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

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# 6

## **Clinicopathological 7T MRI and CSF correlates of iron accumulation, neurodegeneration and neuroinflammation in Huntington's disease: Rationale and design**

This chapter is *to be submitted*.

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## ABSTRACT

**Introduction:** Strong evidence suggests a significant role for iron accumulation in addition to the well-documented neurodegenerative aspects of Huntington's disease (HD). The putative mechanisms by which iron is linked to the HD pathogenesis are multiple, including oxidative stress, ferroptosis and neuroinflammation. However, no study in any neurodegenerative disease so far has linked the observed increase of brain iron accumulation as measured by MRI with direct well-established fluid biomarkers for iron accumulation, or with associated processes such as gliosis. This study is designed to link brain iron levels obtained from 7T MRI of HD patients, in different disease stages, with specific and well-known clinical CSF markers for iron accumulation, neurodegeneration and neuroinflammation. CSF markers will provide quantitative measures of overall iron accumulation, neurodegeneration and neuroinflammation, while MRI measurements on the other hand provide quantitative spatial information on brain pathology and brain iron accumulation, which will be linked with clinical outcome measures.

**Methods:** This is an observational cross-sectional study in premanifest HD patients and patients with manifest HD in early or moderate stage. The study includes a 7T MRI scan, short clinical (i.e. symptom onset, CAG repeat size), motor, functional, and cognitive assessments, and sampling of CSF and blood for the detection of iron, neurodegenerative and inflammatory markers. Age and sex matched healthy subjects are included as a control group.

**Discussion:** Results from this study will provide an important 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.

## 6.1. BACKGROUND

HD is a rare autosomal dominant inherited progressive neurodegenerative disorder, caused by CAG repeat expansion of exon 1 in the HTT gene on chromosome 4 [1]. The disease typically manifests at a mean age at onset of 30-50 years and is characterized by a variety of motor disturbances (typically chorea and dystonia), cognitive impairment and behavioural changes [2]. The size of the CAG repeat expansion accounts for approximately 60 – 70% of variance in disease onset as the CAG repeat expansion length is inversely correlated with age of disease onset and with the rate of disease progression [3, 4].

Neurodegeneration in HD is a direct result of the CAG repeat expansion, which results in intracellular aggregation of the mutant huntingtin protein, thereby causing neuronal dysfunction and neuronal loss of the medium spiny neurons of the striatum, eventually resulting in atrophy of the striatum and the external segment of the pallidum. As the disease progresses, white matter, thalamus, cerebral cortex, and other subcortical gray matter structures also exhibit marked atrophy, resulting in whole brain volume loss [5].

In addition to the well-documented neurodegenerative aspect of HD, strong evidence suggests a significant role for iron accumulation in HD [6–19]. Iron dysregulation can cause neurotoxicity as iron is a redox-active trace element and can catalyse oxidative damage, eventually followed by cell death [12, 13]. Different MRI techniques have been used to measure brain iron levels in HD, all suggesting an increase in iron content in the putamen, caudate nucleus and pallidum [7, 8, 11, 20–22]. Interestingly, brain levels were found to be already increased in premanifest patients and further increase when the disease progresses [7, 10].

Previous studies focusing on iron accumulation in neurodegenerative diseases such as Alzheimer's disease as well as HD have shown that iron appears to be uptaken by activated microglia, the resident macrophages of the brain [23–27]. It is therefore thought that cerebral iron accumulation in humans is mostly explained by iron-accumulating microglia in affected brain regions, linking iron to inflammation, a key pathological mechanism in neurodegenerative diseases [14, 15, 24, 28–30]. Neuroinflammation is also increasingly recognized as a strong component of HD pathogenesis via the cell-autonomous proinflammatory activation of microglia due to the expression of mutant HTT [14–16, 26, 27, 31–33]. This process could contribute to the progression of HD pathogenesis and in conjunction with the known iron accumulation within activated microglia makes iron imaging a potential imaging biomarker for disease progression in HD reflecting neuroinflammation in HD. No study in any neurodegenerative disease so far has linked the observed increase of brain iron accumulation as measured by MRI with neither direct, well-established CSF measures for iron accumulation, nor with CSF markers for neuroinflammation. Previous studies have already shown the clinical utility of CSF markers for iron accumulation and neuroinflammation: for ex-

ample, neuroinflammatory markers YKL-40 and IL-6 were found to be elevated in HD patients, and CSF markers for iron accumulation were shown to predict a more rapid cognitive decline in AD [34–36].

