Multiparametric Magnetic Resonance Imaging Study of Huntington’s Disease

Ricardo Nuno Vieira Leitão

Thesis to obtain the Master of Science Degree in

Biomedical Engineering

Supervisors: Prof. Rita Homem de Gouveia Costanzo Nunes
Dr. Sofia Pereira Coutinho Reimão

Examination Committee

Chairperson: Prof. João Miguel Raposo Sanches
Supervisor: Prof. Rita Homem de Gouveia Costanzo Nunes
Members of the Committee: Prof. Patrícia Margarida Piedade Figueiredo

November 2018
Acknowledgments

Although eventually one presents the thesis by oneself, there is always a group of people in the backstage as important in making the work possible, each of them in a special way.

I would first like to thank my supervisors, Prof. Rita Nunes and Dr. Sofia Reimão, for the excellent opportunity of developing this project, for their availability, patience and continuous support in these past eight months. Thank you for always steering me in the right direction. I would also like to express my gratitude to Dr. Leonor Correia-Guedes for her guidance and for inviting me to attend to medical appointments of patients with Huntington’s Disease. It was a unique opportunity to get closer to the reality of these patients and it truly defined my perspective of the disease.

I would also like to thank the ISR of Instituto Superior Técnico, and especially the folks at LaSEEB, for the warm welcome and for the dynamic and interesting environment. Particularly, I want to express my gratitude to Joana Grilo for all the technical support. A special thanks to Joana Moreira for the countless hours spent together during this period, for the unlimited support and for always helping me keep on track. I could have not asked for a better lab partner. I also want to thank the rest of the team: Carolina Silva, Joana Carmona and Joana Sousa, for all the laughs and for always keeping the mood light and fun through the ups and downs in these eight months.

I want to thank my family, especially my parents, my brother and my godmother Manuela, for providing me with everything I needed and even more; for their unconditional love, support and understanding. May I keep making you proud.

Last but not least, thank you Rafael, Duarte, Filipe, Catarina, Margarida, Teresa, Gonçalo, José, Beatriz, Tânia and Mariana for being a huge part of this unforgettable five-year journey, for all the travels and for all the memories. Here’s to many more years of friendship!
Abstract

Huntington’s Disease (HD) is an autosomal-dominant and fatal neurodegenerative disorder, caused by an expanded trinucleotide repeat in the huntingtin gene. HD displays as a triad of worsening motor, cognitive and psychiatric symptoms, resulting from widespread neuronal dysfunction and cell death. Although genetic testing allows for identification of affected individuals before symptomatic onset, no current therapies can delay HD progression. This cross-sectional study was aimed at comparing multiple structural magnetic resonance imaging parameters in 8 HD patients and 12 healthy controls, in the pursuit of objective and quantitative biomarkers to track disease progression. Firstly, the volumes of several subcortical structures were quantified in T1-weighted images using an automated segmentation approach. Neuromelanin (NM) content in the substantia nigra pars compacta (SNc) was estimated using a semi-automatic segmentation method in NM-sensitive images. The iron content of the basal ganglia was also quantified using R₂* relaxometry. Comparing to controls, HD patients showed significant volume reduction in the caudate nucleus, putamen, globus pallidus, brainstem and midbrain, and an enlargement of the lateral ventricles. Caudate and putamen volumes of HD patients correlated significantly with their total motor and total functional capacity (TFC) scores. R₂* values in the basal ganglia of HD patients were not significantly different from those of controls and did not correlate with volumes of these structures. SNc areas of HD patients were significantly reduced and correlated with their TFC. The SNc areas correlated significantly with all basal ganglia volumes, suggesting that SNc degeneration and basal ganglia atrophy may not be independent processes.

Keywords: Huntington’s disease; structural magnetic resonance imaging; neuromelanin; neurodegeneration; relaxometry; volumetry.
Resumo

A Doença de Huntington (DH) é uma doença neurodegenerativa autossômica dominante e fatal, desencadeada por uma expansão de repetições trinucleotídicas no gene huntingtin. A DH manifesta-se em sintomas motores, cognitivos e psiquiátricos, resultantes da disfunção e morte neuronal generalizadas. Apesar dos testes genéticos permitirem o diagnóstico antes do surgimento de sintomas, as terapias atuais não atrasam a progressão da DH. Neste estudo foram comparados múltiplos parâmetros de ressonância magnética estrutural entre 8 doentes com DH e 12 controlos saudáveis, em busca de biomarcadores quantitativos para monitorizar a progressão da doença. Os volumes de várias estruturas subcorticais foram automaticamente quantificados em imagens ponderadas em T₁. A quantidade de neuromelanina na substantia nigra pars compacta (SNc) foi estimada usando segmentações semi-automáticas em imagens sensíveis à neuromelina. O ferro nos núcleos da base foi quantificado utilizando relaxometria R₂*. Comparando com os controlos, os doentes com DH exibiram reduções significativas nos volumes do núcleo caudado, putamen, globo pálido, tronco cerebral e mesencéfalo, e alargamento dos ventrículos laterais. Os volumes do caudado e putamen dos doentes correlacionaram-se significativamente com o score motor total e com a Capacidade Funcional Total (CFT). Os valores de R₂* nos núcleos da base dos doentes não foram significativamente diferentes dos controlos, não se correlacionando com os volumes dessas estruturas. As áreas da SNc dos doentes estavam significativamente reduzidas, correlacionando-se com a sua CFT e com os volumes dos núcleos da base, sugerindo que a degeneração da SNc e a atrofia dos núcleos da base poderão não ser processos independentes.

Palavras-chave: Doença de Huntington; neuromelanina; neurodegeneração; relaxometria; imagem por ressonância magnética estrutural; volumetria.
# Table of Contents

Acknowledgments .................................................................................................................... iii

Abstract ........................................................................................................................................ v

Resumo ........................................................................................................................................ vii

Table of Contents ....................................................................................................................... ix

List of Acronyms ........................................................................................................................ xiii

List of Figures ............................................................................................................................ xv

List of Tables ............................................................................................................................... xvii

1. Introduction ............................................................................................................................. 1
   1.1. Motivation ......................................................................................................................... 1
   1.2. Aims of this Work ........................................................................................................... 2
   1.3. Thesis Outline ................................................................................................................ 3

2. Background ............................................................................................................................. 5
   2.1. Huntington’s Disease ....................................................................................................... 5
       2.1.1. Historical Background ........................................................................................... 5
       2.1.2. Neuropathology ...................................................................................................... 5
       2.1.3. Corticostrial Dysfunction in Huntington’s Disease .............................................. 6
       2.1.4. Natural History of Huntington’s Disease ............................................................... 8
       2.1.5. Clinical Assessment .............................................................................................. 10
       2.1.6. Iron and its Role in Huntington’s Disease .............................................................. 11
   2.2. Substantia Nigra and Neuromelanin ........................................................................... 11
   2.3. Magnetic Resonance Imaging ....................................................................................... 12
       2.3.1. T1, T2 and T2* Relaxation Mechanisms ................................................................. 13
       2.3.2. Fast Spin Echo Sequences ..................................................................................... 14
       2.3.3. Gradient Echo Sequences ...................................................................................... 17

3. State of the Art ....................................................................................................................... 19
   3.1. Structural Magnetic Resonance Imaging ..................................................................... 19
       3.1.1. Volumetric Magnetic Resonance Imaging in Huntington’s Disease .................. 20
       3.1.2. Neuroimaging Studies of Iron in Huntington’s Disease ...................................... 20
       3.1.3. Neuroimaging Studies of the Substantia Nigra ..................................................... 22
   3.2. Application of other Magnetic Resonance Imaging Modalities in Huntington’s Disease .... 25

4. Sample Description ............................................................................................................... 27
# List of Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two-Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-Dimensional</td>
</tr>
<tr>
<td>CAG</td>
<td>Cytosine-Adenine-Guanine</td>
</tr>
<tr>
<td>CPN</td>
<td>Cortical Pyramidal Neuron</td>
</tr>
<tr>
<td>CR</td>
<td>Contrast Ratio</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DSC</td>
<td>Dice Similarity Coefficient</td>
</tr>
<tr>
<td>ETL</td>
<td>Echo Train Length</td>
</tr>
<tr>
<td>FID</td>
<td>Free Induction Decay</td>
</tr>
<tr>
<td>FSE</td>
<td>Fast Spin Echo</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Amino Butyric Acid</td>
</tr>
<tr>
<td>GE</td>
<td>Gradient Echo</td>
</tr>
<tr>
<td>GP</td>
<td>Globus Pallidus</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy Control</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s Disease</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracranial Volume</td>
</tr>
<tr>
<td>mFFE</td>
<td>multi-echo Fast Field Echo</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic Resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium Spiny Neuron</td>
</tr>
<tr>
<td>MT</td>
<td>Magnetization Transfer</td>
</tr>
<tr>
<td>NM</td>
<td>Neuromelanin</td>
</tr>
<tr>
<td>NM-MR</td>
<td>Neuromelanin-Sensitive Magnetic Resonance</td>
</tr>
<tr>
<td>NM-MRI</td>
<td>Neuromelanin-Sensitive Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>ROI</td>
<td>Region-of-Interest</td>
</tr>
</tbody>
</table>
SAR  Specific Absorption Rate
SE  Spin Echo
SN  Substantia Nigra
SNC  Substantia Nigra pars compacta
SNR  Substantia Nigra pars reticulata
TE  Time of Echo
TFC  Total Functional Capacity
TFE  Turbo Field Echo
TMS  Total Motor Score
TR  Time of Repetition
TSE  Turbo Spin Echo
UHDRS  Unified Huntington’s Disease Rating Scale
List of Figures

Figure 1: Illustration of the genetic mutation of HD and its effect on the huntingtin protein .................. 6
Figure 2: Schematic summary of the corticostriatal pathway model. .......................................................... 7
Figure 3: Distribution and severity of neuronal loss in select brain regions of HD patients. .................. 8
Figure 4: Natural history of adult-onset HD. .................................................................................................. 9
Figure 5: Schematic transverse section through the rostral midbrain at the level of the superior colliculus, showing the SNC and the SNR ................................................................. 12
Figure 6: Schematization of the magnitude values of the longitudinal magnetization $M_z$ and of the transverse magnetization $M_{xy}$ of a sample of spins as a function of time, after being excited with a 90° RF pulse. ................................................................................................................................. 14
Figure 7: Diagram of an FSE/TSE sequence with an ETL of 3. ................................................................. 15
Figure 8: Neuromelanin-sensitive and conventional MR images of the SN of a 42-year old healthy individual, obtained using the sequence developed by Sasaki et al. ........................................... 16
Figure 9: GE sequence diagram. .................................................................................................................. 17
Figure 10: Processed T1-weighted images of an axial slice of an healthy subject and of the same slice with subcortical segmentations performed by FreeSurfer. .............................................................. 30
Figure 11: Processed T1-weighted images of an axial slice of a late-stage HD patient and of the same slice with subcortical segmentations performed by FreeSurfer. .............................................................. 30
Figure 12: Processed T1-weighted images of an axial, coronal and sagittal slices of an healthy subject showing the segmentation of brainstem structures performed with the BrainstemSubstructures tool in FreeSurfer. ......................................................................................................................... 30
Figure 13: Volumes of the basal ganglia relative to the ICV of HCs and subjects with HD. ........... 32
Figure 14: Volumes of the brainstem, midbrain and lateral ventricles relative to the ICV of HCs and subjects with HD. .................................................................................................................. 33
Figure 15: Relative caudate volumes of HD patients plotted against their TMS. ................................. 34
Figure 16: Relative caudate volumes of HD patients plotted against their TFC. ................................. 35
Figure 17: Slice orientation in the NM-MRI sequence used in this work .................................................. 39
Figure 18: Axial slices obtained with NM-MRI at the levels of lower midbrain, centre midbrain and upper midbrain of a healthy control. ......................................................................................... 40
Figure 19: Axial slice at the level of centre midbrain of a healthy subject before and after the application of the Gaussian blur filter. .................................................................................................................. 41
Figure 20: Semi-automatic segmentation of the SNc using the Confidence algorithm after image filtering with a Gaussian blur filter. ................................................................. 42

Figure 21: Examples of a manual segmentation and of a semi-automatic segmentation of the right-sided SNc region of the same healthy subject................................................................. 43

Figure 22: Example of a manual segmentation of the midbrain in a healthy subject.......................... 43

Figure 23: Method used to calculate the CRs of SNc areas in non-filtered NM-MR images.............. 44

Figure 24: SNc areas of HCs and of patients with HD................................................................. 46

Figure 25: SNc ratios of HCs and of HD patients........................................................................ 47

Figure 26: SNc areas plotted against the midbrain areas of the 20 subjects included in this study. .... 47

Figure 27: CR values of the SNc areas in HCs and HD patients................................................... 48

Figure 28: SNc areas of HD patients plotted against their TFC score ........................................... 49

Figure 29: SNc ratios of all subjects plotted against their relative caudate volumes...................... 50

Figure 30: SNc ratios of all subjects plotted against their relative putamen volumes..................... 51

Figure 31: SNc ratios of all subjects plotted against their relative GP volumes............................ 51

Figure 32: Seven T₂*-weighted images at TEs=14, 19, 23, 28, 33, 37 and 42 ms for the same slice of a healthy subject. .................................................................................................................. 55

Figure 33: Example of a monoexponential signal decay curve fitted to the data corresponding to one voxel. .................................................................................................................. 57

Figure 34: Total and basal ganglia R₂* maps in two adjacent axial slices of a late-stage HD subject. 58

Figure 35: Total and basal ganglia R₂* maps in two adjacent axial slices of a HC. ......................... 59

Figure 36: R₂* values in the basal ganglia of HCs and of subjects with HD.................................... 60
List of Tables

Table 1: Summary of several cross-sectional structural MRI studies in HD......................................................... 23
Table 2: Main features of several NM-MRI studies using different MRI sequences and methods for SNc segmentation to quantify NM in the SNc. .................................................................................................................. 24
Table 3: Demographic and clinical characteristics of the sample of subjects. .................................................... 27
Table 4: T1-weighted TFE sequence parameters used in this study. ......................................................................... 29
Table 5: Median (range) volumes of brain structures in the left and right hemispheres of HCs and of subjects with HD. ........................................................................................................................................ 31
Table 6: Median (range) values of the relative caudate, putamen, GP, lateral ventricles, brainstem and midbrain volumes of healthy HCs and subjects with HD .................................................................................. 32
Table 7: Correlations of brain volumes with duration of the disease, TMS and TFC in the HD cohort. .................... 34
Table 8: NM-MRI protocol and sequence parameters used in this study. ............................................................... 39
Table 9: Median (range) area values of the SNc in each side of the brain of HCs and of subjects with HD, obtained with the semi-automatic and manual segmentation methods. ......................................................... 45
Table 10: Median (range) values of the SNc areas, midbrain areas and SNc ratios of HCs and of subjects with HD. ........................................................................................................................................... 46
Table 11: Median (range) CR values measured in the SNc areas for each side of the brain of HCs and of subjects with HD. ........................................................................................................................................... 48
Table 12: Correlations of the SNc areas and of the SNc ratios with disease duration, TMS and TFC in the HD cohort. ........................................................................................................................................ 49
Table 13: Correlations of SNc ratios with the relative volumes of the basal ganglia structures considering all subjects ........................................................................................................................................... 50
Table 14: T2* multi-gradient echo sequence parameters used in this study........................................................... 55
Table 15: R2* (range) values of the basal ganglia structures in the left and right hemispheres of HCs and of subjects with HD. ........................................................................................................................................... 60
Table 16: R2* (range) values of the basal ganglia structures of HCs and of subjects with HD. .............................. 60
Table 17: Correlations of R2* values in the basal ganglia of HD patients with duration of the disease, TMS and TFC. ........................................................................................................................................ 61
Table 18: Correlations of R2* values with the relative volumes of the basal ganglia considering all subjects. ........................................................................................................................................... 61
1. Introduction

1.1. Motivation

Huntington’s Disease (HD) is a progressive, autosomal dominantly inherited and fatal neurodegenerative disorder, triggered by a mutation of Cytosine-Adenine-Guanine (CAG) expansion in the gene that codes for huntingtin, located in chromosome 4 [1]. Neuronal dysfunction and neuronal cell death arise from the toxicity of the mutant protein [2], leading to continuous degeneration of subcortical structures, especially of the basal ganglia, and of cortical gray matter, even years before clinical onset [3],[4]. These features invariably display as a triad of gradually worsening motor (especially chorea), cognitive and psychiatric symptoms in individuals affected by HD. Nonetheless, no current therapies can forestall symptomatic onset nor slow disease progression [5].

Similarly to other neurodegenerative diseases, e.g. Parkinson’s Disease (PD), Lewy body dementia and Alzheimer’s Disease [5], HD results from the accumulation of misfolded proteins. However, HD is unique in the fact that it is caused by a single mutation for which genetic testing is available [6], allowing the preclinical diagnosis to be performed many years before the onset of clinical manifestations. Therefore, the premanifest phase of HD offers a therapeutic window to investigate novel preventing treatments aimed at delaying the clinical onset and at slowing neurodegeneration. Moreover, the genetic foundation of HD allows for the estimation, to some extent, of the age at onset, age at death and even disease progression, based primarily on the powerful tool of quantifying the length of CAG expansion [7].

In order to assess the effectiveness of disease-modifying therapies, it is crucial to identify biomarkers that will longitudinally detect and predict changes in disease progression, namely brain changes, while simultaneously responding to therapeutics. The gold standard used to measure disease progression is the Unified Huntington’s Disease Rating Scale (UHDRS). However, as in any clinical rating scale, UHDRS is subject to inter-rater variability [8].

Neuroimaging methods have offered, so far, the most robust biomarkers for HD [9]. Structural magnetic resonance imaging (MRI) techniques such as voxel-based morphometry and volumetry analysis have been extensively used to measure the volumes of several brain structures, namely of the caudate-putamen (striatum) and of the globus pallidus (GP) nuclei of the basal ganglia [10]. Striatal atrophy, the neuropathological hallmark of HD, begins up to 15 years prior to diagnosable motor onset and progresses steadily through the entire course of the disease [11]. Subsequent to striatal atrophy and in a lesser magnitude, volume loss also occurs in other subcortical structures and propagates to cortical gray matter and white matter, culminating in a widespread neurodegeneration in the later stages of HD. Although striatal volume reduction is well documented in cross-sectional and longitudinal studies of premanifest and manifest individuals with HD, there is currently no validated imaging biomarker for the study of HD progression [12].
Another potential pathological feature of HD is a disruption of brain iron homeostasis caused by the agglomerates of mutant huntingtin, leading to iron accumulation in the basal ganglia. Iron overload generates oxidative stress which results in neuronal damage, ultimately prompting the death of affected neurons. While it is hypothesized that such iron overload may be related to or even precede volumetric changes in HD patients, it is still unclear whether this feature is a primary or secondary event in HD [13].

