

# Nigral iron and neuromelanin imaging studies in Parkinson's disease

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*“You must remember what you are and what you have chosen to become, and the significance of what you are doing. There are wars and defeats and victories of the human race that are not military and that are not recorded in the annals of history. Remember that while you're trying to decide what to do”.*

*— John Williams, Stoner*

# Abstract

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Parkinson's disease (PD) a neurodegenerative condition characterized by the reduction of the pigmented dopaminergic neurons of the substantia nigra pars compacta (SNc) and the loss of the dopaminergic striatal terminals which in turn result in the cardinal motor symptoms including bradykinesia, rigidity and tremor. Iron plays a deleterious role in the pathophysiology of PD due to the generation of reactive oxidative species that leads to oxidative stress and neurodegeneration. In addition neuromelanin is a complex pigment contained in the dopaminergic neurons of the SNc that plays a toxic role in pro-oxidative conditions such as PD. Magnetic resonance imaging (MRI) is a widely available non-ionising technique allowing the study of structural properties of the SN such as iron deposition or neuromelanin pigmentation load in PD. In this thesis I have used state of the art neuroimaging to assess nigral iron accumulation and nigral depigmentation in PD, the correlation between neuromelanin decline and nigrostriatal terminal loss, and the potential clinical use of an iron chelation to improve clinical symptoms. The main findings of this thesis are as follows: A) Increased nigral iron accumulation in PD displays an ascendant stratification according to clinical severity at baseline and it is associated with bradykinesia and rigidity symptoms in cross-sectional and longitudinal settings. B) Nigral depigmentation in PD shows a descendant stratification according to disease duration at baseline and displays significant associations with motor severity at baseline and with bradykinesia at follow-up visits. C) The pattern of nigral pigmentation loss shows a ventro-medial pattern and there was a significant correlation between this loss and nigrostriatal terminals decline in the most affected side, but not in the least affected side. D) Finally, iron chelation with deferiprone in PD shows a trend of motor improvement after six months of treatment being optimally monitored with iron-sensitive MR imaging techniques. The results presented in this thesis support the application of multimodal imaging protocols to further understand the dopaminergic pathophysiology of PD and their usefulness for monitoring the progression of the disease.

# Declaration of originality

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The work presented in this thesis was accomplished in the Centre for Neuroinflammation and Neurodegeneration of the Brain Division of the Department of Medicine at Imperial College London from April 2014.

This is to certify that to the best of my knowledge, the content of the present thesis is my own work.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

# Copyright declaration

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# Dedication

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This thesis is dedicated to my parents.

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# Publications from this thesis

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- **Martin-Bastida A**, Lao-Kaim NP, Loane C, Politis M, Roussakis AA, Valle-Guzman N, Kefalopoulou Z, Paul-Visse G, Widner H, Xing Y, Schwarz ST, Auer DP, Foltynie T, Barker RA, Piccini P. Motor associations of iron accumulation in deep grey matter nuclei in Parkinson's disease: a cross-sectional study of iron-related magnetic resonance imaging susceptibility. *European Journal of Neurology*. 2017 Feb; 24(2):357-365.
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- **Martin-Bastida A\***, Lao-Kaim NP\*, Roussakis AA, Li W, Xing Y, Schwarz ST, Auer DP, Piccini P. Multimodal imaging assessment of nigrostriatal pathways in Parkinson's disease using  $^{11}\text{C}$ -PE2I PET and Neuromelanin-weighted MR. Ready to submission. (\*joint first authors).
- **Martin-Bastida A\***, Xing Y\*, Schwarz ST, Naidu S, Li X, Silverdale M, Bajaj N, Burn D, Grosset DG, Piccini P, Auer DP. Semi-automated segmentation and quantification of the substantia nigra depigmentation in Parkinson's disease - a multicentre case control MRI study. In preparation. (\*joint first authors).

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# Table of contents

---

|                                                                                                  |           |
|--------------------------------------------------------------------------------------------------|-----------|
| <b>Abstract.....</b>                                                                             | <b>2</b>  |
| <b>Declaration of originality .....</b>                                                          | <b>3</b>  |
| <b>Copyright declaration.....</b>                                                                | <b>4</b>  |
| <b>Dedication .....</b>                                                                          | <b>5</b>  |
| <b>Acknowledgements .....</b>                                                                    | <b>6</b>  |
| <b>Publications from this thesis .....</b>                                                       | <b>8</b>  |
| <b>Scientific abstracts presented in national and international conferences from this thesis</b> | <b>9</b>  |
| <b>Table of contents .....</b>                                                                   | <b>12</b> |
| <b>List of tables.....</b>                                                                       | <b>16</b> |
| <b>List of figures.....</b>                                                                      | <b>18</b> |
| <b>Abbreviations .....</b>                                                                       | <b>22</b> |
| <b>1. Introduction .....</b>                                                                     | <b>25</b> |
| <b>1.1. Epidemiology of Parkinson’s disease .....</b>                                            | <b>25</b> |
| <b>1.2. Motor symptomatology of Parkinson’s disease.....</b>                                     | <b>25</b> |
| <b>1.3. Non-motor symptoms in Parkinson’s disease.....</b>                                       | <b>27</b> |
| <b>1.4. Pathophysiology of Parkinson’s disease.....</b>                                          | <b>27</b> |
| 1.4.1. Dopaminergic pathology in Parkinson’s disease .....                                       | 28        |
| 1.4.2. Lewy body pathology .....                                                                 | 29        |
| 1.4.3. Iron accumulation in Parkinson’s disease .....                                            | 31        |
| 1.4.4. Neuromelanin .....                                                                        | 35        |
| <b>1.5. Structural neuroimaging nigral studies in Parkinson’s disease.....</b>                   | <b>40</b> |
| 1.5.1. Iron imaging studies in Parkinson’s disease.....                                          | 40        |
| 1.5.2. Neuromelanin imaging studies in Parkinson’s disease .....                                 | 45        |

|        |                                                                                              |     |
|--------|----------------------------------------------------------------------------------------------|-----|
| 1.6.   | Targeting iron in Parkinson's disease.....                                                   | 49  |
| 1.6.1. | Iron Chelation in Neurodegenerative disorders.....                                           | 51  |
| 1.6.2. | Iron chelation in Parkinson's disease.....                                                   | 51  |
| 1.7.   | Summary of aims and group investigations .....                                               | 53  |
| 2.     | Subjects and Methodology .....                                                               | 55  |
| 2.1.   | Subjects .....                                                                               | 55  |
| 2.1.1. | Parkinson's disease magnetic repository (PAMIR) study .....                                  | 55  |
| 2.1.2. | The TransEuro Study .....                                                                    | 56  |
| 2.1.3. | A pilot clinical trial with the iron chelator Deferiprone in Parkinson's disease.....        | 57  |
| 2.2.   | Clinical assessments.....                                                                    | 58  |
| 2.3.   | Magnetic resonance imaging.....                                                              | 59  |
| 2.3.1. | T1 and T2 .....                                                                              | 60  |
| 2.3.2. | Relaxometry .....                                                                            | 61  |
| 2.3.3. | Susceptibility weighted imaging .....                                                        | 63  |
| 2.3.4. | Analysis of iron-sensitive sequences and imaging protocols .....                             | 65  |
| 2.3.5. | Neuromelanin-weighted MRI imaging.....                                                       | 69  |
| 2.4.   | Positron Emission Tomography .....                                                           | 73  |
| 2.4.1. | Positron Emission Tomography basics.....                                                     | 73  |
| 2.4.2. | The dopamine transporter .....                                                               | 75  |
| 2.4.3. | <sup>11</sup> C-PE2I as PET tracer .....                                                     | 76  |
| 3.     | Studies of iron-related susceptibility imaging in Parkinson's disease .....                  | 78  |
| 3.1.   | Introduction.....                                                                            | 78  |
| 3.2.   | Cross-sectional high-pass filtered susceptibility imaging in Parkinson's disease .....       | 80  |
| 3.2.1. | Subjects and methods .....                                                                   | 80  |
| 3.2.2. | Results.....                                                                                 | 84  |
| 3.2.3. | Discussion .....                                                                             | 92  |
| 3.3.   | Longitudinal high-pass filtered susceptibility imaging in Parkinson's disease .....          | 95  |
| 3.3.1. | Subjects and methods .....                                                                   | 96  |
| 3.3.2. | Results.....                                                                                 | 98  |
| 3.3.3. | Discussion .....                                                                             | 103 |
| 3.4.   | Limitations and future directions .....                                                      | 106 |
| 4.     | Studies of Neuromelanin-weighted magnetic imaging in Parkinson's disease .....               | 108 |
| 4.1.   | Introduction.....                                                                            | 108 |
| 4.2.   | Cross-sectional study of Neuromelanin-weighted magnetic imaging in Parkinson's disease ..... | 109 |
| 4.2.1. | Subjects and methods .....                                                                   | 110 |

|        |                                                                                                                                                      |     |
|--------|------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 4.2.2. | Results.....                                                                                                                                         | 112 |
| 4.2.3. | Discussion .....                                                                                                                                     | 117 |
| 4.3.   | Longitudinal study of Neuromelanin-weighted magnetic imaging in Parkinson's disease.<br>120                                                          |     |
| 4.3.1. | Subjects and methods.....                                                                                                                            | 121 |
| 4.3.2. | Results.....                                                                                                                                         | 122 |
| 4.3.3. | Discussion .....                                                                                                                                     | 125 |
| 4.4.   | Limitations and future directions.....                                                                                                               | 127 |
| 5.     | Brain iron chelation with deferiprone in Parkinson's disease .....                                                                                   | 129 |
| 5.1.   | Introduction.....                                                                                                                                    | 129 |
| 5.2.   | Patients and methods.....                                                                                                                            | 130 |
| 5.2.1. | Study subjects.....                                                                                                                                  | 130 |
| 5.2.2. | Neurological examination.....                                                                                                                        | 133 |
| 5.2.3. | Blood and urine analysis .....                                                                                                                       | 133 |
| 5.2.4. | Quantification of deferiprone and deferiprone glucuronide.....                                                                                       | 133 |
| 5.2.5. | T2* region of interest analysis .....                                                                                                                | 134 |
| 5.2.6. | Summary of investigations.....                                                                                                                       | 134 |
| 5.2.7. | Statistical Analysis .....                                                                                                                           | 135 |
| 5.3.   | Results.....                                                                                                                                         | 136 |
| 5.3.1. | Demographics.....                                                                                                                                    | 136 |
| 5.3.2. | Clinical Parameters .....                                                                                                                            | 138 |
| 5.3.3. | Regional T2* analysis .....                                                                                                                          | 139 |
| 5.3.4. | Blood parameters.....                                                                                                                                | 142 |
| 5.3.5. | Urinary iron content.....                                                                                                                            | 145 |
| 5.3.6. | Plasma Inflammatory markers.....                                                                                                                     | 145 |
| 5.4.   | Discussion.....                                                                                                                                      | 146 |
| 5.5.   | Limitations and future directions.....                                                                                                               | 149 |
| 6.     | Multimodal imaging assessment of nigrostriatal pathways in Parkinson's disease<br>using <sup>11</sup> C-PE2I PET and Neuromelanin-weighted MRI ..... | 151 |
| 6.1.   | Introduction.....                                                                                                                                    | 151 |
| 6.2.   | Subjects and methods .....                                                                                                                           | 153 |
| 6.2.1. | Study subjects.....                                                                                                                                  | 153 |
| 6.2.2. | Imaging acquisition.....                                                                                                                             | 154 |
| 6.2.3. | Imaging procession and region of interest analysis. ....                                                                                             | 155 |
| 6.2.4. | Statistical analyses .....                                                                                                                           | 159 |
| 6.3.   | Results.....                                                                                                                                         | 160 |
| 6.3.1. | Demographics.....                                                                                                                                    | 160 |
| 6.3.2. | Analysis of Neuromelanin Contrast Ratios.....                                                                                                        | 161 |
| 6.3.3. | Clinical correlations.....                                                                                                                           | 162 |