The few studies of iron accumulation in HD with MRI have been qualitative, or semi-quantitative at most, and thus their sensitivity to the association between striatal and cortical iron content with clinical hallmarks of the disease is limited [37, 38]. Quantitative susceptibility mapping (QSM) is an MRI method that allows accurate iron quantification in the brain, based on the quantitative link between iron concentration in tissue and the local phase of the MRI signal. QSM provides a direct quantitative measure of tissue magnetic susceptibility, and thus of iron content [39]. QSM has been shown to correlate linearly with tissue iron content in gray matter, with high sensitivity and specificity to tissue iron changes, as demonstrated by post-mortem studies on brain tissue from healthy subjects and patients with neurodegenerative diseases [40–43]. QSM at ultrahigh field (7T) is particularly advantageous, since MRI at higher magnetic field is more sensitive to tissue-related magnetic field disturbances caused by presence of iron and allows scanning at higher resolution due to the increased signal to noise ratio [44].

In this study, we will link the brain iron levels obtained from QSM of pre-manifest, early and moderate manifest HD patients with specific and well-known clinical CSF markers for iron accumulation, neurodegeneration and neuroinflammation. CSF markers will provide quantitative measures of overall iron accumulation, neurodegeneration and neuroinflammation, while MRI measurements provide quantitative information on the spatial distribution of brain pathology. This will provide an important 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.

Specific objectives include the following:

- (1) To quantify brain iron accumulation in patients with HD using quantitative susceptibility mapping (QSM) at ultrahigh field and compare with healthy controls (7T);
- (2) To link QSM results with specific and well-known clinical CSF markers for iron, neurodegeneration, and neuroinflammation;
- (3) To investigate the relationship between brain iron accumulation as detected by QSM and clinical signs of HD encoded for their symptoms and severity by well-established clinical scales.