The substantia nigra pars compacta (SNc) is a midbrain structure that plays a modulatory role in the corticostriatal pathway, a neuronal circuitry associated with voluntary and involuntary movement regulation [14]. The dopaminergic neurons of the SNc, which provide input to the basal ganglia through the striatum, contain neuromelanin (NM), a highly paramagnetic dark pigment with a major role in iron homeostasis [15]. Considering said features of the SNc, the loss of striatal volume, the possible disruption of iron homeostasis in HD and the debilitation of the motor function in the disease, it would not be surprising if the NM-containing SNc is somehow involved in the pathological cascade of HD. Nevertheless, to the best of our knowledge, no study has quantitatively assessed NM in the SNc of HD patients using MRI.

Given its exclusive genetic foundation, further understanding HD as a disease model may prove to be invaluable to obtain new insights into other more prevalent disorders that share similar features. Furthermore, given the complex pathology of HD, multiparametric studies may prove to be the most adequate in order to assess disease progression and to investigate novel biomarkers in the search for disease-modifying therapies.

1.2. Aims of this Work

The present cross-sectional, multiparametric MRI study of individuals with HD had several objectives. Firstly, this work aimed at investigating the volume changes in the basal ganglia, brainstem, midbrain and ventricles of HD patients by using a fully automated segmentation method in a volumetric analysis.

Secondly, a semi-automatic method was used to segment the SNc in neuromelanin-sensitive magnetic resonance (NM-MR) images and to quantify the NM content in this structure in HD patients. The unique contrast of NM-MR images allows for the identification of the SNc as a high-signal intensity structure due to the contrast generated by the magnetization transfer (MT) effect [16]. More specifically, a two-dimensional (2D) turbo spin echo (TSE) NM-MRI sequence was used to assess single-slice differences in the SNc high-signal areas and contrast ratios of HD patients compared to healthy controls, as indirect measures of the quantity of NM in this brain structure. Similar analyses have been performed for other neurological disorders, such as PD [17], essential tremor [18] and schizophrenia [19]. Studies in these illnesses have shown significant changes in the quantity of NM in the SN of patients. However, as far as known, this technique was never tested in HD patients. If the study of NM content in the SNc proves to be feasible in HD, this parameter may be tested in the future as a novel biomarker to study disease progression and therapeutics.
Additionally, this study also aimed at quantifying the iron deposition in basal ganglia structures of HD patients using quantitative R2* relaxometry. To that end, R2* maps of the caudate, putamen and GP were generated from multi-echo T2*-weighted images.

This work also aimed at investigating the relations of all the parameters examined in this study, i.e. volumes, SNc areas, CRs and R2* values, with the clinical parameters of HD patients: disease duration, Total Motor Score (TMS) and Total Functional Score (TFC). Finally, the relations between the volumes of the basal ganglia and SNc areas were assessed, as well as the relationship between basal ganglia volume and iron.

1.3. Thesis Outline

This dissertation encompasses eight chapters. In this section, after presenting the motivation of this work (1.1), the aims of this study were defined (1.2).

The second chapter, Background (2), includes a more in-depth literature review of HD and its neuropathology (2.1.2), the associated brain changes (2.1.3), the natural history (2.1.4) and clinical assessment of the disease (2.1.5), as well as a brief description of the potential role of iron in this illness (2.1.6). Furthermore, the roles of the SN and of the NM are described (2.2). This chapter also includes a brief overview of the relaxation mechanisms in MRI and of the MRI sequences used in this work (2.3).

The third chapter (3) consists of a review of the main methods and results from several other groups in the analyses of volumetry (3.1.1) and iron of brain structures in HD (3.1.2). A summary of the NM-MRI methods and parameters studied in other neurodegenerative disorders is also included in this State-of-the-Art section (3.1.3).

The fourth chapter (4) comprises the clinical description of the sample of subjects included in this study. The fifth (5), sixth (6) and seventh (7) chapters correspond to the volumetry, NM-MRI and R2* relaxometry analyses, respectively. Each of these sections is further divided into Methods, Results and Discussion.

The eight and last chapter of this thesis is the Conclusion (8), which includes a brief summary of the main results obtained in this study (8.1) and a subsection with respect to the limitations and with some suggestions for future works (8.2).
2. Background

2.1. Huntington’s Disease

2.1.1. Historical Background

Huntington’s Disease (HD) has a rich historic literature stretching back to 1841, since it was first clinically described by Charles Waters as a hereditary combination of cognitive and motor abnormalities, with chorea (rapid dance-like involuntary movements) being its most prominent motor feature [20]. However, it was George Huntington who defined the core clinical features of HD as a separate type of hereditary chorea in his article On Chorea, in 1872 [21]. Through a detailed study of the disease course in several HD patients, the American physician managed to capture the hereditary nature, the onset in adult life, the inevitable and incurable progression of disability, the frequent suicides and the association between chorea and dementia as the main features of the insidious disease, which since then has borne Huntington’s name. Despite such landmark in the study of HD, it was not until 1993 that the HD gene, named HTT (huntingtin) or IT-15 (interesting transcript-15) gene, and its mutation were discovered in the chromosome 4. The finding of the monogenic root cause of HD, a polymorphic expansion of a trinucleotide repeat of CAG, located in the region of this gene that codes for the protein huntingtin [1], paved the way to countless new worldwide studies and changed entirely the understanding of HD.

2.1.2. Neuropathology

HD is a progressive, incurable and fatal neurodegenerative disorder, clinically manifested as a triad of gradually worsening movement (most typically chorea), cognitive and psychiatric disturbances [22]. HD is autosomal dominantly inherited, and each child of one parent with HD has a 50% chance of inheriting the mutation, regardless of gender. The mean age of HD symptomatic onset is about 40 although it can manifest as early as 4 and as late as 80 years of age [23]. Despite being considered a rare disease [24], HD is the most common monogenic neurological disorder in the developed world [5], with a global prevalence of 5.5 cases per 100,000 population, according to a epidemiologic meta-analysis of 22 studies (1993-2015) [25]. However, in North America, Western Europe and Australia, the prevalence of HD ranges from 5.96 to 13.70 cases per 100,000 population, whereas in Asia it is only as high as 0.70 cases per 100,000 population.

The primal source of the abnormalities seen in HD is a mutation in the huntingtin gene, located in the chromosome 4. The mutation consists of a polymorphic expansion of CAG trinucleotides in a region of the gene that codes for the huntingtin protein, such that the number of CAG repeats exceeds 35 trinucleotides. Since the trinucleotide CAG codes for the glutamine amino acid, a subsequent translation of polyglutamine tracts into the protein takes place, resulting in a mutated form of huntingtin (Figure 1). This abnormal polyglutamine expansion results, by unknown mechanisms, in protein misfolding and aggregation of mutant huntingtin fragments into inclusion fibrillar bodies. Such inclusion bodies
constitute a pathological hallmark of the disease that places HD alongside other neurodegenerative disorders also characterized by protein aggregates, such as PD and Alzheimer’s Disease [2].

Figure 1: Illustration of the genetic mutation of HD and its effect on the huntingtin protein. An expansion of over 35 CAG trinucleotides occurs in the HD gene in chromosome 4, resulting in a translation of a polyglutamine chain into the huntingtin protein. Adapted from [26].

The normal function of huntingtin is not yet fully understood [5]. This protein is expressed in all neurons and glial cells of the human brain and can interact with over 200 other cellular proteins [27]. Therefore, it can be easily understood that a mutant form of huntingtin will lead to an extremely complex pathogenicity. The pathological cascade in HD is thought to primarily arise from a toxic gain of function of the mutant huntingtin and its aggregates. Transcription dysregulation of several genes, synaptic dysfunction, abnormalities in cellular proteostasis, mitochondrial dysfunction and increased oxidative stress may be important players in the neuropathology of HD [28]. Whichever may be the mechanisms underlying the HD pathological cascade, it is known to invariably culminate in both neuronal dysfunction and neuronal cell death [2],[29].

2.1.3. Corticostriatal Dysfunction in Huntington’s Disease

Although mutant huntingtin is ubiquitously expressed in the human brain, primary neuronal degeneration occurs in the caudate-putamen (striatum) and in the cerebral cortex. The neuropathological hallmark of HD is a progressive bilateral degeneration of the striatum due to the demise of Gamma-Amino Butyric Acid(GABA)-ergic medium spiny neurons (MSNs) [3]. Striatal atrophy arises years before the symptomatic onset of HD and progresses steadily during the course of the disease, until in its latest
stages up to 95% of MSNs may be depleted [30]. Striatal atrophy also leads to the enlargement of the lateral ventricles [23]. Striatal neuronal loss is usually accompanied by death of cortical pyramidal neurons (CPNs), primarily in motor and premotor areas, albeit to a lesser extent. Moreover, a dysfunction in the patterns of communication between MSNs and CPNs, major players in the corticostratial pathway, is thought to take place even before neuronal death. The corticostratial pathway encompasses the striatum and the GP, which together comprise the basal ganglia, and the cerebral cortex, the subthalamic nucleus, the thalamus and the SN - Figure 2. Among other functions, this neuronal circuitry is responsible for regulating voluntary and involuntary movement.

Figure 2: Schematic summary of the corticostratial pathway model. The direct pathway, comprising the striatum, the internal GP (GPi), the thalamus and the cerebral cortex, initiates voluntary movement. The indirect pathway, encompassing the striatum, the external GP (GPe), the subthalamic nucleus (STN), the GPi, the thalamus and the cerebral cortex, inhibits movement. The SNc has a modulatory role, by exciting the direct pathway and inhibiting the indirect pathway. Adapted from [31].

According to the corticostratial pathway model, shown in Figure 2, the striatal MSNs integrate glutamatergic (excitatory) cortical inputs and relay that information to downstream basal ganglia nuclei, through the direct and indirect corticostratial pathways, which are thought to act in opposite ways to control movement. On one hand, the direct pathway initiates movement. The excitatory input from the cortex to MSNs in the striatum leads to the inhibition of the GABAergic projection in the internal GP, which in turn results in less inhibition on the thalamus glutamatergic neurons. This leads to an excitation of the motor cortex and initiation of voluntary movement. Considering this, dysregulation of MSNs projecting to the direct pathway would result in rigidity. On the other hand, the indirect pathway inhibits movement. Striatal MSNs inhibit GABAergic neurons in the external GP. This in turn results in less inhibition on the excitatory projection in the subthalamic nucleus. The glutamatergic projections from this nucleus excite the GABAergic neurons in the internal GP, which results in greater inhibition to the
thalamus and decreased signaling to the motor cortex. Thus, dysregulation of MSNs in the indirect pathway results in uncontrollable movements, such as chorea and tremor.

Excitatory projections from the cortex are not the only influence on basal ganglia circuitry. Dopaminergic projections from the SNc to the striatum play a modulatory role in the corticostriatal pathway. These neurons excite the direct pathway and inhibit the indirect pathway, ultimately resulting in facilitation of movement. Therefore, a dysregulation of dopamine results in uncontrolled involuntary movements [32].

In HD, MSNs in the indirect pathway are preferentially lost prior to MSNs of the direct pathway, creating an imbalance between both circuits. The consequence is the inability to control voluntary movement, resulting in choreatic hyperkinetic movements. In contrast, direct MSNs are generally affected in the later stages of the disease, resulting in movement impairment such as rigidity. Thus, impaired corticostriatal connectivity and neuronal cell death of MSNs and CPNs beginning early in the disease set the stage for the emergence of HD symptoms [4]. While the most notable atrophy occurs in the striatum, numerous other structures undergo progressive neurodegeneration during the course of the disease, as shown in Figure 3 [11],[33].

Figure 3: Distribution and severity of neuronal loss in select brain regions of HD patients. Brain regions undergoing severe neuronal loss are highlighted in dark blue and brain regions with marked loss of nerve cells in light blue. (A) Schematized frontal section through the cerebral hemispheres at the level of the red nucleus (RD). Abbreviations: C, caudate nucleus; CC, cerebral cortex; CA, corpus callosum; CL, claustrum; P, pallidum; PU, putamen; SN, substantia nigra; STN, subthalamic nucleus; TH, thalamus. (B) Schematized sagittal section through the midbrain (MB), pons (PN), medulla oblongata (MO) and cerebellum (CE). Adapted from [11].

2.1.4. Natural History of Huntington’s Disease

The typical timeline of adult-onset HD, displayed in Figure 4 (a), comprises two main stages: the premanifest period until the diagnosis of motor onset, and the period following such event, designated as the manifest stage. During the premanifest stage, generally up to 10-15 years before an unequivocal diagnosis of manifest HD can be made, individuals with abnormal CAG repeats begin to exhibit slight changes in brain structure and subtle motor, cognitive and behavioral changes. The manifest stage
follows, in which these multimodal symptoms become more pronounced, and inexorably progress over
the remaining course of the disease, invariably resulting in death typically up to 20 years after adult-
onset of HD. The development of motor, cognitive and psychiatric symptoms of HD are accompanied
by the neuronal dysfunction and atrophy of several brain structures, namely the striatum, and other
subcortical nuclei such as the GP, but also of the cortical gray and white matter - Figure 4 (b) [5],[28],[34].

![Graphical representation of the natural history of adult-onset HD.](image)

**Figure 4:** Natural history of adult-onset HD. (a) Progression of clinical symptoms along the several
phases of the disease. (b) Brain changes that accompany and set the stage for clinical symptoms of
HD. Manifest HD is characterized by a slow progression of motor and cognitive declines, and chorea is
usually prominent early, but eventually plateaus later on. Motor impairment progresses more steadily,
often being more present in the later stages of the disease. Adapted from [5].

Motor features of HD include involuntary movements, primarily chorea, and impairment in voluntary
movements, such as incoordination, bradykinesia (slowness of movement), dystonia (repetitive muscle
contractions) and rigidity [35]. On one hand, choreatic movements arise as early as in the premanifest
phase of adult-onset HD, and their severity increases gradually but surely during the course of the
disease, only ceasing during sleep. Eventually, in the later stages of HD, the intensities of choreatic
movements reach a plateau, and ultimately fade. On the other hand, motor impairment does not
supervene until the patient reaches the late stages of adult-onset HD [5]. However, in contrast to adult-onset HD, chorea is not a major manifestation of juvenile-onset HD, and motor impairment along with seizures seem to be the predominant features of this variant of HD [23], also known as Westphal HD. Juvenile HD is defined by the onset of symptoms before the age of 20, by a more severe and rapid neuropathological involvement than adult-onset HD, and accounts for 5 to 10% of HD cases [36].

Similarly to motor symptoms, but with more variability between affected individuals [35], cognitive features subtly arise in initial stages of HD, years before diagnosable motor onset of the disease. Cognitive deficits in HD include cognitive slowing and diminished attention, reduced mental flexibility and less ability to learn new information. Cognitive capacities progressively decline and ultimately culminate in dementia [4], [28]. The neuropsychiatric profile of HD includes disturbances such as irritability, anxiety, apathy and depression. Suicide attempts are not rare in HD patients [35]. The primary cause of death of HD is, however, aspiration pneumonia, a severe pulmonary infection caused by breathing food, saliva or other non-gaseous substances into the lungs or its airways, usually due to choking, which becomes common in later stages of the disease [37].

2.1.5. Clinical Assessment

The current gold standard for determining an accurate diagnosis of HD (with 98.8% sensitivity and 100% specificity) is a simple Deoxyribonucleic Acid (DNA) test showing a CAG repeat of at least 36 trinucleotides in the HTT gene [6]. The CAG repeat size is inversely correlated with the age of HD onset and it is the major determinant of disease progression and age at death in HD [7]. HD is fully penetrant for individuals possessing alleles with 40 or more CAG expansions, meaning they will invariably exhibit unequivocal motor signs of the disease during their lifespan. In the normal population, CAG repeats in the huntingtin gene range up to 35 units. Repeats of 36 to 39 CAG trinucleotides show reduced penetrance, as they do not guarantee that an individual having such expansion range will develop unequivocal symptoms of HD [38].

The Unified Huntington’s Disease Rating Scale (UHDRS) [39] is currently the most widely used tool in clinical practice for the assessment of HD progression. It consists of four components evaluating motor function, cognition, behavior and functional capacities. The onset of HD is considered the point in time when an individual who carries a known CAG-expanded huntingtin allele (or has a family history of HD) develops motor symptoms that are unequivocal signs of HD as defined in the diagnostic confidence level subscale of UHDRS. This subscale comprises five levels of diagnosis confidence from level 0 (no motor abnormalities) up to level 4, indicating motor abnormalities that are unequivocal signs of HD (≥ 99% confidence), with or without psychiatric manifestations or cognitive impairment [40].

The Total Motor Score (TMS) of the UHDRS is a clinical rating subscale assessing multiple domains of motor disability in HD. More specifically, 31 motor signs of the disease are evaluated in this scale, including eye movement, oropharyngeal dysfunction, hand coordination, rigidity, bradykinesia, chorea and balance. Each of these items is rated from grade 0 (not affected) to grade 4 (most severely affected), adding to a maximum of 124 points in the TMS scale. The variety of motor signs classified in the UHDRS-TMS reflect the broad spectrum of motor symptoms observed in HD [41].
One of the most frequent measures of function in HD patients is the Total Functional Capacity (TFC) scale, typically assessed in a brief interview with a patient and/or collateral source. The TFC globally assesses occupation, finances, domestic chores, daily activities and level of care of HD patients. Scores on these five items, attributed by the clinician, are summed to yield a TFC ranging from 0 to 13, with 0 corresponding to severe functional capacity deterioration while greater scores indicate higher functional capacity [34].

2.1.6. Iron and its Role in Huntington’s Disease

Iron is a vital element for normal brain metabolism, as it plays an important role in oxygen transportation, myelin production, neurotransmitter synthesis and in reduction-oxidation reactions, which regulate oxidative stress [42]. Although most of the iron in the human body is bound to haemoglobin for oxygen transport, brain iron is mainly stored in ferritin [43], a protein that acts as a buffer for iron homeostasis in the brain. Ferritin-bound iron accumulates in the brain with normal ageing, especially in structures primarily associated with motor activity, such as the GP, red nucleus and the SN [44]. High concentrations of iron were also reported in healthy putamen, caudate nucleus and thalamus [42].

In HD, protein modification, misfolding and aggregation into inclusion bodies have been associated with a pathological increase of iron content, along with processes such as inflammation and apoptosis [45]. While the wildtype huntingtin protein is involved in iron homeostasis, its mutant form is hypothesized to impair such equilibrium in a number of ways, eventually resulting in an increase in iron concentration in tissues. Iron overload leads to the excessive production of free radicals, which in turn cause oxidative damage to neurons and induce inflammation, ultimately prompting neuronal loss [46],[47]. The most affected structures by iron overload in HD are believed to be the caudate, putamen and GP [48], regions highly related to motor function that undergo neuronal degeneration during the course of the disease. While iron level changes are likely to play an important role in the HD pathological cascade, whether they precede or depend on grey matter loss is not yet known.