|                                                                                                  |                                                                                                                                                            |     |
|--------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 6.3.4.                                                                                           | Nigrostriatal correlations .....                                                                                                                           | 163 |
| 6.4.                                                                                             | Discussion.....                                                                                                                                            | 166 |
| 6.5.                                                                                             | Limitations and future directions.....                                                                                                                     | 170 |
| 7.                                                                                               | Discussion .....                                                                                                                                           | 171 |
| 7.1.                                                                                             | Summary of key results .....                                                                                                                               | 171 |
| 7.1.1.                                                                                           | Assessment of iron accumulation in deep grey matter nuclei of Parkinson's disease using high-pass filtered imaging. ....                                   | 172 |
| 7.1.2.                                                                                           | Assessment of nigral depigmentation in Parkinson's disease using Neuromelanin in Parkinson's disease .....                                                 | 173 |
| 7.1.3.                                                                                           | Iron chelation with deferiprone as therapeutic target in Parkinson's disease. ....                                                                         | 173 |
| 7.1.4.                                                                                           | Assessing nigrostriatal pathways with multimodal imaging assessment using NM-weighted MR imaging and <sup>11</sup> C-PE2I PET in Parkinson's disease. .... | 174 |
| Appendix A: Scientific papers published in peer-reviewed journals arising from this thesis ..... |                                                                                                                                                            | 211 |

# List of tables

---

## Chapter 1:

- Table 1.1: Cross-sectional iron-sensitive MR imaging studies in Parkinson's disease.....42
- Table 1.2: Longitudinal iron-sensitive MR imaging studies in Parkinson's disease.....44
- Table 1.3: Neuromelanin-weighted MR imaging studies in Parkinson's disease.....48

## Chapter 3:

- Table 3.1: Cross-sectional demographic and clinical characteristics of study subjects undergoing high-pass filtered MR imaging.....85
- Table 3.2: Analysis of covariance matrices or regional radiances according to UPDRS-III.....88
- Table 3.3: Longitudinal demographic and clinical characteristics of study subjects undergoing high-pass filtered MR imaging.....99

## Chapter 4:

- Table 4.1: Cross-sectional demographic and clinical characteristics of study subjects undergoing Neuromelanin-weighted MR imaging.....113
- Table 4.2: Longitudinal demographic and clinical characteristics of study subjects undergoing Neuromelanin-weighted MR imaging.....122

## Chapter 5:

- Table 5.1: Recommended volume of oral solution to obtain 20 or 30mg/kg/day according to body weight.....131

- Table 5.2: Summary of investigations, treatment and clinical assessments at baseline and 6 months of deferiprone treatment.....135
- Table 5.3: Demographic and clinical characteristics of Parkinson’s subjects divided in three therapeutic groups.....137
- Table 5.4: Regional T2\* values of PD patients at 0, 3 and 6 months after administration of 20 mg/kg/day, 30 mg/kg/day deferiprone or placebo.....141
- Table 5.5: Haematological and iron measurements at 0, 2, 4 and 6 months after administration of 20 mg/kg/day, 30 mg/kg/day deferiprone or placebo.....143

## Chapter 6:

- Table 6.1: Participant characteristics undergoing multimodal imaging assessment with Neuromelanin-weighted MR imaging and <sup>11</sup>C-PE2I PET.....160

# List of figures

---

## Chapter 1:

- Figure 1.1: Macroscopic and microscopic appearances of midbrain of Parkinson's disease.....28
- Figure 1.2: Stages of Parkinson's disease pathology according to alpha-synuclein inclusions.....30
- Figure 1.3: Neuronal iron homeostasis.....34
- Figure 1.4: Neuromelanin microscopic appearance in SNc.....36
- Figure 1.4: Chemical structure of iron-chelating agents.....50

## Chapter 2:

- Figure 2.1: Magnetic properties of the  $^1\text{H}$  proton nucleus.....60
- Figure 2.2: Spin lattice relaxation ( $T_1$ ) and spin-spin relaxation ( $T_2$ ) times.....61
- Figure 2.3:  $T_2$  and  $T_2^*$  relaxation decay curves.....62
- Figure 2.4: High-pass filtered, magnitude and susceptibility weighted images.....64
- Figure 2.5: Six  $T_2^*$  images acquired at 6.3, 13, 21, 29, 37, and 49 milliseconds of echo time.....65
- Figure 2.6: Regions of interest delineated on the axial plane of high-pass filtered phase images.....68
- Figure 2.7: Regions of interest delineated on the axial plane of Neuromelanin-weighted MR images.....70
- Figure 2.8: Region of interests and threshold on the axial plane of Neuromelanin-weighted MR images.....71
- Figure 2.9: Region of interest on the axial plane of the Neuromelanin-template.....72
- Figure 2.10: Schematic description of  $\beta^+$  annihilation and coincidence detection in positron emission tomography scanner.....74
- Figure 2.11: Coincidence detection in the PET camera.....74
- Figure 2.12: Structural formula of  $^{11}\text{C}$ -PE2I.....76

- Figure 2.13: Striatal dopamine transporter assessed with  $^{11}\text{C}$ -PE2I PET in healthy control and PD volunteers.....77

### Chapter 3:

- Figure 3.1: SNc region of interest placement in the two susceptibility weighted imaging protocols.....83
- Figure 3.2: Comparison of delineated areas between Parkinson's disease and healthy controls.....86
- Figure 3.3: Comparison of baseline radians between Parkinson's disease and healthy controls.....87
- Figure 3.4: SN radians in Parkinson's disease subgroups and healthy controls according to UDPRS III.....89
- Figure 3.5: Comparison of baseline radians of Parkinson's disease subjects according to clinical laterality.....90
- Figure 3.6: Cross-sectional clinical correlations of SN radians in Parkinson's disease.....91
- Figure 3.7: Validation of phase-shift data of deep brain grey matter nuclei from the healthy control group.....92
- Figure 3.8: Comparison of baseline and follow-up radians between Parkinson's disease and healthy controls.....100
- Figure 3.9: Temporal changes of SN radians in Parkinson's disease subgroups according to changes in UDPRS III.....101
- Figure 3.10: Longitudinal clinical correlations of temporal changes in Substantia nigra and dentate nucleus. ....102

### Chapter 4:

- Figure 4.1: Comparison of SNc neuromelanin volumes between Parkinson's disease subgroups and healthy controls.....114
- Figure 4.2: SNc-NM volumes in Parkinson's disease subgroups and healthy controls according to disease duration.....115

- Figure 4.3: Comparison of baseline neuromelanin volumes between most and least affected sides in Parkinson's disease.....116
- Figure 4.4: Cross-sectional clinical correlations of Neuromelanin volumes in Parkinson's disease.....117
- Figure 4.5: Comparison of baseline and follow-up Neuromelanin volumes between Parkinson's disease and healthy controls.....123
- Figure 4.6: Comparison of baseline and follow-up Neuromelanin volumes between most and least side affected in Parkinson's disease.....124
- Figure 4.7: Longitudinal clinical correlations of temporal changes of neuromelanin volumes in Parkinson's disease.....125

## Chapter 5:

- Figure 5.1: Flow chart indicating PD study groups according to deferiprone therapeutic arms.....132
- Figure 5.2: Percentage change of UPRDS III of PD patients receiving placebo, 20 or 30 mg/kg/deferiprone for 2, 4 and 6 mg of treatment.....139
- Figure 5.3: T2\* values in the dentate nucleus and caudate nucleus of subjects receiving placebo, 20 or 30 mg/kg/day deferiprone at baseline, 3 and 6 months of treatment.....140
- Figure 5.4: Plasmatic concentrations of deferiprone and deferiprone glucuronide of subjects receiving 20 or 30 mg/kg/day deferiprone during study.....142
- Figure 5.5: Percentage to change of plasmatic ferritin in subjects receiving placebo, 20 or 30 mg/kg/deferiprone for 2, 4 and 6 mg of treatment.....144
- Figure 5.6: Changes in ferritin levels and T2\* values in two Parkinson's disease patients receiving chelation therapy over 6 months. ....145
- Figure 5.7: Baseline correlations between plasmatic ferritin and IL6 in Parkinson's disease patients.....146

## Chapter 6:

- Figure 6.1: Regions of interest delineated on the Neuromelanin-template MRI and <sup>11</sup>C-PE2I PET.....158

- Figure 6.2: Comparison of neuromelanin contrast ratios between Parkinson's disease and healthy controls.....162
- Figure 6.3: Correlations between SNc-CR of the dorsal tiers with striatal  $^{11}\text{C}$ -PE2I  $\text{BP}_{\text{ND}}$ .....164
- Figure 6.4: Correlations between SNc-CR of the dorsal tiers with striatal  $^{11}\text{C}$ -PE2I  $\text{BP}_{\text{ND}}$ .....165
- Figure 6.5: Correlations between total SNc-CR with striatal  $^{11}\text{C}$ -PE2I  $\text{BP}_{\text{ND}}$ .....166

