflects regional severity of AD pathology[9,35], and it appears that especially cortical regions with the most protracted myelin development are vulnerable to myelin disruption and AD pathology such as Aβ-plaques and neurofibrillary tangles. Therefore, this would provide an explanation for the specific regional distribution of increased iron levels, which appears to coincide with AD pathology.

All in all, detection of intracortical alterations on T2*-w MRI could be an interesting non-invasive biomarker that would potentially be a more accurate marker for iron dyshomeostasis and associated accelerated cognitive decline than quantitative

measurement of net iron concentrations. Nonetheless, further mechanistic understanding of where iron originates from and influences AD pathogenesis is necessary to interpret these biomarker findings. For example, we found that the changes in iron reflected structural myelin alterations and recently it has been shown that myelin degeneration can contribute to both age-related and AD-associated cognitive decline[36,37]. Therefore it could be that the iron is redistributed from iron-rich oligodendrocytes as a consequence of myelin degeneration, which could be the actual driver of accelerated cognitive decline.

2. Microglial iron accumulation

Following from the previous section, where we discussed that increasing iron was potentially coming from the blood, or alternatively redistributed from degenerating myelin, the second crucial question is how iron is subsequently sequestered in the brain. As previously mentioned, iron appears to be inhomogenously deposited in a laminar pattern within the cortex[19,38], predominantly in regions that display more severe AD pathology[9,35]. Iron is also specifically found to accumulate in Aβ-plaques and neurofibrillary tangles[39–41]. In **chapter 4**, we studied which cell types sequester iron in AD, and found significant iron accumulation in microglia in the temporal cortex of AD patients. This confirmed earlier suggestions of microglial iron sequestration in AD[38,42,43]. Preferential sequestering of pathognomonic iron by microglia is also observed in other diseases such as multiple sclerosis; here the source of iron is clearer, as cellular degeneration in lesions leads to iron release from oligodendrocytes, which is predominantly taken up by microglia and macrophages[44–46].

In **chapter 4**, We further characterized these iron-loaded microglia, and found significant expression of FTL suggesting the majority of iron to be stored in an inactive form inside ferritin. These findings coincide with observations made several decades ago, in which researchers observed dystrophic microglia with positive expression for ferritin in AD patients[39,47–49]. Additionally, we conclusively showed that these ferritin^{+} microglia preferentially infiltrate Aβ plaques, which is in agreement with earlier studies[39,47,50–52]. What the potential effect is from a combination of exposure to higher iron-levels in combination with other stimuli such as Aβ fibrils, will be discussed in section 4. Furthermore, we showed these iron-accumulating FTL^{+} microglia to lack TMEM119- and P2RY12-expression, which both are considered homeostatic markers. However, whether specific markers can really differentiate between 'activated' or 'homeostatic' function remains disputed. How microglia should instead be phenotyped and studied in post-mortem tissue will be discussed more elaborately in section 3. When evaluating another feature that can be informative regarding functional status, namely morphology, iron-loaded ferritin^{+} microglia appeared dystrophic, as was reported in earlier studies. This corresponds with the fact that dystrophic, rather than activated microglia are increased in AD[53],

which cannot be attributed to aging and microglia senescence[54]. However, the same applies as for specific protein-based markers; what these morphological subtypes mean for microglial function is still poorly understood.

More evidence pointing towards iron dyshomeostasis as contributor to microglia dysfunction in AD comes from transcriptomic data. Novel single cell or single nuclei RNA-sequencing (sc/snRNAseq) methods performed on both murine AD models and human post-mortem AD brains, identified specific disease-associated microglia (DAM) transcriptomic states[55–58], which are thought to play in important role in AD pathogenesis. Notably, iron-related genes appeared among the top differentially expressed genes in microglia in all studies. Whereas the murine studies characterized these DAM as activated, and found them to specifically infiltrate Aβ plaques, a direct link between transcriptomic states and immunohistochemistry (IHC) findings has not been made for the human studies. Nguyen *et al.* attempted this in human AD tissue, but instead of a DAM-state, identified different transcriptomic microglia states and coined them homeostatic, amyloid responsive and dystrophic states, based on their appearance on corresponding IHC[59]. Contrary to previous literature and our own results (**chapter 4**), they only found infiltration of Aβ plaques from their amyloid-responsive cluster, characterized by CD163 expression, and did not see this association with Aβ plaques in their ferritin^{+} dystrophic microglia. Using more novel high-dimensional techniques, it is now evident that microglia adopt complex functional states, which show only partial overlap with historically defined morphological and single/double marker phenotypes. Therefore, microglia characterized with only one or two markers are still very heterogenous, and studying them as one microglia subtype can lead to conflicting results. Moreover, none of the commonly used microglia markers can be considered a pan-marker that detects all subtypes, as was shown in Chapter 5; studies quantifying microglia populations should be read with this in mind. However, this is not to say that post-mortem IHC has lost its value in research. First of all, in IHC the spatial context is preserved, critical for understanding disease pathology. The advent of spatial transcriptomics now also enables studying the full transcriptome on an almost cellular level. However, additional to the spatial context, the functional and clinical relevance of these defined transcriptomic states needs to be explored. Considering FFPE tissue has been collected for many decades, a wealth of tissue is available from patients that have been followed up clinically and of patients with potentially informative rare genetic variants. Therefore, it is now important to validate and further explore the previously identified high-dimensional transcriptomic or proteomic states in larger clinical cohorts of which FFPE tissue is available, as will be discussed in section 3.