2.2. Substantia Nigra and Neuromelanin

The substantia nigra (SN) is a brain structure located in the midbrain that plays an important role in motor planning, reward, addiction and eye movement. The SN is subdivided into two anatomically and functionally distinct parts: the SN pars reticulata (SNr) and the SN pars compacta (SNC) – schematized in Figure 5. The SNC provides a modulatory input to the corticostriatal pathway through the striatum, by exciting the direct pathway and inhibiting the indirect pathway, ultimately facilitating movement. The SNr, similarly to the internal portion of the GP, relays signals from the striatum and the subthalamic nucleus to several other brain structures, namely to the thalamus [14]. While the SNr is composed of GABAergic neurons, the SNC contains clusters of dopaminergic neurons [49]. A remarkable feature of these nigral dopaminergic neurons is their pigmentation, resulting from an accumulation of NM in SNC. This pigment binds to heavy metal ions, especially iron, and plays an important role in iron homeostasis [50],[51]. The NM of the SNC is thought to have antioxidant properties that contribute to buffering cellular oxidative...
stress, such as trapping potentially toxic iron in an inactive form. Accumulation of NM begins early in life and progresses thereafter [15].

Figure 5: Schematic transverse section through the rostral midbrain at the level of the superior colliculus, showing the SNC and the SNr. Adapted from [52].

However, under iron overload conditions, NM only manages to sequester iron in its reactive form, which can play a role in increasing cellular oxidative stress. Moreover, in this situation, NM may degenerate, releasing metals and toxic compounds, inducing microglia activation and releasing of pro-inflammatory factors, thus triggering neurodegeneration [15]. In fact, the degeneration of SN is the hallmark of PD, and includes the loss of NM dopaminergic neurons and iron deposition in the SNC [49]. Nevertheless, whether such iron accumulation is a primary event or a secondary effect in neurodegenerative diseases is still unclear [53].

2.3. Magnetic Resonance Imaging

The Magnetic Resonance Imaging (MRI) technique relies on the magnetization properties of atomic nuclei. In the absence of any external magnetic field, spins in a tissue are randomly oriented, resulting in a null net magnetization. However, when an external magnetic field $\vec{B}_0$ is applied to the sample, nuclear spins align with $\vec{B}_0$ and begin to precess around their rotation axis with a constant frequency known as Larmor frequency, which is proportional to the strength of $\vec{B}_0$. In other words, the tissue will become magnetized in the presence of an external magnetic field with a net magnetization $\vec{M}_0$. The magnetization of the collection of spins along the direction of $\vec{B}_0$, henceforth named longitudinal magnetization, is positive. On the other hand, in the directions perpendicular to $\vec{B}_0$, spins are still randomly oriented, and therefore their net transverse magnetization is null [54].
2.3.1. T₁, T₂ and T₂* Relaxation Mechanisms

Relaxation is the process by which protons release the energy they previously absorbed from a radiofrequency (RF) pulse as they naturally return to their original conformation of lower energy, with a rate constant known as relaxation time. Relaxation provides the primary mechanism for image contrast.

The T₁ relaxation time is the time required for the longitudinal component of \( \vec{M}_0 \), \( M_z \), to return its equilibrium magnitude following an RF excitation pulse. When an 90º RF pulse is applied to a sample, the net magnetization will tip from the longitudinal to the transverse plane and will exponentially recover as the spins realign with \( B_0 \), according to the tissue-specific time constant T₁. Therefore, T₁ is also known as longitudinal relaxation time or spin-lattice relaxation time, as the excited protons transfer their energy to the lattice in their vicinity rather than to other protons [55]. The magnitude of the longitudinal magnetization \( M_z \) as a function of time, following a 90º excitation pulse, is schematically represented by the blue line in Figure 6, and is given by:

\[
M_z(t) = M_0(1 - e^{-t/T_1})
\]

(1)

where \( M_0 \) is the magnitude of the net magnetization before the application of the RF pulse. The T₂ relaxation time is the time required for the transverse component of \( \vec{M}_0 \), \( M_{xy} \), to decay to 37% of its initial value following a RF pulse. After \( \vec{M}_0 \) is tipped into the xy plane, spins start dephasing and releasing energy as they reorient themselves with \( B_0 \), resulting in an exponential decay of \( M_{xy} \) (black line in Figure 6). During this process, excited spins irreversibly transfer energy to other nearby spins rather to their surroundings [56]. Thus, T₂ is also known as transversal relaxation time, or spin-spin relaxation time. The magnitude of the transverse magnetization \( M_{xy} \) as a function of time, following a 90º excitation pulse, is schematically represented by the black line in Figure 6, and is given by:

\[
M_{xy}(t) = M_0 e^{-t/T_2}
\]

(2)

where \( M_0 \) is the magnitude of the net magnetization before the application of the RF pulse. However, spin-spin interaction is not the only cause for the loss of coherence of transverse magnetization. \( B_0 \) inhomogeneities are unavoidable due to imperfections in magnet manufacturing and result in a constant magnetic field distortion during image acquisition. Furthermore, medium inhomogeneities such as variations in magnetic susceptibility values of brain tissues also produce local magnetic field distortions.

T₂* is a composite relaxation time, often called the ‘apparent’ transverse relaxation time, that dictates the rate at which the spin coherence in transverse magnetization is lost, considering the spin-spin interactions, given by T₂, and the magnetic field inhomogeneities.
Figure 6: Schematization of the magnitude values of the longitudinal magnetization $M_z$ (line in blue) and of the transverse magnetization $M_{xy}$ (line in black) of a sample of spins as a function of time, after being excited with a 90° RF pulse. Following excitation, $M_0$ will rotate into the transverse plane and recover its longitudinal component at a rate dictated by $T_1$, according to eq. (1). $M_{xy}$ will decay due to spin dephasing, at a rate given by $T_2$, according to eq. (2). Note that $T_2$ decay is faster than $T_1$ recovery. Adapted from [55].

During spin dephasing following an excitatory RF pulse, the nuclei release energy that decays exponentially due to transverse magnetization loss, as they return to the lower, more stable energy state [55]. Such unleashed energy induces a voltage in the coil of the MR scanner, displayed as the free induction decay (FID) signal:

$$M_{xy}(t) = M_0 e^{-t/T_2}$$  \hspace{1cm} (3)

Where $M_{xy}$ is the magnitude of the transverse magnetization, $M_0$ is the magnitude of the net magnetization before the application of the RF pulse and $T_2^*$ is the apparent transverse relaxation time.

### 2.3.2. Fast Spin Echo Sequences

A pulse sequence is the measurement technique that contains the hardware instructions (RF pulses, gradient pulses and timings) necessary to acquire an MR image in a specific manner. Two parameters that characterize MR pulse sequences are the time of repetition (TR) and the time of echo (TE). TR is the time between successive excitation pulses, and TE consists of the time between the excitation pulse and the peak of an echo signal. In this work, $T_1$-weighted neuromelanin-sensitive MR (NM-MR) images were acquired with a fast spin echo (FSE) sequence, and both the high-resolution anatomical $T_1$-weighted images and the $T_2^*$-weighted images were obtained with gradient echo (GE) sequences. Some features of these sequences are summarized in this subsection and in the following one.

FSE sequences, also known as turbo spin echo (TSE) sequences, comprise a 90° pulse followed by multiple 180° RF pulses in each TR. The 90° excitation pulse causes spin dephasing by tipping longitudinal magnetization into the transverse plane as aforementioned. Each 180° pulse is then applied
to invert spin magnetization, leading to spin rephasing and to the production of an echo signal (spin echo - SE). The inversion of spin phase will allow the regeneration of signal apparently lost during the FID due to magnetic field inhomogeneities. SEs may be generated as long as $T_2$-relaxation has not completely destroyed the MR signal. The amplitude of each echo is, however, progressively smaller due to $T_2$ decay.

MR signals are digitized in a ‘raw data space’ known as k-space during data acquisition. Through the application of frequency and phase-encoding gradient fields, each k-space point will contain spatial frequency and phase information about every pixel in the final image, which is then reconstructed by applying the inverse Fourier Transform to the k-space. FSE changes the phase-encoding gradient in each echo (Figure 7) so that multiple lines of the k-space may be acquired in a single TR, significantly reducing acquisition time when comparing to conventional SE sequences. More specifically, acquisition duration using an FSE sequence is inversely proportional to the echo train length (ETL), or turbo factor, which represents the number of echoes acquired in a given TR [55].

![Diagram of an FSE/TSE sequence with an ETL of 3. The 90° excitation pulse dephases spins, and each 180° pulse is then applied to invert spin magnetization, leading to spin rephasing and to the production of a SE. The phase-encoding gradient ($G_{PE}$) is changed for each echo, allowing multiple lines of the k-space to be acquired in a single TR. Adapted from [55].](image)

NM-containing structures (SNc and locus coeruleus) can be visualized in vivo using neuromelanin-sensitive magnetic resonance imaging (NM-MRI), a two-dimensional (2D) TSE $T_1$-weighted sequence developed by Sasaki et al. in 2006 for a 3T MRI scanner [17]. The NM-MRI images obtained with this sequence revealed two band-like high signal areas in the posteromedial portion of the cerebral peduncle at the level of the midbrain (Figure 8 (b)) which were confirmed to be the bilateral NM-containing SNc [57]. Conventional SE $T_1$-weighted images hardly exhibit contrast in SN (Figure 8 (c)), possibly because of the small differences in $T_1$ signal between tissues with and without NM. In $T_2$-weighted images, both the SN and the surrounding areas show low signal intensity (Figure 8 (d)) due to physiological iron deposition. In turn, proton density-weighted images do not clearly distinguish the SNc from the SNr (Figure 8 (e)) [58].
Figure 8: Neuromelanin-sensitive and conventional MR images of the SN of a 42-year old healthy individual, obtained using the sequence developed by Sasaki et al. [58]. Axial sections at the level of midbrain. Macroscopic specimen (a) and MR images obtained at 3T with NM-MRI (b), T1-weighted image (c), T2-weighted image (d) and proton density-weighted image (e). In (a) and (b), the arrows point to the SNc. Neuromelanin-sensitive images clearly depict the hyperintense areas corresponding to the locations of the SNc in the macroscopic specimens. In (d), the thick arrow indicates the SN and the thin arrow indicates the peduncular fibres, both of which present low intensity. In (e), the arrowheads point to the SN. Adapted from [58].

The origin of the contrast between NM-containing structures and the surrounding tissues is not yet fully understood. A possible explanation for the high signal intensity of these regions is the T1-shortening effects of the paramagnetic NM. Moreover, the SNc has a high concentration of iron in the form of ferritin, which is suggested to further shorten the T1 in the tissue [17],[59]. Nevertheless, other iron-laden structures such as the red nucleus do not particularly exhibit hyperintensities in NM-MRI [60]. Furthermore, a postmortem study found a significant positive correlation between the signal intensity and the quantity of neuromelanin-containing neurons in SNc, but iron deposition did not seem to influence the increased signal intensity of this structure [57]. These findings suggest that there may be other factors, other than iron concentrations, that more strongly underlie the contrast observed in neuromelanin-containing structures. In fact, it has been shown that the contrast in NM-MRI using FSE sequences results mostly from 'incidental' magnetization transfer (MT) phenomena [16], associated with multi-slice acquisition, while T1 contrast itself is minimal [61].

The MT effect consists in the shifting of energy between the protons contained in macromolecules and membranes of tissues (bound protons) and the mobile water protons (free protons). MRI signals arise solely from free protons, which have sufficiently long T2 times (i.e., greater than 10 ms) so that excitation and acquisition can be performed before the signal completely decays. The signal from bound protons, in turn, decays too quickly to be detected in MRI, as these particles have very short T2 values (of less than 0.1 ms) [62]. In multi-slice FSE sequences, the off-resonance RF radiation resulting from the multiple 180° refocusing pulses selectively saturates bound protons. Bound protons transfer their magnetization to the free protons, accelerating spin dephasing and thus leading to a decrease of the signal produced by the latter. Therefore, in tissues containing a high concentration of macromolecules (and thus of bound protons), the MR signal is reduced. The NM-containing SNc will not be significantly affected by MT effects, thus this structure will exhibit hyperintensity in NM-MR images comparing to the suppressed background tissues - Figure 8 (b) [55],[63],[64]. This background brain tissue signal suppression gives rise to the MT contrast. Of note, since the SNr contains much less dopaminergic NM-containing neurons than the neighboring SNc, it will not present a hyperintense signal, allowing for the distinction between these two structures. Furthermore, iron-rich regions other than the SNc, such as the
putamen, GP and caudate, do not display intense NM-MRI signals, as in these structures the iron is stored in ferritin, which possesses low paramagnetism comparing to NM. [65]

2.3.3. Gradient Echo Sequences

GE sequences do not use a 180° RF pulse to refocus spins following an RF pulse excitation. In this case, the echo signal is produced by a bipolar gradient. After an excitation pulse, a dephasing gradient is applied across the sample, inducing spin dephasing, which accelerates the FID. A second gradient pulse with the same parameters but opposite polarity is then applied to the sample, reversing spin dephasing and producing a gradient echo (Figure 9) [55].

![Diagram of GE sequence](https://via.placeholder.com/150)

**Figure 9:** GE sequence diagram. After the RF pulse, generally with a flip angle ($\alpha$) of less than 90°, a frequency encoding dephasing gradient ($G_{FE}$) is applied across the sample, which accelerates the FID event. A second gradient pulse with the same parameters but opposite polarity is then applied, reversing spin dephasing and producing a GE. Adapted from [55].

In multi-echo GE sequences, the gradient reversal process used to create a single gradient echo may be repeated to produce additional echoes following a single RF excitation pulse. By reversing the frequency-encoding gradient rapidly, multiple individual gradient echoes may be generated at different TEs, as long as $T_2^*$-relaxation has not completely destroyed the MR signal.

The usage of a refocusing bipolar gradient instead of a 180° RF pulse has several important consequences for GE sequences. Firstly, the reversal gradient only refocuses the spins that have been dephased by action of the dephasing gradient itself. Thus, the static sources of proton dephasing such as magnetic susceptibility gradients and $B_0$ field inhomogeneities are not cancelled by the gradient reversal pulse, allowing for a $T_2^*$-weighted acquisition rather than $T_2$-weighting as in SE imaging. Additionally, since only one RF pulse is applied in GE per TR, echoes can be recorded faster in these sequences than in FSE, resulting in shorter TE values. Furthermore, GE sequences, which typically have flip angles smaller than 90°, use short TRs. As a result, signal acquisition with GE sequences is generally faster than with FSE. GE sequences are thus often used to rapidly acquire three-dimensional (3D) $T_1$-weighted images with excellent signal-to-noise ratio and resolution [55].
Iron content in the brain can be measured by means of MRI using GE sequences. $T_2^*$-weighted GE sequences, which do not refocus spins dephased by magnetic field inhomogeneities, are sensitive to gradients in magnetic susceptibility. Tissue accumulations of paramagnetic iron-containing ferritin distort the local magnetic field, causing the transverse magnetization to decay much faster, thus shortening the $T_2^*$ relaxation time. $T_2^*$ values can indirectly quantify the iron content of brain structures, since regions with high concentration of iron will have shorter $T_2^*$ than those having low iron content. More often than $T_2^*$, its reciprocal relaxation time constant $R_2^*$ ($1/T_2^*$) is used to quantify the iron accumulations in brain structures using MRI. This parameter is strongly correlated to iron concentration in brain tissues [42].
3. State of the Art

Regardless of its complex neuropathology, HD is a disease with a well-defined at-risk target population, with a natural history somewhat consistent with the extent of the mutation across patients (especially the progression of motor symptoms and age at onset), and with a monogenic cause which is diagnosable by a simple genetic test. These features characterize HD as a unique model disease for other neuropathologies. Hence, developing tools that assess biological parameters of HD may prove to be invaluable in understanding the disease. Such a tool, or biomarker, is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic processes, or pharmacological responses to a therapeutic intervention” [66]. In other words, biomarkers should allow the objective measurement of disease staging, classification of the extent of the disease, disease prognosis, and should predict and monitor the clinical response to an intervention. There is still no established biomarker for the study of HD progression, nor any effective treatment [12].

The UHDRS is the most widely-used tool in the study of HD progression in clinical practice, by measuring outcomes such as motor, cognitive and behavioral function, and functional limitations [67]. However, as in any clinical scale, UHDRS is subject to inter-rater variability. Additionally, it requires extensive training to achieve reliability among raters. [68]. Neuroimaging techniques, on the other hand, will be able to provide more objective and robust biomarkers of HD, as they more easily achieve reliability across study sites and across time. Neuroimaging biomarkers may be especially useful in premanifest HD, before the onset of rapid neuronal degeneration and the emergence of clinical symptoms. Furthermore, current measures of clinical progression such as motor, cognitive and functional exams and rating scales are less sensitive to changes during this phase [68]. Hence, the premanifest phase of HD offers a therapeutic window to introduce neuroprotective drugs aimed at delaying the clinical onset and slowing the progression of the disease.

A range of neuroimaging techniques has been used to study the many processes involved in HD degeneration. Volumetric MRI assesses macroscopic brain structure; diffusion imaging reveals white matter integrity; functional imaging investigates activity of specific brain regions during mental and motor tasks and positron emission tomography (PET) probes metabolic processes and neuroreceptor levels in specific brain regions. However, structural imaging is the most widely used among the neuroimaging techniques in clinical practice to study the brain changes in HD [9].

3.1. Structural Magnetic Resonance Imaging

This cross-sectional work was aimed at investigating the volumes of the basal ganglia, lateral ventricles, midbrain and brainstem, the NM content in SNc and the iron deposition in the caudate, putamen and GP of HD patients using different structural MRI techniques. Therefore, the literature review of the state-of-the-art, summarized in Table 1, was focused on cross-sectional studies that investigated such parameters and structures in HD.
3.1.1. Volumetric Magnetic Resonance Imaging in Huntington’s Disease

Striatal atrophy, which comprises the neuronal death of both the caudate and the putamen, is the neuropathological hallmark of HD. Thus, it is not surprising that volumetric striatal measurement from MRI has received the greatest amount of attention as the strongest candidate for HD biomarker.

Several cross-sectional MRI studies consistently reported volumetric reductions in the caudate and putamen nuclei of subjects with clinically manifested HD comparing to healthy controls (HCs). Rosas et al. [69] reported significant mean percent reductions in the volumes of striatum (-55%), caudate (-41%) and putamen (-49%) of HD patients when comparing to HCs. Another group found significant mean percent volume changes in the caudate (-50%) and in the SN (-26%) of HD patients [70]. Volumes of the caudate and putamen normalized to intracranial volume (ICV, comprising the gray matter, white matter and cerebrospinal fluid - CSF) were also found to be significantly smaller in manifest HD patients than HCs in another study, and both volume ratios correlated with clinical severity (disease stages) of the disease [71]. Besides disease staging, striatal atrophy also correlated with total functional capacity (TFC) and with several cognitive scores in other studies, implicating striatal degeneration in symptom manifestation in HD [12].