# Abbreviations

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<sup>11</sup>C-PE2I: [<sup>11</sup>C]N-(3-iodopro-2*E*-enyl)-2β-carbomethoxy-3β-(4'-methylphenyl)nortropane

<sup>123</sup>I-F-P-CIT: [I-123] N-ω-fluoropropyl-2β-carbomethoxy-3β-(4-iodophenyl)nortropane

6-OHDA: 6-hydroxydopamine

ANC: Absolute neutrophil count

ANCOVA: Analysis of the covariance

ANOVA: Analysis of the variance

BP<sub>ND</sub>: Non-dispersible binding potential

CN: Caudate nucleus

CP: Cerebral peduncle

CR: contrast ratio

DA: Dopamine

DFO: Deferrioxamine

DFP: Deferiprone

DFS: Deferasirox

DN: Dentate nucleus

FDR: False Discovery Rate

Fe (II): Divalent (ferrous) iron

Fe (III): Trivalent (ferric) iron

Fe: Iron

FSE: Fast spin echo

FSL: Functional MRI of the Brain (FMRIB) centre Software Library

GP: Globus pallidus

H&Y: Hoehn and Yahr Scale

Hb: Haemoglobin

HC: Healthy control

HPLC: High performance liquid chromatography

IL-6: Interleukin 6

KeV: Electronvolt

LB: Lewy body

LC: locus coeruleus

LEDD: Levodopa equivalent daily dosage

MADRS: Montgomery-Åsberg depression rate scale

MMSE: Mini-mental state examination

MNI: Montreal Neurological Institute

MPRAGE: Magnetisation Prepared Rapid Gradient Echo

MRI: Magnetic resonance imaging

MT: Magnetization transfer

MTPT: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NM: Neuromelanin

OFF: Parkinson's disease patients in "off-medication" state

ON: Parkinson's disease patients in "on-medication"

PD: Parkinson's disease

PET: Positron Imaging Tomography

Put: Putamen

ROI: Region of interest

ROS: Reactive oxidative species

SD: Standard Deviation

SEM: Standard error mean

SN: Substantia nigra

SNc: Substantia nigra pars compacta

SNc-CR<sub>dor</sub>: Contrast ratio of dorsal tiers of Substantia nigra pars compacta

SNC-CR<sub>ven</sub>: Contrast ratio of ventral tiers of Substantia nigra pars compacta

SNC-NM: Neuromelanin volumes of Substantia nigra pars compacta

SNr: Substantia nigra pars reticulata

SPECT: Single Photon Emission Computer Tomography

SPIN: Signal Processing in Nuclear Magnetic Resonance software

SRTM: Simplified referenced tissue model

SWI: Susceptibility weighted imaging

TE: Echo time

TNF $\alpha$ : Tumoral necrosis factor alpha

TS: Transferrin saturation

UPDRS III: Unified Parkinson's disease rate motor scale part III.

UPDRS: Unified Parkinson's disease rating scale

VTA: Ventral tegmental area

WBC: White blood count

# 1. Introduction

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## 1.1. Epidemiology of Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (Ascherio and Schwarzschild 2016). PD is expected to be an oncoming economic and social burden in societies with an accelerated and increased aging such as the United Kingdom.

The estimates of the prevalence and incidence of PD vary in regards with the applied methodology across the different cross-sectional studies. The estimated prevalence of PD in populations over 65 years and older was 1.8 (per 100) (de Rijk *et al.* 2000) or 1% in people over 60 years of age (de Lau and Breteler 2006). This prevalence rate shows an increase of 2.6% for population over 85 years (de Rijk *et al.* 2000).

The median annual incidence rates in industrialized countries is 8-18 per 100000 people per year (de Lau and Breteler 2006). The male-to-female ratio showed in several prospective studies varies from 1.1 in Taiwan to 2.6 in Spain, displaying higher incidence in men than in women (de Lau and Breteler 2006).

The age of onset of PD is 60 years and the mean disease duration from diagnosis to death is estimated in 15 years (Katzenschlager *et al.* 2008).

The disease is found in all ethnic groups, although appears to be more prevalent in Caucasians (Van Den Eeden *et al.* 2003) when compared with Asian and blacks ethnicities.

## 1.2. Motor symptomatology of Parkinson's disease

The classical motor symptoms of PD are bradykinesia, rigidity, resting tremor and postural instability (Jankovic 2008, Lees *et al.* 2009). According to UK Parkinson's

Disease Society Brain Bank's clinical criteria for clinical diagnosis PD the presence of bradykinesia must be accompanied by at least one or the following: rigidity, resting tremor and/or postural instability not caused by primary visual, vestibular cerebellar or proprioceptive dysfunction (Hughes *et al.* 2001).

Bradykinesia is the most common motor feature on PD and refers to a fatigable decrement in amplitude and velocity of movement. It reflects difficulties in initiating, planning and sequencing movements (Berardelli *et al.* 2001). This symptom is also manifested in the reduction of facial mimics (hypomimia), difficulties and slowness in handwriting (micrographia), difficulty in swallowing and speech disturbances (hypophonia). Bradykinesia is hypothesized to be the result of dopaminergic degeneration affecting the striatal-cortical pathways (Vingerhoets *et al.* 1997).

Rigidity is defined as increased passive resistance in the joints and can be examined with the “cog-wheel” phenomenon when accompanied by tremor. Rigidity is present in proximal and distal joints and is often associated with pain (Riley *et al.* 1989).

Rest tremor with 4-6 Hz frequency occurs often unilaterally as first symptom in PD. It is described as “pill-rolling” tremor and appears in distal extremities, although it could be also present in lips, jaw and legs (Grosset and Lees 2005). Postural “re-emergent” tremor is often present as well as rest tremor. Tremoric symptoms are absent during the course of PD disease in at least 30% of patients.

Postural instability and axial symptoms due to loss of postural reflexes are often present in later stages of PD.

UK Parkinson's Disease Society Brain Bank's clinical criteria also implies the exclusion of other causes of Parkinsonism such as history of acute vascular disease, traumatic brain injury, early autonomic involvement, pyramidal signs, early cognitive impairment or supranuclear gaze palsy among others.

### **1.3. Non-motor symptoms in Parkinson's disease**

Non-motor symptoms are often present prior to the diagnosis and can contribute to disease disability, impairment of quality of life and morbidity.

Although the diagnosis of PD is based on the presence of motor symptoms, the occurrence of non-motor symptoms can precede the dopaminergic neurodegeneration several years (Chaudhuri and Schapira 2009). Non-motor symptoms do contribute to disease disability, impairment of quality of life and morbidity. Non-dopaminergic neurochemical system including serotonin, norepinephrine and acetylcholine among others are involved in the presence of non-motor symptoms in PD which include:

- Sensory features including olfactory dysfunction (hyposmia and anosmia), visual disturbances (diplopia and blurred vision), pain and other sensory complaints.
- Neuropsychiatric features such as depression, anxiety, apathy, fatigue, cognitive deficits, dementia and visual hallucinations.
- Sleep disorders including excessive sleepiness, vivid dreams and rapid eye movement behaviour disorder (RBD)
- Autonomic dysfunction such as cardiovascular features (orthostatic hypotension), bladder (urinary frequency and urgency) and bowel (constipation, incomplete gastric emptying) dysfunctions.

### **1.4. Pathophysiology of Parkinson's disease**

The pathological hallmark of PD consists in the loss of pigmented dopaminergic neurons of substantia nigra pars compacta (SNc) (Fearnley and Lees 1991), the presence of alpha-synuclein aggregations in the form of Lewy bodies (Spillantini *et al.* 1997), increase of iron (Dexter *et al.* 1987) and subsequent reduction of striatal dopaminergic projections.

#### 1.4.1. Dopaminergic pathology in Parkinson's disease

The main neuropathological finding in PD is the marked loss of dopaminergic neurons of the SNc (*Figure 1.1*). Neuronal loss is greater in lateroventral and caudal calbindin-negative areas with further progression to lateromedial and dorsal areas (Fearnley and Lees 1991, Damier *et al.* 1999). Dopaminergic nigral neurodegeneration leads to dopaminergic terminal loss in dorsal putamen with later involvement in caudate and ventral putamen (Kish *et al.* 1988). Nigral dopaminergic degeneration is directly correlated with striatal dopaminergic loss (Bernheimer *et al.* 1973).

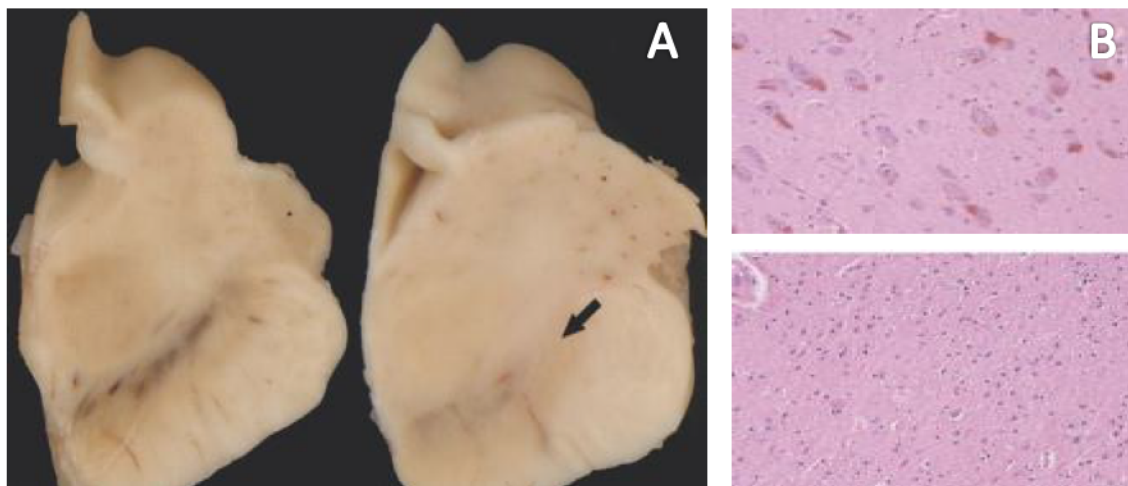


**Figure 1.1:** Macroscopic and microscopic appearances of midbrain of Parkinson's disease. (A) Transverse section of the midbrain in normal control (left) and PD patient (right) showing pigmentation loss of SNc (arrow). (B) Haematoxylin and eosin-staining in normal control (up) and PD patient (down) showing neuronal loss and gliosis in the ventral-lateral tier of the SNc. Images extracted from Petrucelli and Dickson 2008.