3. Tools for studying the heterogeneity of microglia in human tissue

Technological advances have enabled researchers to study many complex cell

processes in parallel and on a single-cell level, and consequently these big data approaches are now transforming many areas of the life sciences[60]. For microglia specifically, an example of the influence big data/single-cell approaches have had on the field, is the transition from a hypothesis of binary microglia polarization (M1 pro-inflammatory vs M2 anti-inflammatory), to multiple functional activation states. Whether microglia can actively switch between functional activation states is still poorly understood. These functional states can be both physiological, or in response to pathological stimuli, and in theory are thought to be both beneficial and detrimental. An enormous advantage of these approaches is that different cell types, pathways and markers can be studied in a largely unsupervised and unbiased fashion.

Nevertheless, even though the identification of functional states such as DAM is very promising, we still do not have a clear understanding of their role in disease, or their clinical relevance. To facilitate this type of research, in **chapter 4** we designed a multispectral immunofluorescence (mIF) panel for FFPE tissue which allows for staining up to six different markers, to more accurately identify functional states identified with -omic approaches, while preserving spatial structure. As stated before, phenotyping with more than two/three markers (which is the current standard) is required to accurately characterize the microglia. Additional to the mIF panel, we developed a pipeline that can segment microglia, including their processes, in 2D images and assign phenotypes. Therefore, mIF with subsequent microglia analysis pipeline will allow for high-throughput evaluation of specific microglia phenotypes in FFPE tissue of many different large cohorts. This will facilitate exploration of the stage at which different phenotypes occur, the association with clinical disease progression and/or cognitive decline, and the interaction of phenotypes with other types of proteins and cell types.

Also other methods are available for more high-dimensional in situ imaging studies. Imaging Mass Cytometry (IMC) (Fluidigm) and Multiplexed Ion Beam Imaging (MIBI) are two relatively recent technologies, enabling simultaneous analysis of up to 40 protein markers[61]. They both rely on the detection of metal-conjugated antibodies, and the instrument uses bright ion sources and orthogonal time-of-flight mass spectrometry to image the metal-conjugated antibodies on tissue sections[62,63]. Although this technique is clearly superior to mIF in its ability to characterize specific subsets with many more markers, there are also a few downsides. Firstly, optimizing an IMC panel is laborious and requires a great deal of expertise and time, evident from the fact that the largest panels for the brain to date only include 7-15 markers[64–66]. Secondly, IMC and MIBI are slow and expensive, enabling only imaging of selected regions of interests, rather than whole slides, making them hardly high throughput. Thirdly, the resolution that can be achieved with time-of-flight mass spectrometry does not allow for accurately identifying microglial cells with complicated morphology (i.e. hyperbranching or process fragmentation). Unfortunately, for both

mIF and IMC/MIBI specialized equipment is necessary in order to image the slides, impeding their use in many standard laboratories.

4. Molecular consequences of microglial iron accumulation

By now we have discussed all the knowledge we have obtained on iron accumulation and cellular distribution, either in an *in vivo* setting or using post-mortem brain tissue. However, post-mortem tissue is limited in the fact that cells are fixed, and the obtained data is correlative. Therefore, to understand how iron influences microglia function on a molecular level one must also adopt other models.