Extra-striatal atrophy in clinical HD patients is also well documented in cross-sectional studies. Volume reductions in the GP [33],[72],[73], nucleus accumbens [33],[73], hippocampus [33], amygdala [33],[72], brainstem [33], thalamus [72],[73] and SN [72] of manifest HD versus HCs were reported. Cerebral white-matter [33], grey-matter [72] and whole-brain [71] volumes were also found to be reduced in HD patients. Moreover, an increase of over 100% in the volume of lateral ventricles of HD patients was found in one study [33]. Curiously, the same work reported larger standard deviations of volumes in the HD cohort than in HCs, which suggest a large heterogeneity of abnormalities in subjects with HD.

Cross-sectional findings have been confirmed and expanded in longitudinal MRI studies, which not only investigate the progression of brain atrophy both in premanifest and manifest HD, but also attempt to correlate the time course of these changes to the clinical scores and to the features of the disease. Large-cohort longitudinal studies have reported that striatal volume of HD gene carriers, while smaller than those of HCs, remains constant until about 15 to 20 years from estimated symptomatic onset, after which atrophy is evidently observable and progressive. Around the time of diagnosis, subjects with HD already have volumes equivalent to 43-67% of normal volume for the putamen, 52-70% for caudate and 59-62% for GP [74]. Furthermore, these studies have shown faster rates of decline in striatal volumes in premanifest and manifest HD compared with age-matched controls, even in those who are distant from estimated clinical onset [10]. Moreover, strong correlations between striatal volumes and estimated years to disease onset (based on age and CAG repeat length) and between striatal atrophy and measures of motor and cognitive impairments have been revealed by these studies.

3.1.2. Neuroimaging Studies of Iron in Huntington’s Disease

Brain iron changes and subsequent oxidative stress are likely to be major players in the HD pathological cascade. However, whether such disruption of iron homeostasis precedes or depends on grey matter
loss is not yet known. Therefore, investigating iron deposition in the brain of HD patients and its timing in relation to neurodegeneration may provide deeper insights into the pathogenesis of the disease. If iron overload is demonstrated to precede atrophy in the basal ganglia, which begins many years before symptomatic onset itself, brain iron quantification might prove to be a reliable novel biomarker in the study of HD. Therefore, over the past years, purely volumetric studies have been replaced by multiparametric studies, which are able to investigate a wider range of phenomena underlying the pathological processes of HD, while relating changes in other several parameters with the well-documented brain atrophy of the disease.

A wide range of approaches, including $R_2^*$ relaxometry and magnetic susceptibility-related measures such as quantitative susceptibility mapping (QSM) have been already used in several neuroimaging studies of iron in HD.

A multiparametric study by Sánchez-Castañeda et al. [73] evaluated the macrostructure (with T1-weighted volumetry), microstructure (using diffusion tensor imaging) and iron content (with $R_2^*$ relaxometry) changes in the basal ganglia of premanifest and manifest HD subjects. $R_2^*$ relaxometry was based on a $T_2^*$-weighted volume series of six echoes (at TEs: 6, 12, 20, 35, 45 and 60 ms), to which a voxel-by-voxel nonlinear least-squares fitting was performed to obtain a monoexponential signal decay curve according to $T_2^*$ relaxation. A similar approach to iron content assessment was performed in this thesis. This group reported significant volume reductions in the caudate, putamen and GP of both the manifest and premanifest HD cohorts when comparing to HCs. Furthermore, a significant increase in iron content was found in the GP of not only mHD, but also of preHD, given by greater $R_2^*$ values in this structure. Such results indicate that iron is abnormally accumulated in the GP in HD well before the first symptoms appear. Regarding microstructure, reduced tissue integrity (given by increased mean diffusivity values) was found in both the caudate and putamen, and strongly correlated with striatal volume loss. In the GP, diffusivity values were not significantly altered despite volume loss, a result that was attributed to the increased iron content in this structure. Finally, no significant correlations were found between $R_2^*$ and volume values of any of the basal ganglia structures of all HD subjects, results which may suggest that iron deposition is an independent process of atrophy in the basal ganglia of HD patients, especially in the GP.

A subsequent study [45] assessed $R_2^*$ values and volumes of the basal ganglia, and the correlation between these two parameters in premanifest and manifest HD subjects. Voxel-based $R_2^*$ relaxometry was performed with similar methods to those of [73]. Significant increases in the values of $R_2^*$ were found in the putamen and GP of both cohorts of HD subjects when compared to HCs, revealing greater iron content in these structures even before symptomatic onset. When comparing HD cohorts, $R_2^*$ values were even higher in the GP of manifest than in premanifest HD subjects, hinting that iron deposition tends to increase in the GP during the course of the disease. Regarding volumetric measurements, basal ganglia volumes were significantly smaller in both preHD and mHD, as previously reported. In contrast to [73], this study found significant inverse correlations between iron concentration given by $R_2^*$ and volume in the putamen ($r = -0.408$) and in the GP ($r = -0.505$), suggesting that atrophy and iron deposition might be, after all, dependent to some extent. The authors also observed that the
longer the disease duration and the higher the CAG repeats length, the greater the iron content and the smaller the volume, especially in the putamen and GP of HD subjects. Finally, increased iron content was also found in several cortical regions of manifest HD patients.

van Bergen et al. [75] made use of both R2* relaxometry and QSM data to study iron deposition in the brain of premanifest HD subjects. This group reported significantly increased values of magnetic susceptibility in the caudate, putamen and GP structures of these asymptomatic subjects compared to healthy controls. Since there is a strong direct correlation between susceptibility values and tissue iron levels in brain grey matter [76], such increase was attributed to HD-related increase of tissue iron in these regions. R2* values, on the other hand, were reported to be increased only in the caudate and putamen. Interestingly, this study showed decreased levels of iron in the SN of premanifest HD subjects. Significant volume decreases were once again observed in the caudate nucleus and putamen of these subjects, but not in GP. Since the iron content of GP is significantly increased before substantial atrophy occurs, the authors suggest that iron changes either precede or have a greater magnitude than volume changes in this structure. Additionally, caudate (r = -0.73) and putamen (r = -0.83) volumes were inversely correlated with magnetic susceptibility.

Domínguez et al. [48] used QSM to demonstrate significantly greater iron deposition in the caudate, putamen and GP of both premanifest HD and manifest HD. This group also found positive correlations between susceptibility values and disease burden scores in all HD subjects in the putamen (r = 0.39) and in the caudate (r = 0.32).

These results are summarized in Table 1. Neuroimaging studies of iron in HD have generally shown greater iron deposition in the basal ganglia during premanifest and/or symptomatic stages, although increases in all basal ganglia structures have not been consistently reported in all stages across studies.

### 3.1.3. Neuroimaging Studies of the Substantia Nigra

NM-MRI has never been used to quantify NM in the SNc of HD patients, as far as known. However, since the development of the NM-MRI sequence for the visualization of the SNc as a high-signal intensity area in vivo by Sasaki et al. [17], an ever-growing number of groups (Table 2) have investigated various parameters that indirectly quantify neuromelanin in SNc in other neurological disorders. The loss of NM-containing dopaminergic neurons in the SNc is the neuropathological hallmark of PD, and recently NM-MRI has been used to study SNc changes as a potential diagnostic tool of this disease.

An NM-MRI parameter widely investigated both in PD patients and HCs is the contrast ratio (CR) of the hyperintense SNc with regard to a given reference region of the midbrain, which indirectly measures the distribution of the NM pigment in the SNc of subjects. The area, maximal length and volume of this structure were also investigated as indicators of dopaminergic neuronal loss. The ratio of SNc area to midbrain area was measured in one study [77], so as to investigate if SNc degeneration was dependent of global midbrain area loss. In summary, all of the aforementioned parameters were found to be reduced in subjects with PD. These results indicate the feasibility of studying SNc degeneration with NM-MRI.
Table 1: Summary of several cross-sectional structural MRI studies in HD.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Subjects</th>
<th>Scanner</th>
<th>Parameters</th>
<th>Main Results (HD vs. HCs)†</th>
<th>Additional Findings†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosas et al. (2001) [69]</td>
<td>27 HD</td>
<td>1.5 T</td>
<td>Volumes</td>
<td>↓ Vol. Striatum (-55%)</td>
<td>CAG repeats correlate with striatal atrophy (0.55)</td>
</tr>
<tr>
<td></td>
<td>24 HCs</td>
<td></td>
<td></td>
<td>↓ Vol. Caudate (-41%)</td>
<td>Rightward asymmetry in striatal volumes of HCs (AI = +0.02, p=0.0044)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Vol. Putamen (-49%)</td>
<td>Leftward asymmetry in striatal volumes of HD (AI = -0.05, p=0.0001)</td>
</tr>
<tr>
<td>Rosas et al. (2003) [33]</td>
<td>18 HD</td>
<td>1.5 T</td>
<td>Volumes</td>
<td>↓ Vol. Caudate (-37%)</td>
<td>TFC correlates with volume of caudate (0.58), putamen (0.52), GP (0.51) and brainstem (0.52).</td>
</tr>
<tr>
<td></td>
<td>18 HCs</td>
<td></td>
<td></td>
<td>↓ Vol. Putamen (-53%)</td>
<td>Standard deviations of volumes were greater in the HD cohort than in HCs.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Vol. GP (-41%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Vol. Brainstem (-14%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Vol. Ventricles (&gt;100%)</td>
<td></td>
</tr>
<tr>
<td>Mascalchi et al. (2004) [71]</td>
<td>21 HD</td>
<td>1.5 T</td>
<td>Volumes</td>
<td>↓ Vol./ICV Caudate Ratio</td>
<td>Volumes of caudate and putamen normalized to each subject’s ICV were reduced in HD patients.</td>
</tr>
<tr>
<td></td>
<td>21 HCs</td>
<td></td>
<td></td>
<td>↓ Vol./ICV Putamen Ratio</td>
<td>Disease stage was correlated to the volume of caudate (-0.63) and putamen (-0.64).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Vol. Whole-Brain</td>
<td></td>
</tr>
<tr>
<td>Douaud et al. (2006) [72]</td>
<td>20 mHD</td>
<td>1.5 T</td>
<td>Volumes</td>
<td>↓ Vol. Caudate (-52% to -60%)</td>
<td>Dorsal and medial striatum exhibited larger atrophy than ventral striatum, evidencing the dorso-ventral gradient of striatal atrophy in HD.</td>
</tr>
<tr>
<td></td>
<td>12 HCs</td>
<td></td>
<td></td>
<td>↓ Vol. Putamen (-48% to -53%)</td>
<td>TFC strongly correlates with striatal atrophy.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Vol. GP (-57%)</td>
<td>Bilateral atrophy of the SN.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ Vol. SN</td>
<td></td>
</tr>
<tr>
<td>Sánchez-Castañeda et al.</td>
<td>17 preHD</td>
<td>3 T</td>
<td>R2* Volumes</td>
<td>↓ Vol. Caudate</td>
<td>All basal ganglia volumes were reduced in both HD and preHD cohorts. R2* values were increased in the GP of HD and preHD, indicating abnormal iron accumulation in this structure before onset of symptoms.</td>
</tr>
<tr>
<td>(2013) [73]</td>
<td>12 mHD</td>
<td></td>
<td>DTI</td>
<td>↓ Vol. Putamen</td>
<td>Reduced tissue integrity (↑ MD values) in the caudate and putamen correlated with volumes (-0.85).</td>
</tr>
<tr>
<td></td>
<td>29 HCs</td>
<td></td>
<td></td>
<td>↓ Vol. / ↑ R2* GP</td>
<td>No correlations were found between R2* and volume values of any of the basal ganglia structures in HD patients.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sánchez-Castañeda et al.</td>
<td>19 preHD</td>
<td>3 T</td>
<td>R2* Volumes</td>
<td>↓ Vol. Caudate</td>
<td>R2* per volume ratios in caudate, putamen and GP of all CAG carriers were higher than those of HCs.</td>
</tr>
<tr>
<td>(2015) [45]</td>
<td>50 HD</td>
<td></td>
<td></td>
<td>↓ Vol. / ↑ R2* Putamen</td>
<td>R2* inversely correlates with volume in the putamen (-0.408) and GP (-0.505).</td>
</tr>
<tr>
<td></td>
<td>73 HCs</td>
<td></td>
<td></td>
<td>↓ Vol. / ↑ R2* GP</td>
<td>↑ R2* in the caudate, ↓ R2* in the Putamen and GP of preHD comparing to mHD.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vol. mHD &lt; Vol. preHD in all basal ganglia structures.</td>
</tr>
<tr>
<td>van Bergen et al. (2016)</td>
<td>15 preHD</td>
<td>7 T</td>
<td>QSM R2*</td>
<td>↓ Vol. / ↑ χ / ↑ R2* Caudate</td>
<td>Susceptibility values in the caudate and putamen are inversely correlated with structure volumes (-0.73 and -0.85 resp.) and directly correlated with both CAG repeat length and age (0.54 and 0.69 resp.).</td>
</tr>
<tr>
<td>(2016) [75]</td>
<td>16 HCs</td>
<td></td>
<td>Volumes</td>
<td>↓ Vol. / ↑ χ / ↑ R2* Putamen</td>
<td>R2* values in the putamen are correlated with CAG repeat length and age (0.52).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ χ GP</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ χ SN</td>
<td></td>
</tr>
</tbody>
</table>

† All represented differences in the parameters between subjects with HD and HCs and correlations between parameters were considered significant with a p-value < 0.05.
χ: magnetic susceptibility, AI: asymmetry index, MD: mean diffusivity, mHD: manifest HD, preHD: premanifest HD, QSM: quantitative susceptibility mapping.
In order to evaluate the NM-MRI parameters of SNc, several groups have resorted to manual methods for segmentation of this region, while others have used semi-automated techniques such as region-growing algorithms [18],[78]. Automated segmentation of SNc was performed by one group [79] which used a reference atlas of manually segmented NM-MRI images.

Most groups have used an FSE sequence similar to that of [17] to obtain NM-MRI images – Table 2. NM-MRI images acquired with this sequence show the SNc as an hyperintense area, due to the ‘incidental’ MT effects induced by the refocusing pulses, which suppress the intensity of background tissues. However, other groups [61],[78],[80],[81] have used MT pulses – off-resonance RF pulses to selectively saturate the bound protons – in their GE sequences to induce the MT contrast.

Table 2: Main features of several NM-MRI studies using different MRI sequences and methods for SNc segmentation to quantify NM in the SNc. While most of these studies were applied in PD, no NM-MRI work was used to study HD.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Scanner</th>
<th>Sequence</th>
<th>MT Pulse</th>
<th>SNc Segmentation</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sasaki et al. (2006)</td>
<td>3 T</td>
<td>2D FSE</td>
<td>No</td>
<td>Manual</td>
<td>CR</td>
</tr>
<tr>
<td>Nakane et al. (2008)</td>
<td>1.5 T</td>
<td>3D GE</td>
<td>Yes</td>
<td>Manual</td>
<td>CR</td>
</tr>
<tr>
<td>Schwarz et al. (2011)</td>
<td>3 T</td>
<td>2D FSE</td>
<td>No</td>
<td>Manual</td>
<td>Area, CR</td>
</tr>
<tr>
<td>Ogisu et al. (2013)</td>
<td>3 T</td>
<td>3D GE</td>
<td>Yes</td>
<td>Semi-Automated</td>
<td>Volume</td>
</tr>
<tr>
<td>Chen et al. (2014)</td>
<td>3 T</td>
<td>2D GE</td>
<td>Yes</td>
<td>Semi-Automated</td>
<td>CR, Volume</td>
</tr>
<tr>
<td>Ohtsuka et al. (2014)</td>
<td>3 T</td>
<td>2D GE</td>
<td>No</td>
<td>Manual</td>
<td>CR</td>
</tr>
<tr>
<td>Castellanos et al. (2015)</td>
<td>3 T</td>
<td>2D FSE</td>
<td>No</td>
<td>Automated</td>
<td>Volume</td>
</tr>
<tr>
<td>Reimão et al. (2015)</td>
<td>3 T</td>
<td>2D FSE</td>
<td>No</td>
<td>Semi-Automated</td>
<td>Area, Length, Area/MB ratio</td>
</tr>
<tr>
<td>Reimão et al. (2015)</td>
<td>3 T</td>
<td>2D FSE</td>
<td>No</td>
<td>Semi-Automated</td>
<td>Area, Width</td>
</tr>
<tr>
<td>Langley et al. (2017)</td>
<td>3 T</td>
<td>2D GE</td>
<td>Yes</td>
<td>Semi-Automated</td>
<td>Volume</td>
</tr>
<tr>
<td>Prasad et al. (2018)</td>
<td>3 T</td>
<td>3D GE</td>
<td>No</td>
<td>Manual</td>
<td>CR</td>
</tr>
</tbody>
</table>

The only study quantifying the loss of dopaminergic neurons and of NM in SNc of subjects with HD found in the literature was that of Oyanagi et al. [85], dating back to 1989. This in vitro study revealed severe atrophy of both the pars reticulata and the pars compacta of the SN in four HD patients. The cross-sectional area of the SN in these subjects was about 57% of that of controls, and the number of neurons was significantly decreased in both regions of the SN. Furthermore, the amount of intraneuronal melanin was reduced, and the pigmented neurons were severely depleted in the medial and lateral thirds of the SN, while neurons in the central third of the SN were relatively well preserved.
3.2. Application of other Magnetic Resonance Imaging Modalities in Huntington’s Disease

Neurodegeneration in HD is likely to be preceded by neuronal dysfunction [27]. Hence, techniques such as functional MRI and Positron Emission Tomography (PET), which measure functional and metabolic changes in brain tissue, might enable the identification of neuronal disturbances even before macroscopic tissue loss. Data from manifest HD patients have revealed reduced task activation in several cortical and subcortical areas, while different cortical regions had increased activation, which were interpreted as compensatory mechanisms for task performances. Impaired brain network connectivity in premanifest and early stage HD subjects was also found, reflecting cognitive dysfunction early in the disease course [12]. One obstacle to the adoption of functional MRI as a biomarker in HD is the uncertainty about the true functional and physiological importance of the altered fMRI activation patterns in this disorder. Presently, patterns of activation still need clarification through further longitudinal studies.

PET has been used to assess glucose uptake and dopaminergic signaling as potential disease markers in HD. In HD patients, striatal glucose hypometabolism was longitudinally associated with motor deficits and reduced TFC, while deficiencies in metabolism in the caudate and cortex correlated with cognitive task performance. PET studies of dopaminergic systems have reported reduced density of dopamine receptors and activity in the striatum of HD patients, correlating with disease duration, cognitive impairment and motor deterioration [8]. Dopamine receptors are highly expressed in MSNs, which are known to be very vulnerable to the neurodegeneration in HD. Moreover, these changes in dopamine receptors were also found in premanifest and early-stage HD subjects [86].