Dopaminergic pigmented neurons in SNc are selectively vulnerable to neurodegeneration due its increased iron content and the toxic role of neuromelanin which could in turn lead to increased oxidative stress (Hirsch *et al.* 1988, Zecca *et al.*

2001). Furthermore dopaminergic degeneration in striatum is strongly associated with disease duration and leads to the appearance of the classical motor symptoms of PD (Rajput *et al.* 2008).

Additional neuronal loss is evident in other dopaminergic regions of the brainstem such as ventral tegmental area (VTA) and retrorubral nucleus (RRN) implicated in the mesocortical and mesolimbic dopaminergic projections (Damier *et al.* 1999). Finally non-dopaminergic neuronal loss is also present in noradrenergic, serotonergic and cholinergic areas including locus coeruleus (LC), raphe nuclei and nucleus basalis of Meynert respectively.

#### **1.4.2. Lewy body pathology**

Dopaminergic neurodegeneration is accompanied distinctive intraneuronal inclusions known as Lewy bodies (LB) and Lewy dystrophic neurites. The increased aggregation of alpha-synuclein is responsible to the formation of LB.

LB pathology first appears in the ventrolateral area of the SN, latter spreading to the medial part and dorsal tier of the SNc (Wakabayashi *et al.* 2006). The occurrence of LB neurons in SN do not correlate with disease duration or motor severity, which could account for the dynamic “turn-over” that is continuously producing and destroying LB at the same rate or the passive “one-pass” phenomenon where the number of LB increases at the beginning of the disease and latter decrease at the same rate of the pigmented nigral loss (Greffard *et al.* 2010).

LB pathology is not only restricted to dopaminergic SN neurons as it is also found in non-dopaminergic nuclei such as olfactory bulb, amygdala, perirhinal cortex, medulla oblongata and autonomic nervous system (Jellinger 1999).

The chronological LB distribution of PD has been proposed by Braak and Braak (Braak *et al.* 2003) from analysing extensive series of brain biopsies (*Figure 1.2*). In pre-symptomatic stages (stage I and II) LB are found in olfactory bulb, medulla oblongata including dorsal motor nucleus, intermediate reticular zone and peripheral autonomic

nervous system with subsequent spreading to pontine tegmentum, LC and raphe nuclei. In the early clinical stages (III and IV) LB pathology is found in SN and midbrain with later spreading to basal ganglia and mesocortex. In the final diffuse cortical LB pathology (stages V and VI) alpha-synuclein pathology spreads to neocortical areas including sensory association areas, prefrontal and sensorimotor cortices.

It is still yet to be ascertained whether alpha-synuclein pathology is directly associated with neurodegeneration. The stereotyped progression of LB pathology described by Braak and Braak does not correlate with disease severity and duration (Jellinger 2009). Lately, the appearance of alpha-synuclein inclusions in embryonic foetal grafted striatal cells could suggest a prion-like transmission (Kordower *et al.* 2008), thus alpha-synuclein acting as a prion protein that could self-aggregate and be transmitted to unaffected cells. The latter may potentially lead to new therapeutic interventions for the treatment of PD, hence the need to developing new drugs directed in reducing the formation and facilitating the clearance of misfolded alpha-synuclein.

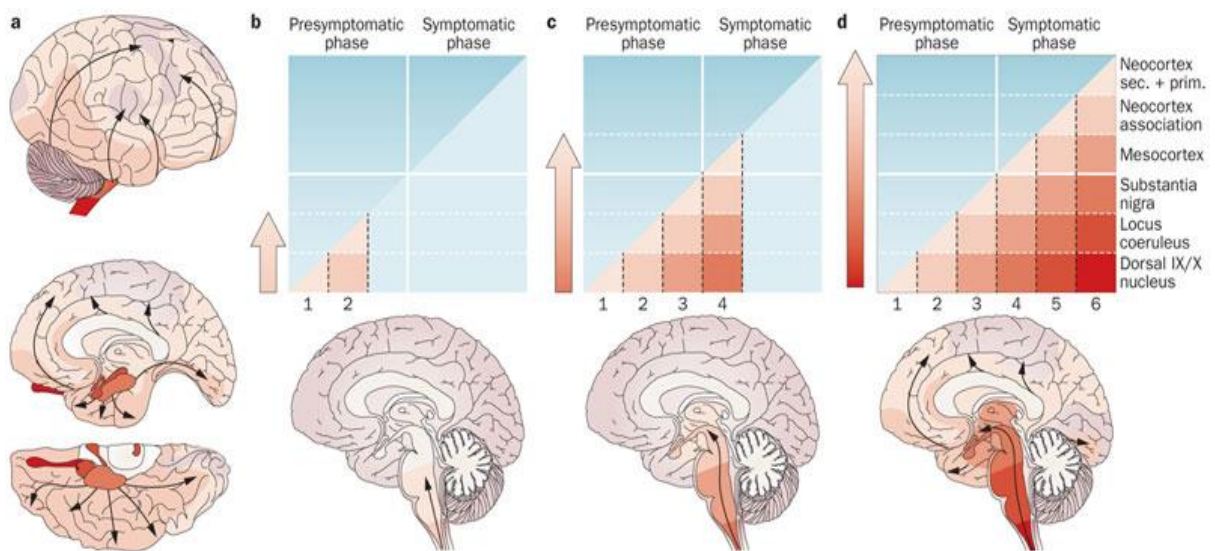


**Figure 1.2:** Six stages of Parkinson's disease pathology according to alpha-synuclein inclusions. Extracted from (Goedert *et al.* 2013). Pathologic alpha-synuclein deposition takes place in olfactory bulb, dorsal IX/X nucleus and LC in pre-symptomatic phase (stages 1 and 2) spreading to SN and mesocortex in the symptomatic motor phase (stages 3 and 4) and ultimately leading to the associative and primary neocortex in the symptomatic cognitive phase (stages 5 and 6).

### 1.4.3. Iron accumulation in Parkinson's disease

Iron is the most abundant transition metal found in the central nervous system (CNS). It plays an important role in many metabolic functions in the CNS including oxygen transportation, myelin synthesis, mitochondrial respiration, DNA synthesis and neurotransmitter synthesis and metabolism (Crichton *et al.* 2011).

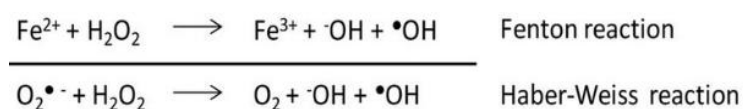
Iron accumulation in healthy ageing shows a selective and heterogeneous pattern of distribution. Hallgren and Sourander (Hallgren and Sourander 1958) determined the pattern of non-haemic iron in 81 post-mortem subjects with ages varying from 30 to 100 years. The highest iron values were found in the structures of the extrapyramidal system nuclei rapidly increasing after the first two decades with later stable plateau phase. In lesser degree motor cortex showed increased iron deposition when compared with the rest of cortical areas.

Increased iron accumulation has been traditionally associated to PD pathology. In 1924 Lhermitte (Lhermitte *et al.* 1924) described an abnormal increase of iron deposition in the globus pallidus with unknown significance in a single autopsy case with PD.

Quantitative techniques have showed increased nigral iron levels in PD (Dexter *et al.* 1987, Sofic *et al.* 1988, Youdim *et al.* 1989, Dexter *et al.* 1991, Sofic *et al.* 1991, Loeffler *et al.* 1995, Galazka-Friedman *et al.* 1996). Dexter *et al.* (Dexter *et al.* 1987, Dexter *et al.* 1991) reported an increase of 31-35% in the total iron content of the parkinsonian SN when compared to control tissue using inductively coupled plasma spectroscopy. Within the SN, the highest iron concentration was found in the SNc, with normal concentrations in SN pars reticulata. Furthermore, iron changes found in SN were increased in the form of ferric iron (III) and total iron (Sofic *et al.* 1988, Sofic *et al.* 1991). In addition, an increase of shift of PD of iron (III)/iron (II) in PD brains when compared to healthy conditions, from 1/1 to 3/1 (Youdim *et al.* 1989) could lead the production of hydroxyl radicals as consequence of a pro-oxidative environment.

### 1.4.3.1. Iron toxicity

Unbound intracellular iron is considered toxic as it is involved in the formation of reactive hydroxyl radicals via Fenton reaction and enhanced Haber-Weiss cycle (Youdim and Riederer 1993). The shift between Fe (II) to Fe (III) through a catalytic reaction with hydrogen peroxide via Fenton reaction can lead to the production of hydroxyl radicals through the Haber-Weiss reaction.



The excessive of production of hydroxyl radicals leads to oxidative stress (Halliwell 1989) subsequently causing lipid peroxidation of cellular membranes (Dexter *et al.* 1989), denaturation or aggregation of proteins, damage of DNA (Gutteridge 1994), proteosomal dysfunction (Reinheckel *et al.* 1998) and apoptosis.

In addition, dopamine can be a major source of reactive oxygen species in SN along with iron. Auto-oxidation of dopamine to its isoquinoline metabolites by monoamine oxidase releases hydrogen peroxide could produce hydroxyl radicals via Fenton's reaction (Halliwell 1989).

Whether iron represents the initiation of the neurodegeneration process or is secondary to nigral degeneration remains to be ascertained. A significant amount of iron induces selective damage to dopaminergic neurons through interaction with dopamine within the cell (Mochizuki 1993). On the other hand injection of MPTP into the caudate or putamen leads to marked increase in ferric iron in the ipsilateral SNc, indicating that injuring the nigrostriatal system can induce iron accumulation in the SN (Mochizuki *et al.* 1994).

The relationship between iron accumulation and dopaminergic degeneration was after MPTP injection was described by He *et al.* (He *et al.* 2003). A significant decrease of number of TH-positive cells occurred from 1 week whereas iron increased after 4.5 months. Iron accumulation was directly associated with dopaminergic loss. The

increased of iron seems to appear after dopaminergic degeneration thus contributing to the neuronal loss.

#### **1.4.3.2. Iron homeostasis alterations**

A number of mechanisms have been proposed in the pathogenesis of increased iron levels in PD. These include alteration of permeability of blood brain barrier, increased intraneuronal iron influx, alterations of ferritin and neuromelanin functions, imbalance of iron efflux and interaction between iron and alpha-synuclein (*Figure 1.3*).

A damaged or "leaky" blood brain barrier could lead a potential source of iron to the extra-neuronal matrix (Oestreicher *et al.* 1994) and might be exacerbated by increased age.