A lot of what we currently know about microglia function is based on studies using murine microglia. Although the use of both immortalized murine microglial cell lines and primary murine microglia have greatly expanded our knowledge, important differences were found between human and murine microglia[67–69]. For instance, 41% of human genes lack convincing mouse orthologs[70], and in AD 15 of the 44 risk genes implicated via GWAS studies lack clear mouse orthologs[71–73]. An alternative is the use of primary human microglia, which can be isolated in limited numbers from surgically resected brain tissue. Next to the limited availability of such tissue, primary microglia were found to undergo rapid transcriptomic and phenotypic changes upon isolation from the brain[68,74]. To tackle these problems, several labs have created protocols to differentiate microglia *in vitro* from induced pluripotent stem cells (iPSC)[75–81]. This has enabled the production of high numbers of microglia-like cells from a single genetic background, in a reproducible manner, and the potential to elucidate the effect of specific disease-associated genetic variants on microglia function. A weakness, however, is these iPSC-derived microglia still lack the crucial microenvironment, required to fully recapitulate the transcriptional signatures that are found in post-mortem isolated microglia. For this purpose, very recently, groups have also developed protocols to study microglia within 3D organoids, or even transplanted progenitors into mouse brains with a human *CSF1* knock-in (essential for microglial growth survival), which enables more accurate recapitulation of human microglia and the potential to study interactions with other cell types[73,82–85]. However, even though the complexity of these advanced models will enable more accurate disease modelling and is required for addressing specific research questions, it is also increasingly difficult to implement across different labs. Therefore, in this thesis we settled for the use of a monoculture of iPSC-derived microglia (**chapter 6**), as this still enables high throughput investigation with enhanced experimental control, while employing a humanized model.

In **chapter 6** we used a human iPSC-derived microglia (iPSC-MG) model, to study the direct effect of iron loading, with and without an additional pro-inflammatory stimulus, on microglia function. Firstly, we found that iron, rather than inflammatory stimuli such as the type two II interferon gamma (IFNγ) or Aβ, induced ferritin^{+}

microglia. Therefore, even though ferritin expression is historically considered to reflect inflammation[86], in line with it being an acute phase reactant in plasma, in microglia it appears to reflect iron loading rather than inflammatory activation per se. This is in line with results from **chapter 4**, where we found ferritin$^+$ microglia to reflect iron-laden microglia, and literature showing that CSF ferritin levels were not associated with an inflammatory response in AD[87]. Vice versa, in our iPSC-MG model iron did not induce proinflammatory activation via activation of the NLRP3 inflammasome following NF-kB pathway activation, as had previously been reported in murine macrophages and microglia[88,89]. However, also in murine macrophages these reports were not consistent, as other studies found iron to inhibit proinflammatory polarization, or to even induce antagonistic anti-inflammatory polarization[90–92]. Instead, our iPSC-MG showed transcriptomic evidence of cellular toxicity and activated oxidative stress pathways such as NRF2, likely as a response to the induction of reactive oxygen species (ROS). Furthermore, we found slower phagocytosis and reduced mitochondrial metabolism capacity in iron-treated ferritin$^+$ iPSC-MG, also indicative of the microglia being more stressed (**chapter 6**). Interestingly, in human post-mortem tissue, ferritin expression is almost exclusively found in microglia with a dystrophic morphological appearance, which is often regarded as the microglia being senescent, meaning they show cessation of cell division. NLRP3 inflammasome activation was potentially expected, considering the increase of intracellular labile iron induced the rise of ROS, which could subsequently result in NLRP3 activation. However, from our results, it appears that following exposure to an increased concentration of iron, iron is stored in a non-toxic ferric form inside ferritin, and the pathways activated even negate potential proinflammatory activation which the increased labile iron pool could induce. However, important to note is that in our study iPSC-MG were only short-term exposed to iron for a short period of time (24 hours), whereas microglia in a neurodegenerative brain are exposed to iron for years.

Microglia have been shown to play a causative role in the development of AD pathology and downstream cognitive impairment. Evidence from murine studies in which microglia are depleted using the CSF1R inhibitor PLX5622 clearly showed that microglia can affect plaque formation and negatively affect cognition[93,94]. Also when inducing more subtle changes to microglia function, for example by crossing AD mouse models with full knock-outs or variants of microglia-specific genetic variants identified in GWAS studies, such as TREM2, or transplanting human iPSC-derived microglia with a TREM2 knock-out, microglia function was found to be altered and subsequentially affect development of Aβ and tau pathology[95–98]. **Chapter 6** provides ample evidence that iron affects microglia phenotype, although likely not via classical proinflammatory activation, but rather via the generation of ROS and subsequent induction of oxidative stress. Nevertheless, one remaining questions is how iron-loaded ferritin$^+$ microglia affect disease progression and lead to downstream neurodegeneration. A previous study demonstrated that iron-induced ROS