The use of functional imaging parameters as biomarkers will require further large-scale, longitudinal studies of premanifest individuals. Furthermore, if these studies were to be integrated in a multimodal evaluation of imaging biomarkers, they could provide new insights into the neuronal dysfunction that sets the stage for the changes observed with other MR techniques such as structural MRI and diffusion tensor imaging.
4. Sample Description

This study consisted of a cross-sectional case-control analysis including 20 subjects: 8 HD patients with abnormal CAG repeats (≥36) and 12 healthy individuals. HD patients were recruited from the Movement Disorders Unit of the University Hospital of Santa Maria-Lisbon. All patients were diagnosed with HD by a movement disorders specialist (Dr. Leonor Correia-Guedes) and were scored for motor (TMS) and functional capacity (TFC) according to the UHDRS. The clinical characteristics of all subjects are shown in Table 3. One patient with 27 years of age had the Westphal variant of HD. Although no significant differences were observed in gender, there was a significant difference in age amongst the two cohorts.

An MRI protocol including T₁-weighted 3D Turbo Field Echo (TFE), NM-MRI 2D TSE and T₂*-weighted multi-echo Fast Field Echo (mFFE) sequences was performed on all subjects, using a Philips 3 T scanner (Philips Achieva; Philips Medical Systems, Best, Netherlands) with an 8-channel head coil.

Table 3: Demographic and clinical characteristics of the sample of subjects. For numerical characteristics the median (range) values are shown.

<table>
<thead>
<tr>
<th></th>
<th>HD (n=8)</th>
<th>HC (n=12)</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>3:5</td>
<td>7:5</td>
<td>0.398</td>
</tr>
<tr>
<td>Age at Study (years)</td>
<td>46 (27–81)</td>
<td>62 (49–83)</td>
<td>0.018</td>
</tr>
<tr>
<td>Disease Duration (years)*</td>
<td>7.5 (1–16)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UHDRS-TMS</td>
<td>20 (4–71)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TFC</td>
<td>12 (1–13)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Disease duration is defined by the length of time between onset of motor symptoms (unequivocal chorea or impairment of voluntary movements due to HD) and date of imaging.
† p-values obtained with the Mann Whitney U test.
5. **Volumetry Analysis**

5.1. **Methods**

5.1.1. **Imaging Protocol**

Structural T₁-weighted MRI data were obtained using a 3D TFE sequence. TFE is an ultra-fast GE sequence with data acquisition after an initial 180º preparation pulse for contrast enhancement. The imaging parameters of this sequence are displayed on Table 4.

Table 4: T₁-weighted TFE sequence parameters used in this study. FOV: Field of View, FA: Flip Angle.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>TR (ms)</th>
<th>TE (ms)</th>
<th>Pixel Size** (mm²)</th>
<th>Slice Thickness (mm)</th>
<th>FOV (mm²)</th>
<th>Num. Slices</th>
<th>FA (º)</th>
<th>Acq. Time (m:s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁ TFE</td>
<td>9.6</td>
<td>4.6</td>
<td>0.49x0.49</td>
<td>1</td>
<td>250x250</td>
<td>151</td>
<td>9</td>
<td>05:25</td>
</tr>
</tbody>
</table>

5.1.2. **Volume Segmentation**

T₁-weighted volumetric analysis was carried out with the version 6.0 of the open-source FreeSurfer [87] software (The General Hospital Corporation, Boston, MA, USA). All T₁-weighted anatomical images were preprocessed with the recon-all FreeSurfer command. This command comprises several streamlines for whole-brain segmentation and cortical parcellation, but only the relevant steps for the volumetric pipeline are hereby mentioned. Whole-brain T₁-weighted 3D images underwent intensity normalization, to correct image intensity variations (or bias fields) due to magnetic susceptibility artifacts and RF-field inhomogeneities. The neck and skull were stripped, and the brain was then registered with the Montreal Neurological Institute (MNI)305 space. Segmentation of several brain structures of the subjects was then automatically performed based on a probabilistic atlas from a manually labeled data set incorporated in FreeSurfer [88].

This segmentation procedure yielded the volumes of the structures of interest for each subject: caudate, putamen, GP, lateral ventricles and intracranial volume (ICV). Examples of the subcortical segmentation are shown in Figure 10 for an HC, and in Figure 11 for an HD patient. To segment and quantify the volumes of the midbrain and of the brainstem, the BrainstemSubstructures tool [89] was used in FreeSurfer. Segmentation of the brainstem structures using this tool is exemplified in Figure 12 for a HC. Images of the segmentation of all subjects were examined for misalignments or any other errors in segmentation using Freeview, FreeSurfer’s image visualization interface. The volumes of the segmented structures were normalized to the ICV to allow for the comparison of volumes between subjects accounting for overall differences in brain volumes.
Figure 10: Processed T₁-weighted images of an axial slice of an healthy subject (a) and of the same slice with subcortical segmentations performed by FreeSurfer (b): head of the caudate nucleus (in light blue), the putamen (pink), the GP (dark blue) and the lateral ventricles (in dark purple).

Figure 11: Processed T₁-weighted images of an axial slice of a late-stage HD patient (a) and of the same slice with subcortical segmentations performed by FreeSurfer (b): head of the caudate nucleus (in light blue), the putamen (pink), the GP (dark blue) and the lateral ventricles (in dark purple). Note the clear reduction on the sizes of the basal ganglia masks, which already indicate a volume reduction in these structures, and the enlargement of the lateral ventricles, when comparing to the HC case in Figure 10.

Figure 12: Processed T₁-weighted images of an axial (a), coronal (b) and sagittal (c) slices of an healthy subject showing the segmentation of brainstem structures performed with the BrainstemSubstructures tool in FreeSurfer: midbrain (in orange), pons (in yellow) and medulla oblongata (in grey).
5.1.3. Statistical Analysis

All statistical analyses were performed with non-parametric statistical tests, using the version 3.5.1 of the R software (R Foundation for Statistical Computing, Vienna, Austria). A p-value lower than 0.05 was considered significant. The Wilcoxon signed rank test for paired samples was used to compare the volumes of structures in both brain hemispheres, in each cohort. Regarding left and right-sided volumes, whenever no statistically significant difference was found between both values, the average volume was used for further analyses. Inter-group comparisons of the relative volumes were performed with Mann-Whitney U test. Finally, the Spearman rank-order correlation was used to investigate correlations between the relative volumes of HD patients with three available clinical variables: disease duration, TMS and TFC.

5.2. Results

5.2.1. Volume Comparison

The median volumes of the caudate, putamen, GP and lateral ventricles obtained with FreeSurfer in each hemisphere and for each cohort (HCs and HD patients) are displayed in Table 5. No statistically significant differences were found among the volumes of the left and right structures in each cohort, using the Wilcoxon signed rank test (p-values > 0.05), except in the case of the caudate nucleus of HCs. The volumes of the right-sided caudate were significantly larger (p < 0.005) than those of the left-sided structure in this cohort.

Table 5: Median (range) volumes of brain structures in the left and right hemispheres of HCs and of subjects with HD. p-values were obtained using the Wilcoxon signed rank statistical test for paired samples, which show statistically significant differences for p<0.05.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Volumes (Range) (cm$^3$)</th>
<th>HCs (n=12)</th>
<th>HD Subjects (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
<td>p-value</td>
</tr>
<tr>
<td>Caudate</td>
<td>3.0 (2.5-3.9)</td>
<td>2.9 (2.4-3.6)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Putamen</td>
<td>4.4 (3.6-5.6)</td>
<td>4.4 (3.6-5.7)</td>
<td>0.13</td>
</tr>
<tr>
<td>GP</td>
<td>1.7 (1.3-2.2)</td>
<td>1.8 (1.2-2.3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Ventrices</td>
<td>9.2 (3.8-25)</td>
<td>9.6 (5.8-19)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Since there were no significant asymmetries in the volumes of the remaining structures, their average volumes, calculated as $(V_L + V_R)/2$ where $V_L$ is the volume of the left-sided structure and $V_R$ is the volume of the right-sided structure, were henceforth considered. Since the left and right caudate volumes were significantly different in the HC cohort, these structures were also separately analyzed. The volumes of the caudate, putamen, GP, ventricles, brainstem and midbrain of each subject were then corrected for
total brain size by dividing each volume by the ICV. The resultant relative volumes of HCs and HD patients are shown in Table 6. To evaluate the relative changes in the volumes of HD patients, the mean relative volume of each brain structure in the HD cohort was divided by that of the HC cohort. The distribution of the relative volumes of the basal ganglia structures of each cohort are also represented in Figure 13. The distribution of the relative volumes of the brainstem, midbrain and ventricles of each cohort are displayed on Figure 14.

Table 6: Median (range) values of the relative caudate, putamen, GP, lateral ventricles, brainstem and midbrain volumes of healthy HCs and subjects with HD. See Figure 13 and Figure 14 for boxplots of these volumes. p-values were obtained with the Mann-Whitney U-test, which show statistically significant differences for p<0.05.

<table>
<thead>
<tr>
<th>Brain Structure</th>
<th>HCs</th>
<th>HD Subjects</th>
<th>p-value</th>
<th>Rel. Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left Caudate</td>
<td>0.22 (0.16-0.27)</td>
<td>0.15 (0.09-0.21)</td>
<td>&lt;0.001</td>
<td>-35</td>
</tr>
<tr>
<td>Right Caudate</td>
<td>0.22 (0.16-0.29)</td>
<td>0.15 (0.08-0.22)</td>
<td>&lt;0.001</td>
<td>-36</td>
</tr>
<tr>
<td>Avg. Caudate</td>
<td>0.22 (0.16-0.28)</td>
<td>0.15 (0.08-0.21)</td>
<td>&lt;0.001</td>
<td>-35</td>
</tr>
<tr>
<td>Putamen</td>
<td>0.31 (0.27-0.42)</td>
<td>0.19 (0.14-0.42)</td>
<td>&lt;0.005</td>
<td>-36</td>
</tr>
<tr>
<td>GP</td>
<td>0.12 (0.09-0.18)</td>
<td>0.09 (0.07-0.15)</td>
<td>&lt;0.01</td>
<td>-28</td>
</tr>
<tr>
<td>Ventricles</td>
<td>0.66 (0.35-1.4)</td>
<td>1.6 (0.62-2.5)</td>
<td>0.02</td>
<td>+96</td>
</tr>
<tr>
<td>Brainstem</td>
<td>1.8 (1.5-2.5)</td>
<td>1.5 (1.2-2.0)</td>
<td>0.02</td>
<td>-18</td>
</tr>
<tr>
<td>Midbrain</td>
<td>0.43 (0.35-0.55)</td>
<td>0.36 (0.30-0.46)</td>
<td>0.01</td>
<td>-17</td>
</tr>
</tbody>
</table>

Figure 13: Volumes of the basal ganglia relative to the ICV of HCs and subjects with HD. Significant reductions were found in the relative volumes of caudate (*** p<0.001), putamen and GP (** p<0.01) of HD patients with the Mann Whitney U test.
Figure 14: Volumes of the brainstem, midbrain and lateral ventricles relative to the ICV of HCs and subjects with HD. Significant reductions in the relative volumes of the brainstem and midbrain and a significant increase in the relative volume of the lateral ventricles (* p<0.05) of HD patients were found with the Mann Whitney U test.

The Mann Whitney U test revealed statistically significant differences in the relative volumes of all structures when comparing HD patients to HCs. More specifically, significant reductions in the left, right and mean caudate (p < 0.001), putamen (p < 0.05), GP (p < 0.01), brainstem (p < 0.05) and midbrain (p < 0.05) volumes were detected in the HD cohort. A significant increase in the volumes of the ventricles (p < 0.05) of HD patients was also detected.

5.2.2. Correlation of Volumes with Clinical Variables

Although significant reductions were detected in the brain volumes of HD patients, a far more important question remains to be addressed, which is how these changes underlie the clinical manifestations of the disease. The core drive to study these brain alterations in HD is not to find parameters that are able to distinguish patients from healthy subjects, since diagnosis of HD may be performed with a simple genetic test, but rather to determine if these parameters correlate with the symptoms and features of the disease. To that end, the relationships between relative brain volumes and the available clinical parameters – disease duration, TMS and TFC, were assessed with the Spearman’s correlation statistical test for the HD cohort. The results from this analysis are displayed in Table 7.

The correlations of the relative volumes of the caudate and of the putamen with the clinical variables are very similar, which is not surprising since the caudate volume was strongly and significantly correlated with the volume of the putamen (r_s = 0.83 and p = 0.02 with the Spearman’s correlation test). Caudate volumes correlated significantly with both the TMS (r_s = −0.71 with p < 0.05; Figure 15) and
the TFC ($r_s = 0.82$ and $p = 0.01$; Figure 16). Putamen volumes also correlated significantly with the TMS ($r_s = -0.76$ with $p = 0.03$) and the TFC ($r_s = 0.81$ and $p = 0.02$), while the correlations of the caudate and putamen volumes with disease duration did not reach statistical significance ($r_s = -0.64$ and $p = 0.09$ for both cases). No significant correlations were found between the remaining volumes and the clinical scores ($p$-values $> 0.05$) considering the sample of eight HD patients included in this study.

Table 7: Correlations of brain volumes with duration of the disease, TMS and TFC in the HD cohort. Correlation coefficients ($r_s$) were obtained with Spearman’s rank order correlation test, which yields significant correlations for $p<0.05$.

<table>
<thead>
<tr>
<th>Structure Volume</th>
<th>Disease Duration</th>
<th>TMS</th>
<th>TFC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_s$</td>
<td>p-value</td>
<td>$r_s$</td>
</tr>
<tr>
<td>Caudate</td>
<td>-0.64</td>
<td>0.09</td>
<td>-0.71</td>
</tr>
<tr>
<td>Putamen</td>
<td>-0.64</td>
<td>0.09</td>
<td>-0.76</td>
</tr>
<tr>
<td>GP</td>
<td>-0.09</td>
<td>0.84</td>
<td>-0.48</td>
</tr>
<tr>
<td>Brainstem</td>
<td>-0.26</td>
<td>0.54</td>
<td>-0.19</td>
</tr>
<tr>
<td>Midbrain</td>
<td>-0.03</td>
<td>0.95</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Figure 15: Relative caudate volumes of HD patients plotted against their TMS. A significant correlation ($r_s = -0.71$ and $p < 0.05$) between both variables was obtained with Spearman’s correlation test.
5.3. Discussion

In the present section, significant volume reductions in the caudate (mean percent change of -35%), putamen (-36%), GP (-28%), brainstem (-18%) and midbrain (-17%), along with a volume increase in the lateral ventricles (+96%), were successfully detected in HD patients when comparing to HCs by means of a fully automated segmentation method.

FreeSurfer’s whole-brain segmentation method, or the assignment of neuroanatomical labels to each voxel in an MRI volume, makes use not only of intensity information, but also of spatial information based on the construction of a probabilistic atlas from a manually labeled training set. Furthermore, this technique employs a registration procedure robust to anatomical variability with enough sensitivity to detect alterations in volumes related to neurodegenerative diseases. Therefore, the use of this automatic segmentation method for the quantification of brain volumes was shown to avoid user-dependent errors and to generally be less time-consuming when comparing to semi-automatic and manual measurements, while yielding results comparable in accuracy to manual labelling [88]. Moreover, only a single command is required for the preprocessing and brain segmentation of T1-weighted images, meaning that virtually no a priori knowledge of brain anatomy is required for the labelling of brain structures.

Interestingly, leftward asymmetry was detected in caudate volumes of healthy subjects, meaning that in this cohort the volume of the caudate nucleus in the left hemisphere was significantly smaller than the
volume of the right-sided caudate - Table 5. In fact, striatal asymmetry of HCs has previously been reported in volumetry studies. Previous studies [90],[91] also found leftward asymmetry in the caudate volumes of HCs, while others [69],[92] have reported rightward asymmetry of this structure in HCs. Asymmetries in the striatal structures apparently do exist in the population, but the direction of the asymmetry varies by sample, thus yielding an inconsistent pattern of results in the literature. Physiological hemispheric asymmetry is often associated with several factors that cause anatomical and functional lateralization of the brain, such as motor activity, aging and genetic factors [93]. In HD patients, no significant volume asymmetries were found in any of the basal ganglia structures, in agreement with the hypothesis that HD is a symmetric disease [69].

The hallmark of HD neuropathology is striatal atrophy, which arises several years before symptomatic onset and progresses steadily over the course of the disease, due to the selective demise of MSNs. In line with this and with the volumetric results from several MRI studies in HD [45],[72],[73], significant volume reductions in the caudate and putamen were detected in HD patients in this work - Table 6; Figure 13. GP atrophy in these subjects was also significant, but in a lesser extent when comparing to atrophy in the caudate and putamen, in agreement with a previous study [33]. Although the selective neurodegeneration of HD is thought to start in the striatum, neuronal loss in GP soon follows [75]. The caudate, putamen and GP are connected as part of the corticostriatal pathway and these structures are cardinal in the regulation of voluntary and involuntary movement. The degeneration of MSNs directly affects the basal ganglia, resulting in an imbalance between the direct and indirect circuits of the corticostriatal pathway, which regulates movement. Since the caudate, putamen and GP are part of both circuits, degeneration of these structures is suggested to play a role in the movement abnormalities seen in HD [32]. In fact, even with the small sample size of HD patients in this study (n=8), the caudate and putamen volumes correlated strongly and significantly with the TMS and the TFC - Table 7, in accordance to previous studies [33],[72]. The UHDRS-TMS assesses multiple domains of motor disability in HD, such as abnormal eye movements, hand coordination, rigidity and chorea. The higher this score, the more affected is the motor function of the HD patient. Thus, a negative correlation of TMS with caudate ($r_S = -0.71$) and putamen ($r_S = -0.76$) volumes indicates that degeneration this basal ganglia structure underlies motor dysfunction. Additionally, a correlation of the volumes of these structures with TFC ($r_S = -0.82$ for the caudate and $r_S = -0.81$ for the putamen) also implicates the atrophy of the striatum in the loss of functional capacity of HD patients. These results corroborate the centrality of striatal atrophy in HD pathology. However, the sample size of HD patients in this study did not suffice for statistical significance of the correlation of disease stage with caudate and putamen volumes ($p>0.05$ with the Spearman’s correlation), although a strong correlation coefficient was obtained between the parameters ($r_S = -0.64$ for the caudate and putamen). Striatal atrophy progresses steadily over the course of the disease [96], thus a correlation between these parameters was to be expected, as reported in [71]. One possible confound factor in this analysis was the presence of a patient with the Westphal variant of HD. In this juvenile-onset form of HD, motor, cognitive and functional debilitating occur at a faster rate than in adult-onset HD, and thus the disease course is shortened and more severe [97]. Therefore, this patient exhibited the smallest relative volume of all the HD cohort for a disease duration of 10 years while other patients with adult-onset HD had larger volumes even after a
longer disease duration. Additionally, the volumes of the lateral ventricles were significantly increased in HD patients, as a result of the loss of brain mass (a condition named *hydrocephalus ex vacuo*) in HD, which is consistent with other studies [33],[94].