Increased neuronal iron transportation leads to elevated nigral iron levels. A down-regulation in the transferrin receptor binding (TfR1) (Faucheux *et al.* 1995) as well as elevation of nigral lactoferrin receptors (Leveugle *et al.* 1996) might cause an increase intra-neuronal iron pool. In addition abnormal activity of iron regulatory proteins (IRP1 and IRP2) and dimethyl transporter 1 (DMT1) (Kaur *et al.* 2009) contributes to an increase of iron transportation.

Ferritin is the most abundant iron storage protein in the brain. It contains heavy (H) and light (L) subunits (Connor *et al.* 1994). Unbound iron is sequestered by ferritin and considered non-toxic. Ferritin is found in microglia, non-pigmented neurons and degenerating pigmented neurons (Jellinger *et al.* 1990). A significant reduction of total ferritin in SN in PD subjects (Dexter *et al.* 1991) along with heavily laden ferritin (Griffiths *et al.* 1999) and reduction of total L-ferritin (Friedman *et al.* 2004) in microglia and neurons could justify the loss of iron storage capacity that potentially makes free iron species available for oxidative stress.

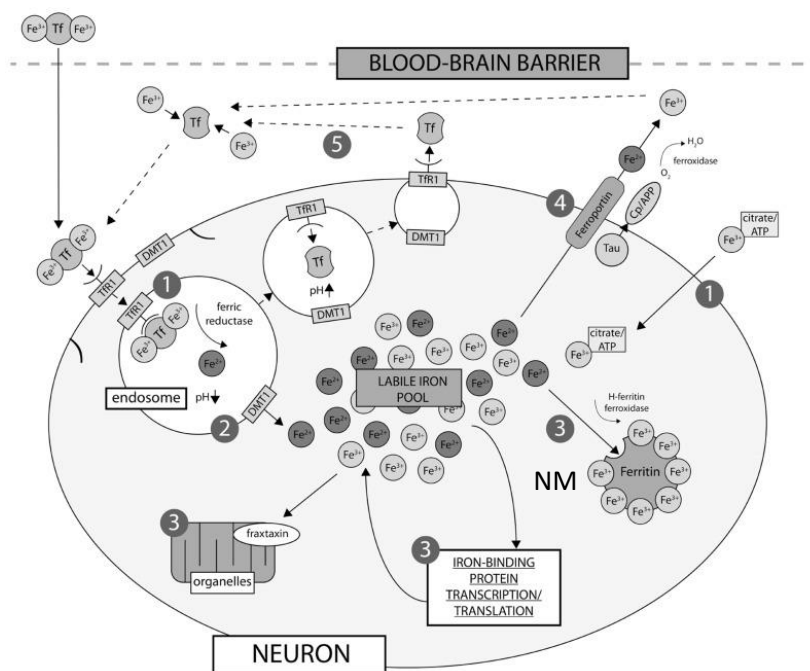


**Figure 1.3:** Neuronal iron homeostasis showing (1) intracellular iron import with citrate/ATP and transferrin receptor (TfR1); (2) intracellular transport with divalent metal transporter-1 (DMT-1); (3) labile intracellular iron is stored in ferritin and Neuromelanin (NM); (4) extracellular export mediated by ferroportin which is stabilized by tau and ceruloplasmin (Cp)/APP; (5) Re-circulation of iron by apo-Tf (5). Graph extracted from (Hare et al. 2013)

Neuromelanin (NM) is a dark complex pigment present in the dopaminergic neurons of SN and VTA and noradrenergic neurons of LC. It has been hypothesized a double protective and toxic role of NM in PD (Zecca et al. 2008). In healthy conditions NM is able to chelate iron and bind in the form of L-ferritin among other protective functions. However in pro-oxidative environments like PD, NM is moved to the extracellular matrix thus releasing the oxidative iron ( $\text{Fe III}$ ) (Double et al. 2003) potentially leading to oxidative reactions and microglial activation (McGeer and McGeer 2004). This pivotal pathogenic step will be explained in its correspondent chapter.

Alternatively, iron accumulation in PD could be caused by reduced neuronal iron efflux, due to loss of ceruloplasmin activity which in turn regulates the intramembrane ferroportin activity (Ayton et al. 2013). It was reported that reduced specific ferro-oxidase activity instead of ceruloplasmin levels is decreased in SN of PD, which in turn may be due to decreased nigral copper levels. In addition, loss of tau protein activity is

also associated to reduced APP-mediated iron efflux hence contributing to iron accumulation (Lei *et al.*, 2010).

Unbound iron directly promotes alpha-synuclein aggregation (Hashimoto *et al.* 1999) and indirectly iron released from NM blocks the mitochondrial ubiquitin proteasome system (McNaught *et al.* 2003). On the other hand redox-active iron accumulates in Lewy bodies in SN (Castellani *et al.* 2000) thus promoting oxidative stress and further neurodegeneration.

#### **1.4.4. Neuromelanin**

Neuromelanin (NM) is a complex, dark and insoluble pigment present in several neurons, specifically concentrated in the dopaminergic neurons of SN, VTA, the noradrenergic neurons of LC and in the ventrolateral reticular formation and nucleus of the solitary tract in the medulla oblongata (Zecca *et al.* 2004, Fedorow *et al.* 2005). Furthermore it was clearly demonstrated with electron microscopy and neurochemical investigations that NM could be also found in putamen, premotor cortex and cerebellum (Zecca *et al.* 2008, Engelen *et al.* 2012).

NM is dark brown to black under visible light pigment and it is similar to other human melanin. At electron microscopy level NM is distributed on irregularly shaped granules of varying electron density and size (0.5 – 2.5 nm). This pigment is located at opposing end of the nucleus in the neuronal soma surrounded by a double membrane. NM pigmented neurons of SN and LC appear as macroscopically dark areas (Sulzer *et al.* 2008) (*Figure 1.4*).

The exact structure of NM is still unknown. However, it seems to have 3D structure composed of melanin groups bound to dolichoic chains of beta-sheet peptides (Zecca *et al.* 2008, Engelen *et al.* 2012, Ferrari *et al.* 2017). In the melanic component there are dihydroxyindole and benzothiazine rings and DA-derived and cysteinildopamine-derived units of proteins that are involved in the melanin synthesis (Zecca *et al.* 2000). These components are sequestered in autophagic organelles and processed to form the final NM (Zecca *et al.* 2000).

It is still not clear whether NM synthesis is due to autoxidation process or it is enzymatically controlled. NM derives from the metabolism of catecholamines, dopamine (DA) in the SN and norepinephrine (NE) in the LC. NM synthesis appears to be activated by an excess of cytosolic catecholamines that are not accumulated into synaptic vesicles by the synaptic vesicular monoamine transporter 2 (VMAT2). In addition, dopamine seems to be oxidized via iron-mediated oxidation catalysis that leads to reaction with beta-sheet proteins and formation of melanin-protein complex (Zucca *et al.* 2015) .

The initiation of pigmentation in human brain starts at approximately 3 years of age and in adulthood NM fills almost half of the cytoplasm (Sulzer *et al.* 2000, Sulzer *et al.* 2008), NM continues to accumulate linearly in the SN during aging with a peak NM concentration between 50 and 90 years (Zecca *et al.* 2004). Fedorow and colleagues (Fedorow *et al.* 2006) suggested three phases in the production of NM consisting in first, initiation of production or synthesis which begin around the age of three, secondly growth with maturation, with implies a certain regulation of NM, and finally sustained maturation without excessive volumetric growth.

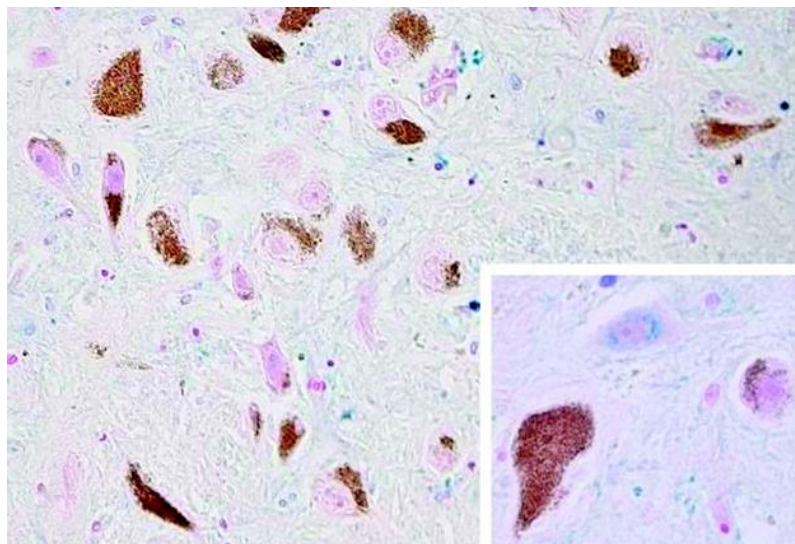


*Figure 1.4: Neuromelanin microscopic appearance and iron histochemistry with Perls' staining of human SNc from a non-PD 88-year-old male subject. Pigmented neurons are displayed as brown granules along with iron deposits as indicated in blue (Scale bar, 100  $\mu$ m). Image extracted from (Zecca *et al.* 2004)*

#### 1.4.4.1. The role of neuromelanin in normal conditions.

NM has a strong affinity for transition metals including iron, copper, lead and zinc (Zecca *et al.* 1994, Zecca *et al.* 2001, Zecca *et al.* 2008). Iron is sequestered by the melanic portion of NM and maintained in a redox inactive form (Shima *et al.* 1997, Zecca *et al.* 2008) hence NM could act as a “black hole” chelating redox-active metals.

NM binds iron in two different high- and low-affinity sites. Most of the iron is bound to high-affinity sites, but under iron overload conditions, the high-affinity sites of NM become saturated, and iron binds to the low-affinity sites (Fasano *et al.* 2006, Zecca *et al.* 2008). In the latter sites, iron is sequestered in a reactive form and could play a deleterious role by promoting redox reactions. Therefore, the binding of iron could have a protective role under physiological conditions, but a toxic gain of function may manifest when iron overload is present in the brain (Zecca *et al.* 2004).

NM appears to have antioxidant properties contributing to regulate cellular oxidative stress (Zecca *et al.* 2004). Furthermore, NM synthesis removes excess of catecholamines from the cytosol (Sulzer *et al.* 2000).