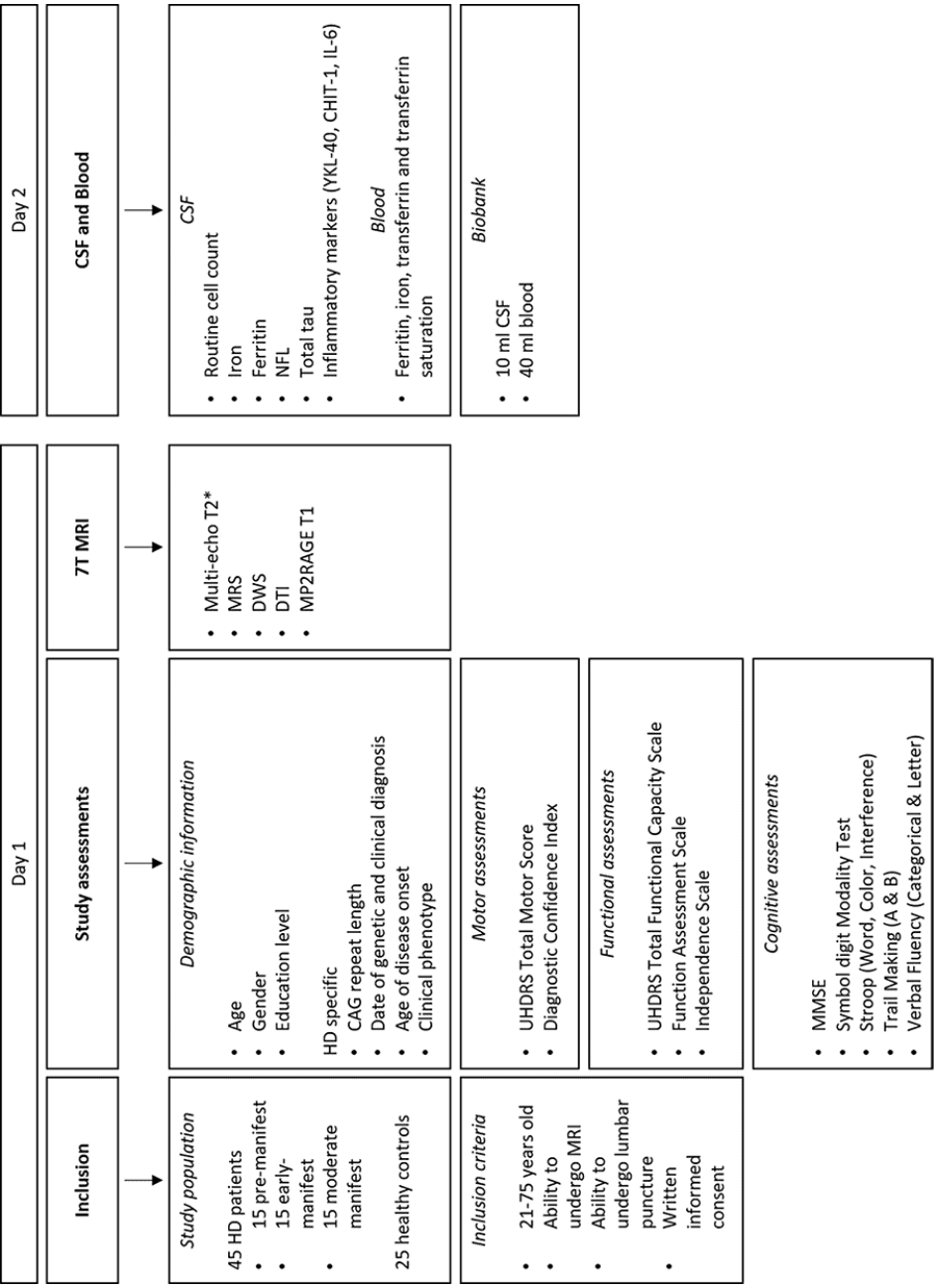


Figure 6.1: Study design and flow chart.

## 6.2. METHODS

### 6.2.1. STUDY DESIGN

This is a 1-year observational cross-sectional study with a multimodal design. The study was approved by the Medical Ethics Committee Leiden Den Haag Delft. This study will involve a two day visit for each participant and will include the following procedures: motor, functional and cognitive assessments of 46-60 minutes, a 7T MRI scan of 60 minutes, and a lumbar puncture and blood sampling. See Fig. 6.1 for a flowchart of the study design.

### 6.2.2. POPULATION

The study population will include 45 HD patients and 25 healthy control subjects consisting of males and females 21 years and older. The two groups will be age and sex matched as much as possible, given the limitation of the rarity of the disease and the difficulty in recruiting healthy controls to a study that include lumbar puncture. The participants will be recruited via the HD outpatient expertise center of the Leiden University Medical Center (LUMC), the Netherlands. We will include 15 pre-, 15 early-, and 15 moderate manifest HD patients, resulting in variation in symptoms and disease severity, ensuring coverage of a broad spectrum of the disease. For individuals diagnosed with HD, a positive genetic test with a CAG repeat expansion of  $> 36$  in the HTT gene is required to be enrolled in this study. For premanifest participants a positive genetic test with a CAG repeat expansion of  $\geq 40$  in the HTT gene is required, to avoid participants that will not become symptomatic with HD, as intermediate repeat lengths are associated with reduced penetrance [4].

Premanifest HD patients are defined by UHDRS Total Motor Score (TMS)  $\leq 5$ , total functional capacity (TFC) = 13 and diagnostic confidence score  $< 4$ . Both early- and moderate manifest HD patients have a diagnostic confidence score equal to 4, but are stratified based on the TFC score. The TFC score is between 11 and 13 (inclusive) for early-manifest patients and between 7 and 11 (inclusive) for moderate manifest patients.

Age- and gender matched healthy individuals will be asked to participate as controls. Controls are preferably partner or spouse of a patient who is not at risk of HD, or a sibling with genetic test results available that show a normal CAG repeat length for both HTT alleles ( $< 36$ ). Controls should have no other known cognitive, neurological or psychiatric disorders.

Exclusion criteria include additional co-morbidities not related to HD, history of severe head injury, use of investigational drug or participation in a clinical drug trial within the last 30 days, current intoxication and inability to understand information about the

protocol. Participants that have severe physical restrictions or severe chorea are also excluded. Participants with common contra-indications for MRI such as pregnancy, claustrophobia, or metal devices are also not included. For lumbar puncture we will perform a screening blood test and participants with results outside normal ranges (white cell count, neutrophil count, lymphocyte count, haemoglobin, platelets, Prothrombin time, activated partial thromboplastin time, C-reactive protein and serum ferritin) in addition to increased intracranial pressure, local skin infections and use of anti-coagulant drugs or platelet-inhibitors are excluded.

### 6.2.3. METHODS – CLINICAL DATA

We will collect basic demographic information including age, gender and education level. For HD patients, we will collect HD related information including CAG-repeat length, date of genetic and clinical diagnosis, age of disease onset according to the patient and family, and clinical phenotype as provided by the clinician.