Significant brainstem volume reduction in HD patients was detected in this study - Table 6; Figure 14, in accordance with the results from previous works [33],[94]. Midbrain volume loss in HD patients was also significant. Brainstem degeneration in HD is not among the established features of this disease, unlike basal ganglia atrophy. Indeed, Rüb et al. [95] have found evidence that brainstem atrophy develops independently from the well-documented striatal degeneration, suggesting that HD is not restricted to neurodegeneration in the striatum, cortex and corticostriatal pathway, but is rather a multisystem neurodegenerative disorder. Brainstem degeneration in HD is thought to play a role in the manifestation of abnormal extraocular eye movements and sleep disturbances in these patients [33].

GP volumes presented only weak and non-significant correlations with the clinical scores. These results are inconsistent with a study of sixteen HD patients that found a significant correlation between GP volume and TFC [33], and with other works which revealed a significant correlation between GP volume and TMS even in premanifest patients [98],[99]. Although statistical significance of such correlations with GP volume might be reached with a larger number of HD patients, the sample size of this study did suffice for significant correlations in the case of the striatum, which suggests that striatal atrophy is more strongly associated with motor and functional disabilities than GP volume in this HD cohort. Additionally, brainstem and midbrain volumes did not correlate with the TMS and TFC scores, indicating that atrophy of these structures is not significantly involved in the deterioration of motor and functional capacities. These results do not agree with the significant correlation reported by Rosas et al. between brainstem volume and the TFC in HD patients [33]. Finally, no significant correlations were found between disease stage and GP, brainstem and midbrain volumes. No studies assessing such correlations were found in the literature. These results suggest that, longitudinally, the volumes of the GP, brainstem and midbrain decrease at a much lower rate than striatal volumes during the course of the disease.
6. NM-MRI Analysis

6.1. Methods

6.1.1. Imaging Protocol

The sequence parameters of the NM-MRI protocol performed in this study are summarized in Table 8, and were similar to those described by Sasaki et al. [17]. The sections were set in the oblique axial plane perpendicular to the fourth ventricle floor with coverage from the posterior commissure to the inferior border of the pons, as displayed in Figure 17.

Table 8: NM-MRI protocol and sequence parameters used in this study. ETL: Echo Train Length, FOV: Field of View.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>TR (ms)</th>
<th>TE (ms)</th>
<th>ETL</th>
<th>Sl. Thickness (mm)</th>
<th>Pix. Size (mm²)</th>
<th>FOV (mm²)</th>
<th>Num. Slices</th>
<th>Acq. Time (m:s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₁ FSE</td>
<td>607</td>
<td>10</td>
<td>3</td>
<td>2.5</td>
<td>0.40x0.40</td>
<td>220x190</td>
<td>20</td>
<td>08:20</td>
</tr>
</tbody>
</table>

Figure 17: Slice orientation in the NM-MRI sequence used in this work, critical to properly identify the SNc signal. Sections were set in the axial plane perpendicular to the fourth ventricle floor with coverage from the posterior commissure to the inferior border of the pons. Adapted from [100].

6.1.2. Image Filtering

The analysis of neuromelanin-sensitive images was conducted using the Lite version 9.5 of the OsiriX software (Pixmeo, Geneva, Switzerland) [101], an application for the MacOS X operating system.
For each subject, from the 20 axial slices obtained with the NM-MRI imaging protocol, only three exhibited the NM hyperintense signal corresponding to the SNc, exemplified in Figure 18. The middle slice, containing the largest area of high-signal SN, was selected for analysis.

Figure 18: Sections of the axial slices obtained with NM-MRI at the levels of lower midbrain (a), centre midbrain (b) and upper midbrain (c) of a healthy subject, displaying the SNc as an hyperintense band-like area (pointed by the white arrows in each slice). The middle slice (b) was chosen for SNc measurements.

A convolution filter was applied to the image to compensate for the typical low signal-to-noise ratio of neuromelanin-sensitive images, and thus to allow for the segmentation of the SNc areas. From the wide range of filters offered by OsiriX, the Gaussian blur filter yielded the best results in segmentation of SNc areas in a previous work [102] that compared the relative error and the Dice Similarity Coefficient (DSC) between semi-automatic and manual segmentations of the SNc after applying several different filters to the images. Therefore, the Gaussian blur convolution filter was chosen for this work. The application of an image-blurring filter reduces both image noise and detail, as shown in Figure 19, when using a Gaussian kernel. During image convolution, the new value of each pixel is set to a weighted average of that pixel’s neighborhood. The original pixel receives the heaviest weight, and neighboring pixels receive lighter weights as their distance to the original pixel increases according to a normal distribution. The Gaussian blur filter used in this work had a 5 x 5 pixels of kernel dimension and a full width at half maximum (FWHM) of 2 pixels [103].

6.1.3. Segmentation Methods

The Confidence segmentation algorithm, available in the OsiriX segmentation tool, was chosen for this study, as it was proven to be the most efficient method for the segmentation of high-signal SN areas in [102]. This region-growing algorithm starts from the selection of a seed point, which consists of a single pixel included in the ROI to be segmented. Then, the algorithm progressively appends neighboring pixels to the current region if their intensities $I(X)$ are included in a confidence interval iteratively updated by the statistics of the current region [104]:

$$I(X) \in [m - f \sigma, m + f \sigma]$$

(4)

where $m$ and $\sigma$ are, respectively, the mean and standard deviation of the intensities for all pixels in the current region, and $f$ is a multiplicator factor defined by the user.
Figure 19: Axial slice at the level of centre midbrain of a healthy subject before (a) and after (b) the application of the Gaussian blur filter, showing a clear blur effect of the applied filter.

The iteration stops when the algorithm visits all pixels neighboring the current region. Then, $m$ and $\sigma$ of the new ROI are calculated, and another iteration begins. In this way, the Confidence algorithm does not require predefined intensity limits for the segmentation. Besides seed point placement, the algorithm requires three input parameters: the multiplier factor, the number of iterations and the so-called initial radius (in pixels). The initial radius defines the number of pixels neighboring the seed point that the algorithm will visit in its first iteration. The same work [102] focused on optimizing the values of these parameters for the optimal segmentation of SNc areas using the same NM-MRI TSE sequence. Accordingly, the values of the multiplicator factor, the number of iterations and of the initial radius used in this study were set to 2, 2 and 1, respectively, in the OsiriX segmentation tool.

To begin the 2D segmentation of SN areas, a user-defined seed pixel is chosen. Despite image filtering, it was observed that variations on the seed point placement still lead to different segmentation results. In order to reduce the effect of such variability, the same method of seed point selection was applied to every image, which consisted of drawing a line crossing the interpeduncular fossa as shown in Figure 20 (a) and selecting a hyperintense SNc pixel along such line. This procedure was previously used in [102] and in [103].

After the application of the algorithm on the selected seed point of the filtered image, SNc segmented regions usually presented small gaps of pixels within the ROI, as evidenced in Figure 20 (b). To avoid anatomically implausible discontinuities in the SNc, a Brush Closing filter was applied whenever required, resulting in continuous ROIs, as displayed in Figure 20 (c). The corrected SNc segmented areas were finally saved in OsiriX, along with their mean intensity and area values. For each subject’s single-slice filtered image, the semi-automatic segmentation procedure was executed once for each side of the brain, adding to a total of 40 SN areas.
Figure 20: Semi-automatic segmentation of the SNc using the Confidence algorithm after image filtering with a Gaussian blur filter. Firstly, a line is drawn crossing the interpeduncular fossa (a). Then, a hyperintense pixel along the line is chosen and the Confidence algorithm generates the ROI accordingly (b). At last, the brush closing filter is applied to ensure that the selected SNc area is completely filled (c). Adapted from [103].

Although this semi-automatic segmentation method was proven to be accurate for PD patients in [102] and in [103] comparing to manual segmentations, its accuracy was never tested in HD patients. Furthermore, in the course of this work, there still was variability of the segmented area values associated with seed point selection, despite image filtering. Therefore, manual segmentation of the SNc was also performed in this study to confirm the accuracy of the semi-automatic segmentation method when applied to this patient group. Manual segmentation was done for each SNc in a different day from the corresponding semi-automatic segmentation to prevent biasing. SNc manual delineation was performed with a tool in OsiriX that allows the user to freely draw over the image. A manual SNc segmentation is exemplified in Figure 21, along with a semi-automatic segmentation of the same structure of the same subject, for comparison. The manual SNc ROIs were confirmed by a certified neuroradiologist (Dr. Sofia Reimão) and these segmentations were considered as the ground truth for semi-automatic segmentation validation. Additionally, the midbrain was manually segmented so as to obtain the global midbrain area, as exemplified in Figure 22. The ratio of SNc area to midbrain area was calculated for each side of the brain as $\frac{SN_{area}}{M_{area}}$, where $SN_{area}$ is the SNc area and $M_{area}$ is the
midbrain area. This parameter, hereafter termed SNc ratio, allowed for a comparison of SNc areas while taking into account anatomical variability.

![Figure 21](image1.png)

Figure 21: Examples of a manual segmentation (a) and of a semi-automatic segmentation (b) of the right-sided SNc region of the same healthy subject performed on a non-filtered NM-MR image using the OsiriX software.

![Figure 22](image2.png)

Figure 22: Example of a manual segmentation of the midbrain in a healthy subject, performed on a non-filtered NM-MR image using the OsiriX software.

### 6.1.4. Similarity Analysis

In order to validate the semi-automatic image segmentation method, it was necessary to compare each resulting high-intensity area with the corresponding manually defined SNc area, not only regarding their values, but also their similarity. The evaluation of the accuracy of semi-automatic image segmentation was carried out using the Dice Similarity Coefficient (DSC) [105], which quantitatively measures the spatial overlap between the two segmentations:
\[
DSC = 2 \frac{|SN_{sa} \cap SN_m|}{|SN_{sa}| + |SN_m|}
\]

where \(SN_{sa}\) represents the SNc area obtained using the semi-automatic segmentation and \(SN_m\) is the SNc area manually delineated. The DSC ranges from 0, when there is no intersection between the areas, up to 1, when there is total overlap between the two segmentations.

Images containing the SN areas were binarized, so that the intensity value of one is assigned to every pixel comprised in the ROI, while the intensity of remaining pixels is set to zero. Binarized images were exported from OsirIX and the DSC values for the 40 pairs of SN areas were determined using MATLAB (version 9.1 R2016b; The Mathworks, Natick, MA, USA).

### 6.1.5. Image Contrast Analysis

Another method frequently used to estimate NM content in the SNc is to determine the contrast ratio (CR) between the hyperintense SNc area and a background reference ROI. This measurement estimates the spatial distribution of neuromelanin in the SN. In this work, circular ROIs of 8 mm² were placed bilaterally in the crus cerebri as references, as shown in Figure 23.

![Figure 23: Method used to calculate the CRs of SNc areas in non-filtered NM-MR images. For each side, an 8 mm² circular ROI (in yellow) was placed in the central region of the crus cerebri. The CR is determined by dividing the mean intensity of the pixels contained in each SNc area (in green) by the mean intensity of the pixels included in the corresponding reference ROI.](image)

The right and left CRs of each subject, \(CR_r\) and \(CR_l\) respectively, were calculated as:

\[
CR_r = \frac{I_{SN_r}}{I_{CC_r}}
\]

\[
CR_l = \frac{I_{SN_l}}{I_{CC_l}}
\]

where \(I_{SN_r}\) and \(I_{SN_l}\) are, respectively, the mean intensities of all the pixels contained in the right and left SNc areas, and \(I_{CC_r}\) and \(I_{CC_l}\) are the mean intensities of pixels included the left and right reference ROIs.
The CR analysis was performed in the same slices where SNc areas were measured, with no filter applied to the image.

### 6.1.6. Statistical Analysis

A p-value lower than 0.05 was considered significant, as in previous statistical tests. The statistical analyses of the comparison of SNc areas, SNc ratios and CRs were performed with non-parametric statistical tests. Pair-wise comparisons between semi-automated and manually segmented SN areas, between the left and right-side areas and SN ratios, and between the left and right CRs were carried out with the Wilcoxon signed rank test for paired samples. Regarding left and right-sided parameters, whenever no significant difference was found between both values, the average value of the parameter was henceforth used. Inter-group comparisons of SNc areas, SNc ratios and CRs between the HC and HD patient cohorts were performed with Mann-Whitney U test. Finally, the Spearman rank-order correlation was used to investigate correlations of the SNc areas and ratios in HD patients with the disease duration, TMS and TFC score, and with the relative basal ganglia volumes obtained in 5.2.1.

### 6.2. Results

#### 6.2.1. SNc Area and Ratio Analysis

The values of SNc area obtained for each side of the brain for each cohort according to the segmentation method are displayed on Table 9. Considering the healthy subjects, no significant differences were revealed between their left and right-sided SNc areas, obtained with either semi-automatic ($p = 0.08$) or manual ($p = 0.97$) segmentation methods. Regarding HD patients, no significant side-wise differences were found in their SNc area values as well, delineated using the semi-automatic ($p = 0.20$) or manual ($p = 1.0$) segmentations.

Table 9: Median (range) area values of the SNc in each side of the brain of HCs and of subjects with HD, obtained with the semi-automatic and manual segmentation methods. p-values obtained using the Wilcoxon signed rank statistical test for paired samples, with statistically significant differences for p<0.05.

<table>
<thead>
<tr>
<th>Segmentation Method</th>
<th>HCs</th>
<th>HD Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Semi-Automatic</td>
<td>36 (23-55)</td>
<td>34 (20-48)</td>
</tr>
<tr>
<td>Manual</td>
<td>35 (25-43)</td>
<td>34 (22-48)</td>
</tr>
</tbody>
</table>

For all subjects, the DSC obtained between the semi-automatic and manual SNc segmentations was 0.78 [0.59 – 0.88] (median; range), which reveals a very good spatial overlap between the areas. Furthermore, no statistically significant difference was found when comparing the semi-automatically and manually segmented SNc area values ($p = 0.83$) using the Wilcoxon signed rank test. Given the
excellent agreement between semi-automatic and manual SNC areas revealed by the similarity analysis and by the comparison of area values, only the SNC areas obtained with the semi-automated segmentation method were henceforth considered. Moreover, no side-wise differences were found between the SNC areas in any case. Therefore, it was considered feasible to study the average SNC areas obtained using the semi-automated segmentation method from this point on.

The SNC and midbrain areas are represented in Table 10, along with the SNC ratio, for each cohort of subjects. Significant differences between HCs and subjects with HD were detected in all parameters, resorting to the Mann Whitney U test. More specifically, there were significant reductions in the SNC areas ($p < 0.005$; Figure 24), in the midbrain areas ($p < 0.01$) and in the SNC ratios ($p < 0.01$; Figure 25) of subjects with HD comparing to HCs.

Table 10: Median (range) values of the SNC areas, midbrain areas and SNC ratios of HCs and subjects with HD. p-values were obtained with the Mann-Whitney U-test, which show statistically significant differences for $p<0.05$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HCs</th>
<th>HD Subjects</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNC Area (Range) (mm$^2$)</td>
<td>35 (22-50)</td>
<td>25 (17-36)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MB Area (Range) (cm$^2$)</td>
<td>6.6 (5.6-7.6)</td>
<td>5.7 (5.0-7.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SNC Ratio (Range) (%)</td>
<td>5.2 (3.5-7.4)</td>
<td>4.4 (3.1-5.1)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 24: SNC areas of HCs and of patients with HD. A significant reduction (**) p-value<0.01 was found in HD patients with the Mann Whitney U test.
Figure 25: SNC ratios of HCs and of HD patients. A significant reduction (** p-value<0.01) was found in HD patients with the Mann Whitney U test.

The Spearman’s rank order statistical test was performed to investigate the relationship between SN and midbrain areas in each subject. A strong significant correlation ($r = 0.77, p < 0.0001$; Figure 26) was found between the two parameters.

Figure 26: SNC areas plotted against the midbrain areas of the 20 subjects included in this study. A statistically significant strong positive correlation between both variables was found resorting to the Spearman’s correlation test ($r = 0.77$ and $p < 0.00001$).
6.2.2. Contrast Ratio Analysis

The CR values in the left and right SNc areas of HCs and HD patients are displayed on Table 11. No significant asymmetry in the CR values were found either in HCs ($p = 0.20$) or in subjects with HD ($p = 0.74$) with the Wilcoxon signed rank test for paired samples. Therefore, the average CR values were analyzed for each cohort.

Table 11: Median (range) CR values measured in the SNc areas for each side of the brain of HCs and of subjects with HD. p-values were obtained using the Wilcoxon signed rank statistical test for paired samples, with statistically significant differences for $p<0.05$.

<table>
<thead>
<tr>
<th>SN Area</th>
<th>HCs</th>
<th>p-value</th>
<th>HD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>13 (10-17)</td>
<td>0.20</td>
<td>13 (9-18)</td>
<td>0.74</td>
</tr>
<tr>
<td>Right</td>
<td>15 (10-18)</td>
<td></td>
<td>12 (7-21)</td>
<td></td>
</tr>
</tbody>
</table>

Regarding HCs, a SNc CR value of 14% (11%-17%) [median; range] was obtained, while for the HD cohort, a value of 13% (8%-16%) was found. The average CR values did not allow, however, to significantly distinguish controls from patients, as the p-value obtained with the inter-group Mann Whitney U-test was of 0.5714. Nevertheless, Figure 27, which shows the CR values for the two cohorts, reveals that the CR values of the SN segmented areas of HD patients tended to be smaller than those of HCs.

Figure 27: CR values of the SNc areas in HCs and HD patients. No statistically significant difference was found with the Mann Whitney U-test. However, HD patients tended to have smaller CR values.
6.2.3. Correlation of SNc Areas and Ratios with Clinical Variables

The Spearman’s correlation test was used to evaluate the relationship between the SNc areas and SNc ratios with disease duration, TMS and TFC of HD patients. The Spearman’s correlation coefficients ($r_S$) and respective p-values are displayed in Table 12. Firstly, correlation with the clinical variables was stronger for the values of SNc areas than for SNc ratios in all cases, as the correlation coefficients obtained for the latter were consistently smaller. Adding to this, there was no statistical significance in the correlation of SNc ratios to disease duration ($p = 0.56$), TMS ($p = 0.29$) and TFC ($p = 0.12$).

Table 12: Correlations of the SNc areas and of the SNc ratios with disease duration, TMS and TFC in the HD cohort. The correlation coefficients ($r_S$) were obtained with Spearman’s rank order correlation test, with correlations considered to be significant for $p<0.05$.

<table>
<thead>
<tr>
<th>Clinical Variables</th>
<th>$r_S$</th>
<th>p-value</th>
<th>$r_S$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease Duration</td>
<td>- 0.60</td>
<td>0.12</td>
<td>- 0.25</td>
<td>0.56</td>
</tr>
<tr>
<td>TMS</td>
<td>- 0.67</td>
<td>0.07</td>
<td>- 0.43</td>
<td>0.29</td>
</tr>
<tr>
<td>TFC</td>
<td>+ 0.79</td>
<td><strong>0.02</strong></td>
<td>+ 0.60</td>
<td>0.12</td>
</tr>
</tbody>
</table>

There were considerable correlations of the values of SN areas with disease duration ($r = -0.60$) and TMS ($r = -0.67$), but these correlations did not reach statistical significance ($p = 0.12$ and $p = 0.07$, respectively). On the other hand, a statistically significant, strong positive correlation between the SNc segmented areas and the TFC score of subjects with HD was found ($r = 0.79$, $p = 0.02$; Figure 28).