NM is able to bind a variety of organic molecules including lipids, pesticides and toxic compounds (Zecca *et al.* 1994, Zecca *et al.* 2001, Ito 2006, Zecca *et al.* 2008). The herbicide paraquat, which has a similar structure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has a strong affinity with NM (Lindquist *et al.* 1988). Antipsychotic drugs such as chlorpromazine and haloperidol, the antidepressant imipramine and the antimalarial chloroquine have a strong affinity for NM (Karlsson and Lindquist 2016). In addition, NM may also be associated with lipids like cholesterol and other high molecular poly-unsaturated complex, as lipids are part of more than 20 % of its structure (Zecca *et al.* 2000, Engelen *et al.* 2012).

#### 1.4.4.2. Neuromelanin in Parkinson's disease

In PD brains extracellular NM granules are preceded by nigral neurodegeneration, hence NM released from dying neurons is phagocytised by activated microglia. Microglia activation releases pro-inflammatory factors initiating an adaptive autoimmune response that leads to a vicious cycle of neurodegeneration and neuroinflammation (Wilms *et al.* 2003, Zecca *et al.* 2008, Zhang *et al.* 2011, Zhang *et al.* 2011). Extracellular NM also releases metals and toxic compounds stored in normal conditions intraneuronally, potentially resulting in increased oxidative stress. NM has also been reported to inhibit the 26S proteasome (Shamoto-Nagai *et al.* 2004), which may obstruct protein degradation causing accumulation of abnormal proteins such as alpha-synuclein aggregates (Figure 1).

Hirsch and colleagues (Hirsch *et al.* 1988) by using a quantitative analysis of NM pigmented neurons and Tyrosine Hydroxylase (TH) immunohistochemistry demonstrated the link between the pigmentation of SN and the vulnerability of nigral neurons to neurodegeneration in PD. They reported that healthy subjects showed a dense TH neuronal distribution in SN and in tegmental extranigral A8 and A10 while PD cases showed a marked reduction of TH neurons in SNc (77%) with moderate reduction of extranigral tegmental nucleus (A8 and A10 43% and 48% respectively) and a strong correlation with the number of NM-pigmented neurons. These findings suggest the direct correlation between PD cell loss and the percentage of NM pigmented neurons, thus showing a selective vulnerability of pigmented subpopulation of dopamine containing neurons.

Yamada and colleagues (Yamada *et al.* 1990) studied the distribution of calbindin-D28K (CaBP), a calcium binding protein, which may have a protective function in the pathological process of PD, in healthy volunteers and PD patients. The CaBP positive SN regions were found preserved when compared with controls, but a substantial reduction of NM pigmented neurons was found in the PD group and (70%) with many extra-neuronal deposits of NM due to neurodegeneration.

Furthermore, Gibb and Lees (Gibb and Lees 1991) examined the pigmented cell distribution healthy controls and PD subjects within the SNc. Authors reported the

heavily and lightly pigmented neurons were located in the dorsal and the ventrolateral tiers laying parallel to each other which in turn project to caudate and putamen respectively showing dorsal to ventral inverted relationship in healthy controls. Additionally, there was a complete loss of lightly pigmented neurons in the ventrolateral with moderate reduction of highly pigmented cells in dorsal SNc in the PD group reflecting greater dopamine depletion in the dorsal putamen compared to the relative indemnity of caudate nucleus.

The micro-architecture of SN in PD patients was studied by Fearnley and Lees (Fearnley and Lees 1991). In healthy controls a linear decrease of NM pigmented neurons in SNc was associated with age, showing a reduction of 4.7% per decade with regional selectivity in the dorsal and medial tiers. In the PD cases there was an exponential decrease of NM cells with 45 % of loss in the first decade of disease and a significant regional selectivity in the lateral ventral tier, thus showing an opposite pattern to that found in ageing processes.

Damier (Damier *et al.* 1999) used compartmental patterns of calbindin immunostaining to subdivide the pattern of loss of dopamine containing neurons in PD. The authors identified within the SNc, dopamine-containing neurons in the calbindin-rich regions named matrix and in five calbindin-poor neuronal subgroups which were labelled as “nigrosomes” and identified as 1, 2, 3, 4 and 5 according to their caudo-rostral localization. The degree of neuronal loss was significantly higher in the nigrosomes than in the matrix. Depletion was maximum (98%) in the main subgroup (nigrosome 1), located in the caudal and mediolateral part of SNc. Neuronal loss was also detectable in more medial and rostral nigrosomes, with nigrosome 1 > nigrosome 2 > nigrosome 4 > nigrosome 3 > nigrosome 5, therefore following a caudo-rostral gradient.

More recently, Kordower and colleagues (Kordower *et al.* 2013) studied the extent of nigrostriatal degeneration in 28 PD cases with different disease duration. A progressive reduction of 50% to 90% of TH dopaminergic cells in SN was found from the first year from diagnosis to more advanced disease. In addition 30 to 60% variable NM pigmented cell loss was observed, with greater NM /TH cells ratios at all the time points. This finding suggests that loss of dopamine markers precedes the loss of

pigmented cells implicating that there may be two different neuronal subpopulations in SNc with different neurodegenerative vulnerability.

## **1.5. Structural neuroimaging nigral studies in Parkinson's disease.**

### **1.5.1. Iron imaging studies in Parkinson's disease.**

A total of 27 cross-sectional studies using MRI iron sequences in PD, from them 13 with relaxometry techniques, 7 with Susceptibility Weighted Imaging and 8 with Quantitative Susceptibility Mapping) have been published to date (see *table 1.1*). Relaxometry techniques (T2\* and R2\*) are based in the molecular interactions of the T2 or R2 ( $=1/T2$ ) magnetic effect and variations in the magnetic field inhomogeneities (see chapter 2). Susceptibility weighted imaging (SWI) is a new contrast imaging that exploits the magnetic susceptibility from the susceptibility of neighbouring tissues using the phase images to detect these differences. Quantitative Susceptibility Mapping (QSM) provides a different contrast mechanism than SWI as it uses phase images solving the magnetic susceptibility inverse source problem, generating a three-dimensional susceptibility distribution. The majority of the aforementioned studies demonstrated a significant elevated nigral iron accumulation in PD when compared to HC (Antonini *et al.* 1993, Gorell *et al.* 1995, Ryvlin *et al.* 1995, Graham *et al.* 2000, Kosta *et al.* 2006, Martin *et al.* 2008, Zhang *et al.* 2009, Baudrexel *et al.* 2010, Gupta *et al.* 2010, Peran *et al.* 2010, Zhang *et al.* 2010, Du 2011, Jin *et al.* 2011, Du *et al.* 2012, Jin *et al.* 2012, Loftipour 2012, Wang *et al.* 2012, Lewis *et al.* 2013, Ulla *et al.* 2013, Wu *et al.* 2014, Barbosa *et al.* 2015, He *et al.* 2015, Murakami *et al.* 2015, Du *et al.* 2016, Hopes *et al.* 2016, Langkammer *et al.* 2016, Acosta-Cabronero *et al.* 2017, Guan *et al.* 2017) which is in agreement with the previous post-mortem studies. Increased nigral mineralization is an invariable finding in PD that is subsequently associated with dopaminergic degeneration. More importantly nigral iron accumulation is associated with disease severity as assessed by UPDRS-III motor score (Martin *et al.* 2008, Zhang *et al.* 2009, Baudrexel *et al.* 2010, Zhang *et al.* 2010, Du 2011, Jin *et al.* 2011, Jin *et al.* 2012, Lewis *et al.* 2013, He *et al.* 2015, Langkammer *et al.* 2016) and in lesser degree with Hoehn and Yahr staging (Jin *et al.* 2012, Wu *et al.* 2014, Langkammer *et al.* 2016) and disease duration (Lewis *et al.* 2013, He *et al.* 2015, Du *et al.* 2016) thus being considered a feasible biomarker of clinical severity in PD.

Results pertaining striatal iron accumulation are heterogeneous, with several studies reporting increases (Antonini *et al.* 1993, Graham *et al.* 2000, Hopes *et al.* 2016, Langkammer *et al.* 2016), reductions (Ryvlin *et al.* 1995, Kosta *et al.* 2006) or normal values in PD (Gorell *et al.* 1995, Martin *et al.* 2008, Peran *et al.* 2010, Zhang *et al.* 2010, Barbosa *et al.* 2015). One possible explanation for this heterogeneity could be due to the presence of calcifications in the striatum, which may cause SWI and relaxometry techniques to underestimate iron levels as well as the rostro-caudal gradient of striatal mineralisation, which in turn may be dependent on disease duration. A recent meta-analysis (Wang *et al.* 2016) examining post-mortem and in vivo imaging studies including relaxometry and SWI techniques concluded that imaging techniques may not authentically reflect iron changes in striatal regions other than SN. Interestingly two studies (Ryvlin *et al.* 1995, Graham *et al.* 2000) describe an inverse association between putaminal iron deposition and disease duration above of ten years.

More importantly, validation of the in vivo imaging techniques with the previously published iron concentration regions in post-mortem healthy volunteers (Hallgren and Sourander 1958) has been scarcely reported in the aforementioned studies (Graham *et al.* 2000, Martin *et al.* 2008, Zhang *et al.* 2010, Jin *et al.* 2011, Barbosa *et al.* 2015, He *et al.* 2015). A significant correlation between in vivo and post-mortem regional iron accumulation is mandatory to further undergoing cross-sectional imaging studies.