In addition, motor, functional and cognitive assessments are performed. The motor assessments include the Unified Huntington's Disease Rating Scale (UHDRS) total motor score (TMS) and the, Diagnostic Confidence Index to assess the motor dysfunction characteristics of HD. The functional assessments include the UHDRS total functional capacity scale (TFC), function assessment scale and independence scale to assess general functioning. The cognitive assessments include the symbol digit modality test, Stroop word reading, Stroop color naming, Stroop interference, Trail making A & B, categorical verbal fluency, letter verbal fluency and the Mini Mental State Examination (MMSE).

### 6.2.4. METHODS - MRI

All scans are collected locally at the LUMC using a 7T Philips Achieva MRI scanner (Philips Healthcare). The standardized scan protocol consists of a short survey scan and a sensitivity encoding (SENSE) reference scan followed by 3D MP2RAGE T1-weighted scan for anatomical information [45]. Our central MRI tool for estimation of iron in the brain is QSM. Therefore the protocol includes a multi-echo flow-compensated gradient echo scan. For additional read-outs for putative markers for inflammation and neurodegeneration, we acquire single-volume MR spectroscopy (MRS) and diffusion-weighted MRS (DWS), and diffusion tensor images (DTI).

Established pipelines for basic segmentation of anatomical images, co-registration and basic motion and intensity corrections are applied, using SPM [46], FSL [47] and/or MIPAV/JIST toolbox [48, 49]. We use the STI-Suite toolbox [50] which provides the pipeline necessary to generate QSM maps from single and multi-echo data: phase unwrapping, background phase removal and dipole inversion. Using established pipelines we ob-

tain voxel-wise susceptibility values indicative for iron content of the whole brain in addition to susceptibility values for predefined structures, namely: frontal cortex, motor cortex and the striatum. MRS data analysis is performed with LCModel [51]. DWS data is pre-processed and analysed with in-house MATLAB tools prior to analysis with LCModel. DTI data will be analysed with FSL: individual DTI data sets will be co-registered to the T1-weighted image, and maps of DTI metrics will be generated per subject and co-registered with a common template (MNI 152). Data will be analysed in subject space using ROIs drawn from the MNI template and transformed to subject space, as well as in MNI space using tract-based spatial statistics (TBSS).

### 6.2.5. METHODS - CSF

CSF will be drawn after overnight fasting by lumbar puncture under standardised conditions by experienced physicians. CSF for the proposed biomarker assays will be collected in polypropylene tubes, centrifuged, divided into 0.5 ml aliquots, and stored at -80°C until analysis. In addition to routine cell count, which will be analysed locally within the LUMC CSF lab, we selected the following markers for CSF analyses: iron and ferritin (iron storage protein) as markers for brain iron accumulation, neurofilament light protein (NFL) and total tau as reflecting neuronal damages, YKL-40, CHIT-1 and IL-6 as inflammatory markers. Analyses for CSF concentrations of ferritin, YKL-40, CHIT1, IL-6 and NFL will be done using ELISA and will be performed in the Neurochemical Laboratory of Ulm University, Ulm, Germany. Measurements to determine CSF iron levels will be done in the Research Unit Analytical BioGeoChemistry Helmholtz Zentrum, Munich, Germany using inductively coupled plasma – mass spectrometry.

### 6.2.6. METHODS - BLOOD

Before or immediately after the lumbar puncture, up to 10 ml of venous blood will be drawn according to clinical standards and procedures. Blood samples will be used for a local routine laboratory test for glucose to confirm participants being sober and full blood count. Blood samples will also be used to measure markers related to systemic iron (ferritin, iron, transferrin and transferrin saturation). These analyses will be performed in the Neurochemical Laboratory of Ulm University.

### 6.2.7. STATISTICS

One-way ANOVA will be used to assess baseline intergroup differences. Potentially confounding demographic variables (age, gender) will be examined and those found significant will be included as covariates for subsequent analyses. The distributions of both tested MRI and CSF markers will be tested for normality. If applicable, appropri-

ate non-parametric test will be used and corrections for multiple comparisons will be done. For all statistics the significance is set at  $p < 0.05$ .

The first objective is to quantify brain iron accumulation in patient with HD using QSM at 7T and compare these values with healthy controls. Differences in QSM values and CSF markers for iron accumulation, neurodegeneration and neuroinflammation between controls and HD patients will be analysed using parametric (one-way ANOVA) or non-parametric tests when applicable.