![Figure 28](image)

$r_S = 0.79$

$p = 0.02$

Figure 28: SNc areas of HD patients plotted against their TFC score. A statistically significant strong positive correlation between both variables was found resorting to Spearman’s correlation test ($r = 0.79$ and $p = 0.02$).
6.2.4. Correlation of SNc Ratio with Basal Ganglia Volumes

The Spearman's correlation test was used to evaluate the correlations between SNc ratios and the relative volumes of the basal ganglia structures of all subjects, in order to assess if there was an association between depletion of NM-containing neurons of the SNc and volume atrophy of the caudate, putamen or GP in this cohort. The results from this analysis are displayed on Table 13.

Table 13: Correlations of SNc ratios with the relative volumes of the basal ganglia structures considering all subjects (n=20). Correlation coefficients ($r_s$) were obtained with Spearman's correlation test, with correlations considered to be significant for $p<0.05$.

<table>
<thead>
<tr>
<th>Brain Structure</th>
<th>$r_s$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudate</td>
<td>0.58</td>
<td>0.009</td>
</tr>
<tr>
<td>Putamen</td>
<td>0.66</td>
<td>0.002</td>
</tr>
<tr>
<td>GP</td>
<td>0.67</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Statistically significant correlations were detected between all basal ganglia relative volumes and the SNc ratios. More specifically, a correlation with the SNc ratio of 0.58 ($p = 0.009$) was found for the caudate - Figure 29, a correlation of 0.66 ($p = 0.002$) for the putamen - Figure 30, and a correlation of 0.67 ($p = 0.002$) was obtained for the GP - Figure 31.

Figure 29: SNc ratios of all subjects plotted against their relative caudate volumes. A statistically significant positive correlation between both variables was found with the Spearman's correlation test ($r = 0.58$ and $p = 0.009$).
Figure 30: SNc ratios of all subjects plotted against their relative putamen volumes. A statistically significant positive correlation between both variables was found with the Spearman’s correlation test ($r = 0.66$ and $p = 0.002$).

Figure 31: SNc ratios of all subjects plotted against their relative GP volumes. A statistically significant positive correlation between both variables was found with the Spearman’s correlation test ($r = 0.67$ and $p = 0.0015$).
6.3. Discussion

In this section, significant reductions in SNC areas and in SNC ratios of HD patients comparing to controls were, for the first time, successfully detected by means of a semi-automatic segmentation method in NM-MRI. Such outcomes are consistent with the results obtained in a previous in vitro study [85], which reported a significant decrease in the number of NM-containing neurons and a reduction in cell body and nuclear areas of pigmented neurons in HD patients comparing to HCs. Furthermore, the reduction of SNCs in HD patients of this study correlated significantly with the relative volumes of all the basal ganglia structures.

SNC segmentation performed with the Confidence region-growing algorithm proved to be accurate when compared to manual segmentation, given the high DSC (0.78) and the similarity of area values between both segmentations. The only operator-dependent step required by the semi-automatic segmentation was the seed point selection, which involved drawing a straight line crossing the interpeduncular fossa and selecting a hyperintense pixel belonging to the SNC along such line. SNC segmentation in NM-MRI using this seed point selection procedure was shown to be reproducible in a previous work [103], which revealed excellent agreement between three repeated measurements of the each SNC hyperintense area obtained with the Confidence algorithm. For these reasons, the semi-automatic segmentation method used in this work was less user-dependent and reduced the time required for image analysis while yielding accurate segmentations of the SNCs as compared to the manual segmentation technique.

The volumetry results obtained in the previous section (5.2.1) showed a significant reduction of volume in the midbrain of HD patients with respect to HCs. Furthermore, manually segmented midbrain areas, measured in the same slices where SNC segmentation was performed, were significantly smaller in HD patients than in HCs (Table 10). Hence, it may not come as a surprise that the area of SNC is depleted in HD patients as a consequence of midbrain atrophy. In order to evaluate the impact of the midbrain atrophy in NM measurements, the ratio of NM-containing SNC hyperintense area to global midbrain area was calculated for each group of subjects. This ratio was significantly reduced in HD patients when comparing to HCs, suggesting that although midbrain atrophy plays a role in the reduction of SNC areas (given the significant strong correlation between SNC and midbrain areas - Figure 26), it does not solely account for the observed depletion of NM-containing neurons in the SNC of HD patients. These results hint that there may be alternative pathological mechanisms underlying neuronal degeneration in the SNC, other than midbrain atrophy.

While a reduction of SNC area in HD patients represents dopaminergic neuronal loss and thus NM depletion in this structure, other processes may be involved in the pigment loss in these patients. Neurons that survive SNC atrophy in HD might still present reduced content of NM. In this case, the remaining SNC areas of HD patients could exhibit a lower contrast when compared to those of HCs. As such, the mean intensities of the bilateral SNC areas were normalized with the intensities of reference regions placed in the bilateral crus cerebri in the midbrain of each subject, yielding the CR parameter. This normalization procedure was performed to account for intra and inter-subject intensity variabilities, characteristic of FSE NM-MRI images. NM-MRI hyperintense signal areas have been shown to correlate
with the local density of NM-containing neurons [57], and thus the CR was used to quantify the
distribution of this pigment in the segmented SNc of HD and healthy subjects. Results obtained in this
study revealed no significant differences in the CR values of the SNc between the HD and HC groups -
Figure 27. Nevertheless, SNc CR values of HD patients tended to be smaller than those of HCs. One
of the possible factors affecting this analysis is the choice of reference ROI. The central regions of the
bilateral crus cerebri were selected for the placement of two circular ROIs as in [77] and [102], while in
other studies a single and larger reference ROI was placed in the decussation of the superior cerebellar
peduncles [17],[78],[83]. Nonetheless, studies with larger sample sizes of HD patients may be required
for the differences in CRs between groups to reach statistical significance.

The significant correlations found between SNc ratio and the relative volumes of the caudate ($r_s = 0.58$
- Figure 29), putamen ($r_s = 0.66$ - Figure 30) and GP ($r_s = 0.67$ - Figure 31) were key outcomes of this
analysis. These results suggest that a reduction in SNc area, and thus a depletion of NM-containing
neurons, is somehow related to basal ganglia atrophy in HD patients, which is a hallmark of the disease.
However, it is not clear if this NM depletion in SNc would be a primary or secondary event in HD, nor if
these changes would precede basal ganglia atrophy or result from such process. The striatum is a
central component in the corticostriatal pathway, as it connects with several other subcortical and cortical
regions with the main purpose of regulating voluntary and involuntary movement. The striatum receives
neuronal input from the cortex, thalamus and from the SNc. Loss of glutamatergic CPNs, which project
from the cortex to the striatum, is known to occur in HD from early on, even preceded by a dysfunction
in the pattern of communication between these neurons and MSNs of the striatum [4]. On the other
hand, dopaminergic projections from the SNc to the striatum play a modulatory role in the corticostriatal
pathway, ultimately facilitating voluntary movement. Hence, a dysregulation in CPNs or dopaminergic
projections of this pathway could result in uncontrolled involuntary movements or bradykinesia [32].
Indeed, in PD, selective degeneration of NM-containing nigrostriatal neurons, which is the hallmark
of this disorder, leads to abnormalities in the dopamine metabolism, manifesting as bradykinesia, tremor
and rigidity [106]. While the neuropathology and symptoms of HD differ from those of PD, both disorders
arise from the accumulation of misfolded protein aggregates, suggesting at least some degree of
similarity between underlying pathological processes, perhaps involving SNc degeneration and NM loss.

Correlations between both SNc areas and SNc ratios with the available clinical parameters – disease
duration, UHDRS TMS and TFC – were assessed to investigate if any of the neuroimaging parameters
(partly) explained the disease staging, motor or functional capacities of HD patients - Table 12. However,
the small number of patients in this study did not allow for statistical significance to be reached for the
correlations between the SNc ratios and the three clinical variables considered. Regarding the
correlations of SNc areas with disease duration ($r_s = -0.60$) and TMS ($r_s = -0.67$), only a trend towards
significance was observed in both cases. On the other hand, despite the small sample size, a significant
positive correlation ($r_s = 0.79$) was found between SNc areas and TFC in HD patients - Figure 28. This
result suggests that depletion of dopaminergic NM-containing neurons in the SNc of HD patients might
be one of the factors responsible for the decrease in functional capacity of these subjects. Nevertheless,
it must be taken into account that in this cohort of eight HD patients, TFC values (ranging from 0 to 13)
were quite discrepant, as four of these subjects were assigned a score of 13 (indicating the highest functional capacity in this scale), while two patients were scored with a 1 (meaning severe loss of functional capacity). As such, only the two remaining subjects had TFC scores in between. Therefore, while there is a clear correlation between TFC and SNc area values in this cohort, testing the relationship between these two variables in a larger cohort of HD patients with more uniform TFC scores is needed to confirm the results obtained in this study.

A 2D FSE sequence similar to that of Sasaki et al. was used in this study to obtain NM-MRI images. This sequence most likely relies on incidental MT effects to generate contrast associated with the multi-slice acquisition, as each refocusing pulse induces off-resonance saturation to other slices, leading to background signal suppression [80]. The MT effect in the SNc is reduced. Consequently, this structure displays in NM-MRI images as an hyperintense area. Although the contrast generated by MT effects in FSE NM-MRI is enough to distinguish the SNc from the surrounding tissues, its source is still considered to be incidental. This means that MT effects are not controllable in FSE and thus these sequences may not be optimal for generating NM-sensitive contrast. MT contrast can also be produced by applying an off-resonance RF pulse (with a frequency several kilohertz shifted from the free water frequency) to selectively saturate the bound protons, inducing the MT effect. However, prepared MT pulses further increase the specific absorption rate (SAR), and thus FSE sequences with MT pulses may not always be feasible due to SAR constraints. Chen et al. [80] have used GE sequences prepared with an explicit MT pulse, which were more sensitive in imaging the SNc than TSE sequences, while simultaneously inducing a lower SAR. However, the image protocol used by this group included only 9 slices. Nevertheless, MT-prepared GE sequences are likely to be considerably faster than FSE sequences, leading to an overall reduction in acquisition time. While the 2D FSE sequence used in this work allowed the measurement of significant differences in the single-slice SNc areas between HD patients and HCs, a more complete approach would be to evaluate high-signal SNc volumes and to calculate their CR three-dimensionally in NM-MR images. While the MT contrast of NM-containing structures is lower using 3D GE than in 2D FSE [80], MT pulses might be included in 3D GE sequences to enhance this contrast. This was performed by Ogisu et al. [78], who managed to use a NM-MRI 3D GE sequence with preparation MT pulses to distinguish PD patients from HCs based on a significant difference in SNc volumes between both cohorts.

Although the slice thickness of the NM-MRI images obtained with the 2D FSE sequence did not allow for the measurement of SNc volume, these images still presented satisfactory contrast, and the analysis methodology followed in this work resulted in significant differences in the relative SNc areas of HD patients when comparing to controls, and in significant correlations between these SNc ratios and the relative volumes of the three basal ganglia structures.
7. \( R_{2}^{*} \) Relaxometry Analysis

7.1. Methods

7.1.1. Imaging Protocol

The \( T_{2}^{*} \) relaxation data were acquired with a mFFE sequence, with the parameters summarized in Table 14. Seven \( T_{2}^{*} \)-weighted image volumes were acquired with equally spaced TEs, with a spacing of 4.7 ms, starting from 13.8 ms, as exemplified in Figure 32. Images were acquired in an axial orientation parallel to the commissures line and covering the whole brain. A partial Fourier of 80% was used.

Table 14: \( T_{2}^{*} \) multi-gradient echo sequence parameters used in this study. APS: Acquired Pixel Size, FOV: Filed of View, IS: Interslice Gap, NS: Number of Slices, ST: Slice Thickness.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>TR (ms)</th>
<th>TEs (ms)</th>
<th>ST (mm)</th>
<th>IG (mm)</th>
<th>APS (mm²)</th>
<th>FOV (mm²)</th>
<th>NS</th>
<th>Acq. Time (m:s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{2}^{*} )-mFFE</td>
<td>1405</td>
<td>13.8/18.5/23.2/27.9/32.6/37.3/42.0</td>
<td>4</td>
<td>1</td>
<td>0.90x1.12</td>
<td>240x180</td>
<td>28</td>
<td>03:48</td>
</tr>
</tbody>
</table>

Figure 32: Seven \( T_{2}^{*} \)-weighted images at TEs=14, 19, 23, 28, 33, 37 and 42 ms for the same slice of a healthy subject.
7.1.2. Data Processing

The R2* relaxometry analysis was performed on the T2* data of a sample of 11 HCs and 5 HD patients. The reasons for having smaller group sizes are that T2* mFFE data were not acquired for two HD patients, and the poor quality of the T2*-weighted images of a third patient and of a HC made their analyses unfeasible due to motion artifacts.

Processing of the T2*-weighted images was performed with FSL 6.0 (FMRIB Software Library; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/). For each subject, FSL’s brain extraction tool (BET) was used to strip the skull and neck of the first echo (TE=13.8 ms), which had the highest contrast-to-noise ratio of the T2*-weighted volume series. A resulting binarized brain mask was then applied to the volumes of the six remaining echoes. This method was observed to be more accurate in extracting the brain of the T2*-weighted images than independently applying BET to the volumes of all seven echoes. FMRIB's Automated Segmentation Tool [107] (FAST) was then applied to each skull-stripped volume to correct for bias field. Afterwards, the resulting volumes were merged into 4D image files, which contained the seven volumes of the pre-processed echoes.

For each subject, this 4D image was used to perform a voxel-wise nonlinear least squares fitting of the data acquired at the seven TEs with MATLAB, in order to obtain a monoexponential signal decay curve according to the T2* relaxation:

\[ S(TE) = S_0 e^{-\frac{TE}{T_2^*}} \]  

(8)

where \( S \) is the measured signal intensity for each echo, \( S_0 \) reflects the proton density and the scanner receiver gain, TE is the echo time and T2* is the apparent relaxation time. More specifically, the signal intensities measured in each voxel in all the seven T2*-weighted volume series were fitted with the exponential function in eq. (8), as exemplified in Figure 33, so as to obtain the T2* value for that voxel. Ultimately, an image containing a T2* value for each voxel, or a T2* map, was obtained for each subject.

Previous studies assessing the iron content of brains structures through relaxation methods have used the R2* relaxation rate rather than T2*. Therefore, to facilitate the comparison with the results of such studies, the R2* was also the metric used in this work to analyse iron content in brain structures. R2* maps were thus computed for each subject from the previously obtained T2* maps.

In parallel, using the boundary-based registration of the epi_reg function in FSL, the pre-processed first echo was registered to the high-resolution skull-stripped T1-weighted volume previously generated by FreeSurfer, so as to obtain the registration matrix for the T2*-to-T1 transformation. Once again, the first echo was chosen for this procedure since it had the highest contrast-to-noise ratio of the T2*-weighted volume series. Subsequently, the segmentation masks of the basal ganglia formerly obtained with FreeSurfer were transformed to the space of the first echo of the T2*-weighted volume series using FMRIB's Linear Image Registration Tool [108] (FLIRT) with the inverse of the T2*-to-T1 transformation matrix, calculated in the previous step.
Figure 33: Example of a monoexponential signal decay curve fitted to the data corresponding to one voxel. The red circles correspond to the measured signal values in arbitrary units for each of the seven TEs. The dashed line represents the exponential decay as a function of TE, obtained after nonlinear fitting of the measured data in this voxel ($T_2^* = 39$ ms, $R_2^* = 26$ s$^{-1}$).

Afterwards, the basal ganglia masks in the $T_2^*$-space were binarized and multiplied with the total $R_2^*$ maps, yielding regional $R_2^*$ maps of the caudate, putamen and GP of each subject. Since the caudate is adjacent to the CSF in the lateral ventricles, some voxels included in the caudate mask might have overlapped with the CSF, which has much lower $R_2^*$ values. Therefore, voxels belonging to this tissue were removed from the caudate masks in order to avoid underestimation of $R_2^*$ values in this structure. Lastly, the mean values of $R_2^*$ in the putamen, GP and restricted caudate masks were calculated.

### 7.1.3. Statistical Analysis

The Wilcoxon signed rank test for paired samples was applied to assess differences in the mean $R_2^*$ values of the basal ganglia in each brain hemisphere for HCs and subjects with HD. The Mann Whitney U test was then used to investigate differences in the mean $R_2^*$ values of the basal ganglia between HC and HD patients. Finally, the Spearman’s correlation statistical test was used to assess relationships between iron deposition on the basal ganglia with the relative volumes of this structures and with the clinical variables.
7.2. Results

7.2.1. Iron Quantification in the Basal Ganglia

Figures Figure 34 and Figure 35 show examples of the total and regional R$_2^*$ maps in two slices of a HD patient and of a HC, respectively. Hyperintensities can be readily observed in the regions corresponding to the caudate, putamen and GP structures in the R$_2^*$ maps of both subjects (Figure 34 (a) and (b); Figure 35 (a) and (b)). These observations suggest that there is substantial iron content in the basal ganglia structures both in healthy subjects and in patients with HD.

Figure 34: Total and basal ganglia R$_2^*$ maps in two adjacent axial slices of a late-stage HD subject. (a), (b): R$_2^*$ maps displaying a clear hyperintense signal in the caudate, putamen and GP, which indicates high R$_2^*$ values and significant iron content in the basal ganglia of this patient. (c), (d): Regional R$_2^*$ maps of the caudate (coloured in red), putamen (in blue) and GP (in green) overlapped with the total R$_2^*$ map in each slice of the HD patient.
Figure 35: Total and basal ganglia $R_2^*$ maps in two adjacent axial slices of a HC. (a), (b): $R_2^*$ maps displaying a clear hyperintense signal in the caudate, putamen and GP, which indicates high $R_2^*$ values and significant iron content in the basal ganglia of this HC. (c), (d): Regional $R_2^*$ maps of the caudate (coloured in red), putamen (in blue) and GP (in green) overlapped with the total $R_2^*$ map in each slice of the HC.

The $R_2^*$ values of each basal ganglia structure for each hemisphere of HCs and HD patients are displayed in Table 15. No statistically significant differences were found between the $R_2^*$ values of the left and right structures in each cohort, using the Wilcoxon signed rank test ($p$-values $> 0.05$), except in the case of the caudate nucleus of HCs. The statistical analysis revealed that the $R_2^*$ values of the left-sided caudate were significantly larger ($p < 0.01$) than those of the right caudate in this cohort. Moreover, the values of $R_2^*$ in the left caudate of HD patients tended to be larger than those of the right-sided structure in this cohort, but this difference did not reach statistical significance.