Clinical associations of motor iron accumulations in the PD baseline cohorts have been poorly described in the literature. Bunzeck *et al.* found a significant association between striatal iron accumulation and tremor symptomatology, suggesting that putaminal iron may be used as a predictor for tremor dominance in early PD (Bunzeck *et al.* 2013). However, their measures were collected in the “on” medication state hence not avoiding the confounding effect of variable clinical benefit with respect to tremor. More recently Guan *et al.* (Guan *et al.* 2017) showed significant associations of tremor subcores with dentate (DN) and red nuclei (RN) in a cohort of tremor-dominant PD patients suggesting that iron might play an important role in the dentato-thalamo-cortical pathways of tremor.

| Authors                    | Subjects        | Technique  | Field Strength | SN | Striatum  | Clinical associations                                                     |
|----------------------------|-----------------|------------|----------------|----|-----------|---------------------------------------------------------------------------|
| <b>Relaxometry studies</b> |                 |            |                |    |           |                                                                           |
| Antonini et al (1993)      | 30PD,<br>33HC   | T2         | 1.5            | ↑  | ↑ Put, CN | -                                                                         |
| Ryvlin et al. (1995)       | 45PD,<br>45HC   | T2         | 2              | ↑  | ↓ Put     | Put inversely correlates with DD                                          |
| Gorell et al (1995)        | 13PD,<br>10HC   | R2,R2*,R2' | 3              | ↑  | ↑↓        | SN correlates with motor performance (SRT, SMT)                           |
| Graham et al (2000)        | 21PD,<br>13HC   | R2, R2'    | 1.5            | ↑  | ↑ Put     | Put inversely correlates with DD                                          |
| Kosta et al (2006)         | 40PD,<br>40HC   | T2         | 1.5            | ↑  | ↓ Put, GP | -                                                                         |
| Martin et al (2008)        | 22PD,<br>11HC   | R2*        | 3              | ↑  | ↑↓        | SN and GP correlate UPDRS III                                             |
| Baudrexel et al (2010)     | 20PD,<br>20HC   | T2*        | 3              | ↑  | ↑↓        | SN correlates UPDRS III                                                   |
| Peran et al (2010)         | 30PD,<br>22HC   | R2*        | 3              | ↑  | ↑↓        | SN correlates LEDD                                                        |
| Du et al (2011)            | 16PD,<br>16HC   | R2*        | 3              | ↑  | ↑↓        | SN correlates UPDRS III and DD and LEDD.                                  |
| Du et al (2012)            | 40PD,<br>29HC   | R2*        | 3              | ↑  | ↑↓        | -                                                                         |
| Lewis et al (2013)         | 38PD,<br>23HC   | R2*        | 3              | ↑  | ↑RN       | SN correlates UPDRS III and DD and LEDD.<br>RN correlates with dyskinesia |
| Ulla et al (2013)          | 27PD,<br>26HC , | R2*        | 1.5            | ↑  | ↑↓        | -                                                                         |
| Hopes et al (2016)         | 70PD,<br>20HC   | R2*        | 3              | ↑  | ↑ Put, CN | -                                                                         |
| <b>SWI studies</b>         |                 |            |                |    |           |                                                                           |
| Zhang et al (2009)         | 42PD,<br>30HC   | SWI        | 3              | ↑  | ↑RN, CN   | SN correlates UPDRS III                                                   |
| Zhang et al (2010)         | 40PD,<br>26HC   | SWI        | 3              | ↑  | ↑↓        | SN correlates UPDRS III                                                   |
| Gupta et al (2010)         | 11PD,<br>11HC   | SWI        | 1.5            | ↑  | ↑ RN, Put | -                                                                         |

|                               |               |     |     |   |                              |                                                          |
|-------------------------------|---------------|-----|-----|---|------------------------------|----------------------------------------------------------|
| Wang et al (2012)             | 16PD,<br>44HC | SWI | 1.5 | ↑ | ↑ Put                        | -                                                        |
| Jin et al (2011)              | 45PD,<br>45HC | SWI | 3   | ↑ | ↑↓                           | SN correlates UPDRS III                                  |
| Jin et al (2012)              | 87PD,<br>50HC | SWI | 3   | ↑ | ↑↓                           | SN correlates UPDRS III and HY scales                    |
| Wu et al (2014)               | 54PD,<br>40HC | SWI | 3   | ↑ | ↑ Put, GP, CN, RN            | SN correlates with HY scale                              |
| <b>QSM studies</b>            |               |     |     |   |                              |                                                          |
| Loftipour et al (2012)        | 9PD,<br>11HC  | QSM | 7   | ↑ | -                            | -                                                        |
| Barbosa et al (2015)          | 20PD,<br>30HC | QSM | 3   | ↑ | ↑↓                           | -                                                        |
| Murakami et al (2015)         | 21PD,<br>21HC | QSM | 3   | ↑ | -                            | -                                                        |
| He et al (2015)               | 44PD,<br>35HC | QSM | 3   | ↑ | ↑ RN                         | SN correlates UPDRS III and DD                           |
| Du et al (2016)               | 47PD,<br>47HC | QSM | 3   | ↑ | ↑↓                           | SN correlates DD and LEDD                                |
| Langkammer et al (2016)       | 66PD,<br>58HC | QSM | 3   | ↑ | ↑ GP, Tha, RN                | SN correlates UPDRS III, LEDD and HY                     |
| Guan et al (2017)             | 62PD,<br>40HC | QSM | 3   | ↑ | ↑ DN, RN in TD<br>↑ CN in AD | DN and RN correlates tremor. CN correlates with akinesia |
| Acosta-Cabronero et al (2017) | 25PD,<br>30HC | QSM | 3   | ↑ | ↑ RN                         | -                                                        |

**Table 1.1:** Cross-sectional iron-sensitive MR imaging studies in Parkinson's disease. PD= Parkinson's disease; HC= Healthy Controls;  $R2' = R2^* - R2$ ; SWI= Susceptibility weighted imaging; QSM= Quantitative susceptibility mapping; SN= Substantia nigra; Put= Putamen; GP= Globus pallidus; CN= Caudate nucleus; RN= Red nucleus; Tha= Thalamus; DN= Dentate nucleus; DD= Disease duration; SRT= Simple reaction time; SMT= Simple movement time; UPDRS III= Unified Parkinson's disease rate motor scale; HY= Hoehn and Yahr scale; TD= Tremor dominant; AD= Akinetic dominant.

Four longitudinal relaxometry studies in PD have been published to date (*Table 1.2*). Increases in nigral iron accumulation have been shown to correlate with global motor severity of PD (Ulla *et al.* 2013, Rossi *et al.* 2014, Wieler *et al.* 2015, Hopes *et al.* 2016) with heterogeneous results pertaining to striatal nuclei. The inconsistency in previous findings may be a result of heterogeneity within the PD cohorts, including small sample size, variable disease duration and suboptimal clinical evidence (one study performed in on-medication state). The heterogeneity of iron deposition in the midbrain measured longitudinally might be also due to the effect of water content in the T2\*/R2\* signal, offering a technical bias for monitoring the progression of the disease.

| Authors             | Subjects   | Technique | Field Strength | SN | Striatum  | Clinical associations   |
|---------------------|------------|-----------|----------------|----|-----------|-------------------------|
| Ulla et al (2013)   | 19PD, 11HC | R2*       | 1.5            | ↑  | ↑ Put     | SN correlates UPDRS III |
| Rossi et al (2014)  | 20PD, 30HC | R2*       | 3              | ↑  | ↑ GP, Tha | -                       |
| Wieler et al (2015) | 21PD, 21HC | R2*       | 3              | ↑↓ | ↑↓        | SN correlates UPDRS III |
| Hopes et al (2016)  | 44PD, 35HC | R2*       | 3              | ↑  | ↑ Put, CN | SN correlates UPDRS III |

**Table 1.2:** Longitudinal iron-sensitive MR imaging studies in Parkinson's disease. PD= Parkinson's disease; HC= Healthy Controls; SN= Substantia nigra; Put= Putamen; GP= Globus pallidus; CN=Caudate nucleus; Tha= Thalamus; UPDRS III= Unified Parkinson's disease rate motor scale.

### 1.5.2. Neuromelanin imaging studies in Parkinson's disease

Enochs and colleagues (Enochs *et al.* 1997) were the first to quantitate the binding of metals to synthetic melanin in vitro, and to test whether such binding by natural melanin can be detected in cultured melanoma cells in vivo with MR imaging. They indeed proved that melanotic melanomas are hyperintense on T1-weighted images because of paramagnetic metal scavenging.

Many of the recent imaging studies have used high resolution T1 weighted (T1w) with Fast Spin Echo (FSE) on 3 Tesla MR to visualize NM-weighted contrast in midbrain and brain tissue of the NM-pigmented nuclei. The contrast obtained in Neuromelanin-weighted MRI seems to be directly related to reduction of T1 from the melanin-compounds. In addition magnetization transfer MT also helps distinguishing NM-rich areas from surrounding brain tissue, however it is not directly affected by the presence of NM (Trujillo *et al.* 2016).

A total of 11 cross-sectional and one follow up studies using NM-weighted MRI in PD have been published to date (see *table 1.3*). A common finding of the aforementioned studies is the reduction of NM contrast in the SN in PD subjects when compared to HC (Montgomery and Asberg 1979, Sasaki *et al.* 2006, Kashihara *et al.* 2011, Schwarz *et al.* 2011, Ogisu *et al.* 2013, Ohtsuka *et al.* 2013, Castellanos *et al.* 2015, Reimao *et al.* 2015, Reimao *et al.* 2015, Matsuura *et al.* 2016, Fabbri *et al.* 2017). Results pertaining LC depigmentation are heterogeneous, with several studies reporting reductions (Sasaki *et al.* 2006, Castellanos *et al.* 2015) or normal values in PD (Schwarz *et al.* 2011, Ohtsuka *et al.* 2014).

The first approach to measuring NM-sensitive T1w FSE contrast was performed by Sasaki and colleagues (Sasaki *et al.* 2006). In this study PD patients and age-matched healthy volunteers were scanned with T1 weighted FSE and then the imaging compared with macroscopic specimens at the level of midbrain and upper pons from two cadavers, one with pathologically diagnosed PD and one without any neurological disease. Measurement of high T1 signal intensity was performed in SNc and LC with circular Regions of Interest (ROIs) along with measurement of the surrounding pontine

tegmentum and decussation of the superior cerebellar peduncles. Contrast ratios of LC/pontine tegmentum and SNc/superior cerebellar peduncles were significantly reduced in PD groups when compared to healthy volunteers. T1w FSE findings in the midbrain showed NM distribution in the SNc in a band-like high signal similar to the post-mortem specimen. In addition radiological findings at the pons level of LC showed high signal “spots” due to NM deposition in LC identical to the gross specimen.

Ohtsuka and colleagues studied regional differences of NM-contrast within the SNc according to the rostral-caudal gradient of neuronal loss in PD (Ohtsuka *et al.* 2013). Significant reduction in lateral and central SNc contrast ratios were found in the PD patients group when compared to healthy controls. These results support the neuropathological finding that neurodegeneration in SNc in PD progresses from lateral to central to medial regions.

Several works have described automated and semi-automated approaches of NM-weighted MRI (Ogisu *et al.* 2013, Castellanos *et al.* 2015, Reimao *et al.* 2015, Reimao *et al.* 2015). Ogisu *et al.* (Ogisu *et al.* 2013) reporting that at higher thresholds, the area under the curve and likelihood ratios, based on receiver operative characteristic (ROC) analysis, increased with balanced sensitivity and specificity at earlier stage of PD. Furthermore Castellanos *et al.* (Castellanos *et al.* 2015) showed that contralateral atrophy in the SNc resulting from decreased NM contrast showed the highest power to discriminate PD subjects from controls. Reimao *et al.* (Reimao *et al.* 2015) also reported high specificity in discriminating essential tremor (ET) from early stage PD (93%) using semi-automated MRI analysis.