production in microglia was regulated via NADPH oxidative (NOX) signaling[92], and it was recently suggested that the generation of ROS can initiate a self-perpetuating cycle of ROS generation by microglia via NOX signaling, causing oxidative stress, inflammation and downstream neurotoxicity[99]. Alternatively, as proposed in section 2, iron can potentially contribute to development of DAM or another dysfunctional microglia subtype. We showed with targeted gene-expression analysis of iron-treated microglia, that overlap with the DAM transcriptomic signature exists. Moreover, one important gene which was found to be upregulated in iron-loaded microglia was *CTSB*. *CTSB* encodes Cathepsin B, which is a mediator of the iron-dependent cell-death pathway ferroptosis[100]. Ferroptosis is important to highlight, considering it has repeatedly been proposed as potential mechanism for neurodegeneration[17,101,102] and microglia were also found to be susceptible to ferroptotic cell death[103]. All in all, microglia phenotypes in AD are likely a result of responses to many different pathogenic stimuli present in AD brains, and iron loading could serve as additional stressor and contribute to a dysfunctional state. Nevertheless, as emphasized in section 3, although the recent identification of disease-associated transcriptomic subtypes of cells such as DAM is very promising, we still poorly understand how these specific transcriptomic disease-associated states or other dysfunctional phenotypes such as dystrophic, ferroptotic or oxidatively stressed microglia, play a role in the pathological cascade of AD and can lead to neurodegeneration. Further investigation is warranted to study the consequence of these microglia states on the function of other cell types, such as neurons and astrocytes, and on AD disease progression. This would require a variety of models, such as stem-cell co-culture models, but also slice culture and murine AD models, where ferritin^{+} microglia or other dysfunctional subtypes of microglia can be induced in a controlled setting and the microglia-autonomous and non-autonomous effects can be studied.

As mentioned in section 2 and crucial to highlight again, on top of mechanistic insight we need to research the clinical relevance of these findings, to establish whether iron influences disease progression by affecting microglia phenotype and function. What is interesting about the hypothesis of microglia-mediated iron toxicity, is that it could potentially also be applicable to other neurodegenerative diseases. Although outside of the direct scope of this thesis, iron accumulation, and more specifically ferritin^{+} microglia, have also been observed in Huntington's disease and Multiple Sclerosis[44,46,104]. Additionally, although not in the same disease, the clinical observation that iron levels correlate with accelerated cognitive decline have now also been reported in Parkinson's disease and Amyotrophic lateral sclerosis[105–107]. Similar findings on iron-associated cognitive decline across different neurodegenerative diseases increase the likelihood of a common mechanism being responsible for the observed clinical effect, independent of disease-specific pathologies such as Aβ and tau. Microglia-mediated iron toxicity would be a potential candidate for such a common mechanism. Finally, this is not to say that iron accumulation plays an important role in

all patients with AD. There is great heterogeneity in the extent of iron accumulation between patients, and the observed negative clinical effect is primarily observed in subpopulations of patients with higher iron loads [16,17]. Especially for these patients, iron-targeting therapies would be promising, and different validated iron biomarkers will be essential in identifying these subpopulations.

5. Concluding remarks

Thanks to the genetic revolution in recent decades, considerable advances in understanding AD pathogenesis, development of useful biomarkers and identification of potential therapeutic targets have been made. Nevertheless, no effective drugs have been developed that can halt or reverse neurodegeneration and its associated cognitive decline. Therefore, the main aim of this thesis was to gain understanding in one potential factor that can contribute to AD pathogenesis, namely iron, and explore whether it could be utilized as potential biomarker or therapeutic target.

We and others showed that iron-associated changes can be identified on 7T MRI (**Chapter 3**). However, to date crucial information with regard to where the iron is coming from, and whether it is actually accumulating in the brain or primarily redistributed between (cellular) compartments, is missing. This would be essential in order to develop appropriate biomarkers that should detect specific stages of disease pathogenesis and/or therapies that aim to prevent the initial influx or redistribution of iron. We are starting to understand what the consequences are of brain iron accumulation, although there are still some important missing links. On one hand, on a clinical level, increasing iron levels have been shown to be associated with accelerated disease progression from MCI to AD and accelerated cognitive decline[16,17,108]. This is accompanied by findings of increased iron in specific layers and regions of the cortex, associated with structural myelin alterations (**Chapter 3**), and cellular iron accumulation in microglia (**Chapter 4**). On the other hand, experimental models have shown iron to activate specific molecular oxidative stress and cellular detoxification pathways in microglia (**Chapter 6**), that can lead to functional cellular changes. Nevertheless, this does not conclusively mean that these *in vitro* pathways directly lead to the observed clinical effect. It is now crucial to translate these experimental findings back to the patient, to dissect its clinical relevance. For example, by applying novel techniques, such as multispectral immunofluorescence (**Chapter 4/5**) or snRNAseq, to well-characterized clinical cohorts, one can gain understanding of the clinical relevance of our experimental findings. Once we have a better understanding of how iron contributes to disease pathogenesis, it will also enable us to identify appropriate therapeutic targets and subpopulations of patients that could benefit from these therapies, for AD but also other neurological disorders that show iron accumulation.

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