The Mann Whitney U test did not reveal any significant differences in the $R_2^*$ values between the left caudate structures ($p = 0.6612$) and between right caudate structures ($p = 0.3773$) when comparing HD patients to HCs. Therefore, the average $R_2^*$ values between left and right-side structures for each cohort were considered for the inter-group comparison. These values are displayed in Table 16 and represented in Figure 36. No significant differences were found for the mean $R2^*$ values of the caudate ($p = 0.4409$), putamen ($p = 0.5833$) and pallidum ($p = 0.3196$) between HCs and subjects with HD.
Table 15: $R^*_2$ (range) values of the basal ganglia structures in the left and right hemispheres of HCs and of subjects with HD. p-values were obtained using the Wilcoxon signed rank statistical test for paired samples, which show statistically significant differences for $p<0.05$.

<table>
<thead>
<tr>
<th>Brain Structure</th>
<th>HCs</th>
<th>HD Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Caudate</td>
<td>25 (22-35)</td>
<td>28 (22-37)</td>
</tr>
<tr>
<td>Putamen</td>
<td>33 (27-48)</td>
<td>31 (28-46)</td>
</tr>
<tr>
<td>GP</td>
<td>44 (28-58)</td>
<td>42 (30-53)</td>
</tr>
</tbody>
</table>

Table 16: $R^*_2$ (range) values of the basal ganglia structures of HCs and of subjects with HD. p-values were obtained with the Mann-Whitney U-test, which show statistically significant differences for $p<0.05$.

<table>
<thead>
<tr>
<th>Brain Structure</th>
<th>HCs</th>
<th>HD Subjects</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudate</td>
<td>25 (22-27)</td>
<td>25 (24-29)</td>
<td>0.44</td>
</tr>
<tr>
<td>Putamen</td>
<td>31 (28-34)</td>
<td>31 (29-32)</td>
<td>0.58</td>
</tr>
<tr>
<td>GP</td>
<td>41 (34-53)</td>
<td>42 (41-52)</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Figure 36: $R^*_2$ values in the caudate, putamen and GP of HCs and subjects with HD. No significant differences between cohorts were found in the values of $R^*_2$ for any of the basal ganglia structures using the Mann Whitney U test (NS.: p-value greater than 0.05).
7.2.2. Correlation of Basal Ganglia Iron with Clinical Parameters

The median age of the HCs in this analysis was 63 (ranging 49-83), while HD patients had a median age of 41 (ranging 27-81). In order to assess the relation of age with iron content in basal ganglia structures, the Spearman’s correlation test was applied between the R2* values in the caudate, putamen and GP, and the age of healthy subjects included in this analysis. Only the R2* values in the caudate significantly correlated with age (r = 0.65 and p = 0.03).

Correlations between R2* values in the basal ganglia structures with the clinical variables obtained with the Spearman’s correlation are displayed in Table 17. The only statistically significant correlation detected was between iron content in the GP with the TFC score (r = -0.95 and p = 0.01). A trend towards significance was, however, revealed between the R2* values in the GP and disease duration (r = 0.87 and p = 0.05).

Table 17: Correlations of R2* values in the basal ganglia of HD patients (n=5) with the duration of the disease, TMS and TFC. Correlation coefficients were obtained with Spearman’s rank order correlation test, with correlations considered to be significant for p<0.05.

| Structure | Disease Duration | | | TMS | | | | TFC | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | rs | p-value | rs | p-value | rs | p-value | | | |
| Caudate | 0.15 | 0.80 | 0.20 | 0.78 | 0 | 1.0 | | | |
| Putamen | -0.05 | 0.94 | 0.30 | 0.68 | -0.32 | 0.60 | | | |
| GP | 0.87 | 0.05 | 0.80 | 0.10 | -0.95 | 0.01 | | | |

7.2.3. Correlation of Basal Ganglia Iron and Volumes

The Spearman's correlation test was used to evaluate the correlations between R2* values and the relative volumes of the basal ganglia structures of all subjects, in order to assess if there was an association between iron content and volume atrophy of the caudate, putamen and GP in this cohort. The results from this analysis are displayed on Table 18.

Table 18: Correlations of R2* values with the relative volumes of the basal ganglia considering all subjects (n=16). Correlation coefficients (rs) were obtained with Spearman’s rank order correlation test, with correlations considered to be significant for p<0.05.

| Structure | R2* vs. Vol. | | |
| --- | --- | --- | |
| | rs | p-value | |
| Caudate | -0.49 | 0.06 | |
| Putamen | -0.07 | 0.80 | |
| GP | -0.25 | 0.35 | |
No significant correlations were found between $R_z^*$ values and relative volumes of the basal ganglia, although a trend towards statistical significance was detected in the correlation between the iron content and relative volume of the caudate in this cohort ($p = 0.06$).

7.3. Discussion

The aims of this section were to investigate iron distribution in the basal ganglia of HD patients and to evaluate the correlations of iron content with atrophy of these structures and with the clinical variables of these subjects. $R_z^*$ relaxometry was used as an indirect marker of iron deposition, since the values of this relaxation rate are known to be highly correlated to iron content [42]. This is a robust and established method that has been applied in several clinical studies [76]. Firstly, no statistically significant differences were revealed in the $R_z^*$ values of the basal ganglia of HD patients when comparing to HCs - Table 16; Figure 36, which indicates that there was no significant increase in iron content in these brain structures of HD patients. These results are inconsistent with the observations of several previous studies, which have collectively reported greater iron accumulation in the caudate, putamen and GP in manifest HD. It is worth noting, however, that partly due to the use of different MRI-based iron measures, previous findings on iron level changes in HD have not always been consistent.

For instance, Domínguez et al. [48] found increased iron in all of the basal ganglia of manifest HD patients using QSM, while Rosas et al. [109] reported increased iron only in the caudate of HD patients using field mapping evolution. Furthermore, Vymazal et al. [110] measured increased iron content only in the GP of HD patients with $R_z$ relaxometry, but not in the caudate or putamen. Regarding $R_z^*$ relaxation studies, Sánchez-Castañeda et al. [73] reported increased $R_z^*$ values only in the GP of manifest HD patients, while in [45] increased iron content in both the GP and the putamen were detected.

In the present work, only the $R_z^*$ values in the GP of HD patients tended to be greater than those of HCs, but still this difference was not statistically significant. One possible confound factor in this analysis was the effect of age on iron content in the basal ganglia. Iron deposition in the brain increases physiologically with age, especially in the caudate, putamen and GP [111]. While the median age of the HD patients included in this analysis was 41, the median age of HCs was 63. Therefore, the $R_z^*$ values in the basal ganglia of HCs might have been inflated due to age-related iron deposition, hampering the detectability of differences in $R_z^*$ values in these structures when comparing to HD patients. This effect was even visible in the caudate of HCs, as a significant correlation ($r = 0.65$) was detected between age and $R_z^*$ values in this structure, despite the small sample of subjects in this group (n=11). While no significant correlations of $R_z^*$ values of the putamen and GP with age were found in this cohort, other groups have confirmed the effect of age in iron deposition in these structures [111].

In HD, the mutant huntingtin is thought to impair iron homeostasis in various ways, ultimately resulting in the increase of iron content in brain tissue and subsequent oxidative stress, inflicting neuronal damage and triggering inflammation processes, especially in the basal ganglia and its neuronal tracts. However, whether iron deposition in these brain structures is a primary outcome of tissue damage or a secondary result of neuronal loss remains unclear [48]. In other words, it is still not known if iron overload precedes basal ganglia atrophy or whether it results from such process. In this study, no significant correlations
were found between $R_2^*$ values of basal ganglia and their relative volumes, although there was a trend towards significance in the case of the correlation between caudate iron and volume - Table 17. These findings suggest that basal ganglia atrophy and iron accumulation might be independent processes, in agreement with an $R_2^*$ relaxometry study in HD [73] and with a study using magnetic field correlation maps to study iron in patients with the same disease [112]. On the other hand, significant inverse correlations were reported between iron and volume in the putamen and in the GP of manifest HD patients in a third $R_2^*$ study [45], while significant inverse correlations were found between the same parameters but in the caudate and putamen of premanifest HD patients in a QSM study [75].

Finally, in order to estimate if iron content in the basal ganglia increases during the disease and if it plays a role in motor and functional abnormalities observed in the HD patients included in this analysis, correlations between $R_2^*$ values of the caudate, putamen and GP with disease duration, TMS and TFC scores were tested. Regarding the caudate and the putamen, no statistically significant correlations with any of the clinical variables were found. These results are congruent with a previous study [112]. On the other hand, a strong significant correlation was detected between $R_2^*$ values in the GP and the TFC ($r_S = -0.95$), indicating that an increase in iron in this structure may underlie the debilitation of functional capacity of HD patients. No significant correlations were, however, found between $R_2^*$ values in the GP with either disease duration or TMS. These outcomes are partly consistent with an $R_2$ relaxometry study in the GP of HD patients [113], which did not find any significant correlation between the iron content of this structure and the disease duration and UHDRS-TMS. However, the same study reported that no significant correlation was found between iron content in the GP and the TFC score of HD patients.
8. Conclusion

8.1. Summary

The main objective of this cross-sectional neuroimaging study in HD was to investigate changes in multiple structural MRI parameters and to relate such changes to the clinical variables of HD patients, in the pursuit of objective and quantitative measures that might track progression across the disease spectrum.

Firstly, using a fully automated segmentation approach in high-resolution T1-weighted images, significant volume reductions were observed in the caudate, putamen, GP, brainstem and midbrain of HD patients, while a significant enlargement of the lateral ventricles was detected. Significant atrophy in striatal and extrastriatal structures of HD patients when comparing to HCs supports the thesis that HD is a multisystem neurodegenerative disorder. Nevertheless, the striatal volumes were the parameters that most strongly correlated with the MTS and TFC scores, emphasizing the role of the bilateral degeneration of the caudate and putamen in the debilitation of motor and functional capacities of these patients. The volumes of the GP, brainstem and midbrain did not significantly correlate with the clinical variables of this HD cohort, suggesting that atrophy of these structures occurs at a lower rate than striatal atrophy and with no significant implications in the motor and functional abnormalities.

R2* regional maps of the basal ganglia were obtained using a monoexponential fitting to the T2*-weighted data acquired at different TEs. No significant increases in R2* values were verified in the caudate, putamen and GP of HD patients, perhaps partly due to the effect of age on iron accumulation in the basal ganglia, which was not taken into account in this study. Furthermore, the iron content did not significantly correlate with the relative volumes of the caudate, putamen and GP of the subjects included in this analysis, suggesting that basal ganglia atrophy and iron accumulation might be independent processes. On the other hand, the R2* values of the GP and the TFC of HD patients correlated significantly, indicating that iron accumulation in this structure might underlie abnormalities in the functional capacities of these subjects.

Significant reductions in the SNc area and in the SNc/midbrain ratio of patients with HD were, for the first time, successfully detected by means of a semi-automatic segmentation method in NM-MRI. A reduction of the SNc ratio, which takes into account the variability in midbrain area, suggests that there may be alternative mechanisms to midbrain atrophy underlying the depletion of dopaminergic NM-containing neurons in the SNc of HD patients. Furthermore, the SNc ratio correlated significantly with the relative volumes of the caudate, putamen and GP considering all subjects. These key results suggest that loss of NM-containing neurons in the SNc is related to the basal ganglia atrophy, which is the hallmark of HD. The CR, which quantifies the distribution of NM in the SNc, tended to be smaller in the SNc of these subjects comparing to HCs, but this reduction did not reach statistical significance. Moreover, a significant and strong correlation was found between the SNc areas and the TFC of HD
patients, suggesting that depletion of NM-containing neurons might be one of the factors responsible for the decrease in functional capacity of these subjects.

8.2. Limitations and Future Work

The main limitation of this study was the small number of HD patients included in the clinical sample. Since HD is a rare disease, data from HD patients is generally scarce. Regardless, most cross-sectional MRI studies of the disease have a sample of over 20 patients. The small sample size of 8 HD patients may have hindered several comparisons in this study, especially the correlations between the analysed parameters and the clinical variables of these subjects, which did not reach statistical significance, in contrast to other works. On the other hand, despite this limitation, brain atrophy of several structures and correlations between striatal volumes and clinical scores were significant, in agreement to multiple previous studies. Furthermore, the sample size of HD patients was sufficient for the detection of significant differences in SNc areas and SNc/midbrain ratios comparing to HCs in NM-MR images, which is noteworthy. Reproducing these analyses in larger HD sample size is of upmost importance to confirm the results hereby obtained and, most of all, to verify the reliability of using NM-quantification parameters as biomarkers in this disease. An analysis of larger cohort of subjects will raise the statistical power and robustness of the findings obtained in this work.

A confound factor transversal to all analyses performed in this study was the effect of age on the measured parameters. Firstly, the caudate, putamen, GP and brainstem volumes decrease with age [114]. Moreover, there is a progressive accumulation of NM in the SNc [15] and an increase in the iron content of basal ganglia with age [111]. Since the mean age of HCs was significantly greater than that of HD patients, these effects might have introduced bias in the performed analyses. In future studies, age should be taken into account as a covariate to avoid such bias effects.

This is the first study in HD using NM-MRI analysis techniques. A depletion of NM-containing dopaminergic neurons of the SNc of HD patients, partly independent of midbrain atrophy as given by the SNc ratio, was reported in this cross-sectional work. In the future, it would be crucial to evaluate these parameters longitudinally in HD, from the early stages of the disease and even before symptomatic onset so as to assess the reliability of measuring changes in NM of SNc during the course of HD as a potential biomarker of the disease. Alternatively, cross-sectional NM-MRI studies with HD patients subdivided into premanifest, early-stage and late-stage groups could lead to similar answers. The sample size of HD patients in this study (n=8) was too small for its division into subclinical groups.

A histological study reported that the loss of pigmented neurons in the SNc of HD patients was more severe in the medial and lateral thirds of the SNc, while neurons in the central third of this structure were relatively spared [85]. In the future it would be interesting to study separately the CR in each of these subregions of the SNc in HD patients with NM-MRI and investigate possible differences when comparing to HCs in search for a possible pattern of NM depletion.

The FSE NM-MRI imaging protocol used in this work had some limitations. Signal intensities of FSE acquisitions are not homogeneous due to the sensitivity to the RF field [78], which represents a subject-
dependent confounding factor that cannot be accounted for with image filtering. Longer acquisition time and larger slice thickness were also required to account for the relatively low signal-to-noise ratio of images obtained with this sequence. Furthermore, this imaging protocol had a long scan time (8 minutes), which may pose a critical problem when imaging patients with intense choreatic movements. In this study, 4 of the patients had early-stage HD (5 years or less of disease duration) and thus did not present strong involuntary movements, while the remaining 4 subjects had a disease duration of 10 or more years, corresponding to the disease stage when rigidity and movement impairment supervene. In the future, if 2D FSE sequences are to be used in NM-MRI analysis, the acquisition protocol should be further optimized to reduce acquisition time and to improve image contrast, which might increase the sensitivity of CR measurements. Alternatively, 3D GE sequences with prepared MT contrast pulses, which generally have a much lower acquisition time, may be used to evaluate high-signal SNc volumes and to calculate their CR three-dimensionally in NM-MR images as a more complete approach to NM quantification. While the measurement of SNc volumes with such GE sequences in NM-MR images has been applied in at least one study in PD [80], its feasibility still needs to be further confirmed and validated in HD.

NM-MRI methods for quantitative estimation of SNc area, volume and CR parameters and for SNc segmentation have not yet been standardized. Differences in image acquisition parameters, image processing, ROI selection methods, and clinical characteristics of patients hamper the quantitative comparison of NM-MRI studies. Despite being accurate, less user-dependent and less time-consuming comparing to manual segmentation, the SNc segmentation region-growing algorithm used in this work required manual seed point selection. While segmentation variability associated with seed point selection was not investigated, future studies should confirm the inter-rater reproducibility of this method. Furthermore, midbrain area measurements were performed manually, as well as the placement of the reference ROIs for the calculation of the CR. Fully automated NM-MRI segmentation and image analysis approaches are required to eliminate the inter-operator inaccuracies of manual and semi-automatic methods, while retaining measurement accuracy and reducing analysis time. For instance, an automated SNc segmentation with registration to a previously labelled atlas of the midbrain, as it is done in the FreeSurfer software for other brain structures on high-resolution T1-weighted images, could prove to be feasible in NM-MR images.

Pathological iron accumulation in the basal ganglia structures and its relations to atrophy and to symptomatic manifestation in HD are still not fully understood. Regarding the R2* relaxometry analysis, no significant increases in iron content were detected in the basal ganglia of HD patients. However, reports from other groups using several different MRI-based iron measures inconsistently indicate that one, two or all of these structures undergo significant iron deposition in HD patients. Electing a single method for MRI-based iron quantification such as R2* relaxometry, QSM or a combination of both, might aid in dissipating the variability of the results reported in the literature.

The CAG repeat number of HD patients and the scores of clinical scales other than the UHDRS MTS and TFC were not available in this study. CAG repeat size is the major determinant of age at onset, disease progression and age at death in HD, thus it would be interesting to relate the changes revealed
in this study, especially the degeneration of the SNc, with this clinical parameter. Furthermore, assessing correlations between the multiple parameters studied and the cognitive and neuropsychiatric clinical scores might also reveal new processes underlying the manifestation of cognitive and psychiatric dysfunction. Finally, it might also be interesting to study the iron content in the SN of HD patients and its relation to NM-quantifying parameters in NM-MRI.

To date, the most widely used tool for clinical assessment in HD is the UHDRS. However, this scale tends to have high inter-rater variability, is susceptible to floor and ceiling effects and is fairly insensitive in the premanifest stages of the disease [10]. Nonetheless, HD research is entering a critical phase where promising novel disease-specific therapies are on the horizon [29]. There is, therefore, a pressing need for a panel of biomarkers capable of monitoring disease progression and ultimately determining drug efficacy in clinical trials. Neuroimaging techniques provide the most robust biomarkers to this end. The use of high-resolution T1-weighted imaging to quantify the decrease in brain volumes has so far been considered the most suitable and sensitive MRI technique for the cross-sectional and longitudinal study of early HD. However, in view of the clinical heterogeneity of the disease, a multimodal approach such as this study may offer a novel means of detecting simultaneous neurological changes that occur in the HD brain. The predictability of HD makes it perhaps the most governable of the neurodegenerative diseases from the standpoint of early intervention. NM-MRI techniques previously applied for the quantification of NM in the SNc in PD patients were for the first time employed in this work in the study of brain changes in HD. In turn, findings from future HD studies may have key relevance for other more prevalent neurodegenerative disorders which share some pathological features with HD, such as Alzheimer's Disease and PD, for which highly predictive tests are not yet available.
9. References


C. A. Ross et al., “Huntington disease: Natural history, biomarkers and prospects for


[88] B. Fischl et al., “Whole Brain Segmentation: Neurotechnique Automated Labeling of


[102] M. Carvalho, “Estudo Imagiológico em Ressonância Magnética de Doentes com Doença de Parkinson Late Stage,” Faculty of Sciences and Technology, NOVA University of Lisbon, 2016.