To investigate the link between brain iron as detected with QSM and CSF markers for iron, neurodegeneration and neuroinflammation we will use linear or logistic regression analyses. The same statistical analyses will be used to investigate the link between brain iron as detected with QSM and clinical signs as for example the disease stage (pre-, early- and moderate manifest), TFC and UHDRS total motor score.

## 6.3. DISCUSSION

Despite its monogenetic origin, the pathogenesis of HD is complex, resulting in multiple potential biomarkers ranging from markers reflecting the huntingtin protein to the devastating final pathway of neuronal death. Although a variety of clinical tools exist to assess disease progression in HD, the gold standard for disease severity of HD remains neuropathological confirmation upon post-mortem examination using the Vonsattel score [5]. This score, however, only gives information on the end stage of the disease, whereas a non-invasive tool to assess disease severity over the course of the disease in vivo is needed. Neuroimaging is appealing as a potential biomarker due to its ability to provide putative markers for several pathological mechanism in a predominantly non-invasive manner. To date, several MRI studies in neurodegenerative diseases, including HD, showed the relevance of iron accumulation [6, 12, 52]. Specific for HD, increases of iron levels in putamen, caudate nucleus and pallidum have been reported [7, 10, 21]. Some reports suggest elevated iron levels in premanifest patients compared to healthy controls and correlations with disease severity, pointing to MRI readouts of iron accumulation as a potential early biomarker [7, 10]. Nevertheless, before such biomarker can be used in the clinic, more research is needed to examine the clinical value and study its correlation with well-accepted biofluid markers. This study is especially designed to provide basis for the evaluation of brain iron levels as an imaging biomarker for disease state in HD.

While biofluid markers for disease progression, especially CSF, are not generally currently used in clinical HD setting, CSF can provide a set of objective markers for the interpretation of MRI findings. Studies on HD strongly suggest that CSF neurofilament light protein (NFL), a marker for axonal damage, and total tau (t-Tau), reflecting damage of neurons, are promising biomarkers for overall neuronal damage in HD [53–55].

Especially for NFL, a close correlation with disease progression has been described<sup>56</sup>. Inflammatory markers have been less studied in HD, but CSF YKL-40 / chitinase-3-like-protein 1 (CHI3L1) secreted by astrocytes [56, 57] and chitotriosidase-1 (CHIT-1) secreted by activated microglia [58, 59] are increased in many inflammatory central nervous system disorders. Elevated levels of YKL-4036 as well as of the proinflammatory cytokine IL-6 have been found in CSF of HD patients [35]. Previous studies on iron accumulation in several neurodegenerative diseases have also shown the clinical utility of CSF iron markers. CSF-ferritin levels could serve an index of brain iron load, and in Alzheimer's disease (AD) for example, it has been shown that CSF ferritin levels have similar clinical utility compared with more established AD CSF biomarkers in predicting various outcomes of AD [34]. More specifically, higher CSF ferritin predicted a more rapid cognitive decline in AD and is positively correlated with the rate of clinical disease progression [34]. As this remains to be proven in HD, we chose these CSF markers because of their potential to reveal new biomarkers in HD.

If future pharmaceutical trials including HTT lowering therapies become effective, establishing a biomarker to measure treatment effect becomes a highly important goal [60]. Ideally, in order to prevent neuronal damage, treating HD mutation carriers starts as early as possible. It is hoped that results from this study, which includes both pre-manifest and manifest HD patients, give more insight in potential biomarkers involved early in the disease. The information obtained in this study may potentially benefit the development of new treatments and, when treatments are available, these markers could also guide the timing for treatment initiation and evaluation of effect.

## 6

One limitation of this study, as well as of several previous studies, is the cross-sectional nature. Currently lacking are longitudinal data of brain iron accumulation in a well-characterized HD cohort covering different disease stages. This study would therefore ideally be extended into a longitudinal study and thus serves as a pilot for future studies. A potential future direction could be for example the effect of iron on HD phenotypes or lateralization of HD symptoms. Also, standardization of protocols is important to allow comparison between different research sites. Therefore, the CSF and blood protocols follow the same guidelines as described for HD Clarity. In addition, if given permission by the participant, additional CSF and blood samples are stored in a local biobank which can be used for future scientific studies on promising biomarkers related to HD.

In conclusion, this study will provide an important 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.

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