Clinical associations of nigral depigmentation in PD showed heterogeneous results. Reduction of nigral NM load inversely correlates with disease duration (Kashihara *et al.* 2011, Schwarz *et al.* 2011, Matsuura *et al.* 2013), Hoehn and Yahr staging (Kashihara *et al.* 2011, Matsuura *et al.* 2013, Fabbri *et al.* 2017), total UPDRS scale (Schwarz *et al.* 2011) and UPDRS part II (Fabbri *et al.* 2017). Surprisingly no associations with UPDRS motor scale have been previously shown with the reduction of nigral NM contrast.

To date only one longitudinal study using NM-weighted MRI (Matsuura *et al.* 2016) has showed significant reductions in NM load in PD over time (annual decline of 12%).

Longitudinal reductions of nigral pigmentation in PD were associated with temporal changes in disease duration. The authors however included different follow-up time-points in the study as well as no healthy controls.

The relationship between NM distribution and iron accumulation in SN is yet to be ascertained. Reimão et al (Reimao *et al.* 2016) reported marked reduction in NM load was found along SN in PD patients with no significant differences in the nigral mineralization when assessed with T2\*. The authors confirm the lack of correlation between iron and NM in SNc suggesting that both pathophysiological pathways present in neurodegeneration might not be parallel in PD.

Nigral pigmented load might be useful to explore the nigrostriatal pathway integrity in PD in combination with striatal dopamine transporter SPECT imaging. Recently Isaias et al (Isaias *et al.* 2016) assessed the relationship between NM load and iron accumulation in SN with dopaminergic striatal markers in equally sized PD and healthy volunteers groups. Nigral contrast ratios and NM volumes positively correlated with striatal DAT uptake values, however no other associations with nigral iron accumulation were demonstrated. These results suggest that NM-weighted MRI could be potentially used in combination with Positron Emission Imaging to further study nigrostriatal pathway abnormalities in PD.

| Authors                  | Subjects                                 | Technique   | Field Strength | SN | LC | Clinical associations                 |
|--------------------------|------------------------------------------|-------------|----------------|----|----|---------------------------------------|
| Sasaki et al (2006)      | 17PD, 22HC                               | T1w FSE     | 1.5            | ↓  | ↓  | -                                     |
| Kashikara et al (2011)   | 80PD, 54HC                               | T1w FSE     | 3              | ↓  | -  | SN correlates with DD and HY          |
| Schwarz et al (2011)     | 10PD, 12HC                               | T1w FSE, MT | 3              | ↓  | ↑↓ | SN correlates with DD and UPDRS total |
| Otshuka et al (2013)     | 37PD, 22HC                               | T1w 3D FSE  | 3              | ↓  | ↑↓ | Reduced in lateral/central SN         |
| Matsuura et al (2014)    | 32PD, 23HC                               | T1w FSE     | 3              | ↓  | ↓  | SN correlates with DD and HY          |
| Reimão et al (2015)      | 22PD (12 de-novo, 10 ES)<br>30HC         | T1w FSE     | 3              | ↓  | -  | -                                     |
| Reimão et al (2015)      | 12PD, 10ET, 10HC                         | T1w FSE     | 3              | ↓  | -  | -                                     |
| Castellanos et al (2015) | 23 iPD,<br>8 LRRK2,<br>5 Parkin,<br>23HC | T1w FSE     | 1.5            | ↓  | ↓  | No differences between PD groups      |
| Matsuura et al (2016)    | 14 PD follow-up                          | T1w FSE     | 3              | ↓  | -  | Correlation with DD.                  |
| Fabbri et al (2017)      | 35 PD (12 DN, 10 ES, 13 LS), 10HC        | T1w FSE     | 3              | ↓  | -  | Correlation with UDPRS II and HY      |

**Table 1.3:** Neuromelanin-weighted MRI studies in Parkinson's disease. *PD= Parkinson's disease; HC= Healthy Controls; DN= De novo; ES= Early stage PD; LS= Late stage PD; ET= Essential tremor; iPD= Idiopathic PD; LRRK2= Leucine-rich repeat kinase 2 mutation; SN= Substantia nigra; LC= Locus Coeruleus; UPDRS= Unified Parkinson's disease rate scale; HY= Hoehn and Yahr scale.*

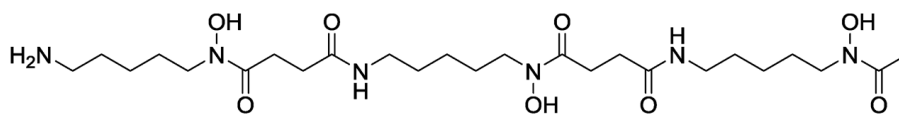
### 1.6.Targeting iron in Parkinson's disease.

Chelation aims at removing local iron accumulation from toxic labile iron that promotes oxidative damage without interfering with essential local functions(Cabantchik *et al.* 2013). The link between chelation and neurodegenerative diseases has been strengthened by the clinical benefit of copper chelation in Wilson's disease and manganese chelation in SLC30A10 manganese transporter deficiency.

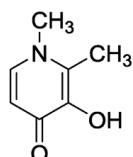
Traditional chelation strategies for systemic siderosis are based on removing the excess of metal with subsequent excretion via faecal and urinary pathways (Ma *et al.* 2012). Neurodegenerative conditions such as PD could be considered regional siderosis thus needing a different chelation approach aiming to preserve either regionally and systemically chelated iron by redeploying it to its original site (Cabantchik *et al.* 2013). Iron redeployment strategies depend on the scavenging of labile-iron pools responsible of oxidative stress, by delivering to extracellular apotransferrin and redeploying it to cell acceptors (Cabantchik *et al.* 2013). Chelators are meant to access to the intracellular labile unbound iron pool which is directly involved in oxidative stress (Gaeta and Hider 2005).

Several orally available iron chelators such as desferrioxamine, deferiprone and deferasirox (*Figure 1.5*) are effectively used in the clinical management of systemic iron overload siderosis such as haemachromatosis or thalassemias, with the occurrence of minimal side effects in the long term use.

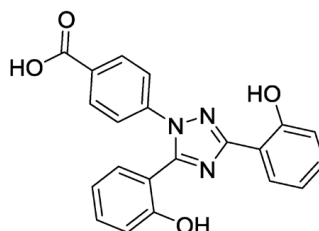
Deferiprone (DFP) is a bidentate iron chelator which binds iron in a 3:1 ratio (Cohen *et al.* 2003) thus three molecules of deferiprone are required to bind each ferric iron. DFP is a small lipophilic molecule with short half-life that is able to cross the blood-brain barrier. This chelator can readily penetrate several cells including neurons and removing intracellular labile pools of iron to extracellular transferrin (al-Refaie *et al.* 1995). DFP also facilitates transfer of iron from extracellular matrix and endosomes to nuclei and mitochondria. DFP is metabolised by glucuronidation in the liver. The resulting deferiprone glucuronidate is a stable complex that is not able for chelating iron, is excreted into the urine through the kidneys. A minimal amount of the chelation is excreted in the faeces (al-Refaie *et al.* 1995).



Desferrioxamine B (1)



Deferiprone (2)



Deferasirox (3)

**Figure 1.5:** Chemical structure of iron-chelating agents: desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFS). Image taken from (Ward *et al.* 2015).

The most severe side effect of deferiprone is agranulocytosis and mild neutropenia, which are defined as absolute neutrophil count (ANC) of less than  $0.5 \times 10^9/L$  and  $500-1500 \times 10^9/L$  respectively. The rate of agranulocytosis has been estimated at 0.2 per 100 patient-years whereas for neutropenia it is 2.8 per 100 patients-year (Ceci *et al.* 2002, Cohen *et al.* 2003). These haematological side effects disappear with the total cessation of the drug. For this reason weekly blood counts are required for strict monitoring of ANC.

Other adverse effects of deferiprone include gastrointestinal discomfort, arthralgia and transient elevation of alanine transaminase (ALT). These symptoms usually disappear after a few weeks of deferiprone's withdrawal.

### **1.6.1. Iron Chelation in Neurodegenerative disorders**

Several attempts of iron chelation in neurodegenerative conditions have been previously described in the scientific literature. Systemic improvement with variable neurological outcomes after chelation with DFO, DFP and DFS has been extensively reported in aceruloplasminemia (Miyajima *et al.* 1997, Fasano *et al.* 2008). Moderate improvement of dystonic symptoms when DFP is combined with intrathecal baclofen was described in some case controls of Pantothenate kinase-associated neurodegeneration (PKAN) (Zorzi *et al.* 2011, Cossu *et al.* 2014). Furthermore chelation treatment showed moderate improvement of ataxia, dystonia and chorea in idiopathic Neurodegeneration with brain iron accumulation (NBIA) (Forni *et al.* 2008).

Importantly, DFP was used in patients with Friedreich ataxia (Boddaert *et al.* 2007, Velasco-Sanchez *et al.* 2011) with clinical improvement of neurological symptoms and subsequent reduction of cerebellar dentate nucleus iron deposition as assessed with relaxometry MR techniques. This drug was well tolerated in a relative small group of young patients with low and high dosages (20 and 30 mg/kg/day respectively).

### **1.6.2. Iron chelation in Parkinson's disease.**

The important link between iron deposition and pathophysiology in PD leads to iron chelation as a possible disease-modifying and neuroprotective therapeutic option. The neuroprotective effect of oral iron chelators was examined in a 6-hydroxydopamine (6-OHDA) animal model (Dexter *et al.* 2011). In this study intraperitoneal administration of DFP, DFO and DFS showed a reduction of nigral dopaminergic total neurons by 6-OHDA as well as decreased dopamine striatal turnover in the lesioned side when compared to the contralateral non-lesioned side. Furthermore the formation of hydroxyl radical species (OH-TA) was significantly reduced after the administration of chelators. The post-hoc analyses performed within the three oral chelators demonstrated DFP as the most significant changes in total loss of dopaminergic nigral neurons differences and striatal dopaminergic turnover when compared with DFS and DFO.

So far only one study has investigated the efficacy and safety of deferiprone in a double-blinded placebo clinical trial in PD (Devos *et al.* 2014). The authors recruited 20 early PD volunteers administering DFP (30 mg/kg/day) in a delayed start paradigm over twelve months. The therapeutic effect of the iron chelator was assessed with R2\* MRI imaging as well as examining the motor severity with UPDRS III. Reduction of nigral iron deposition as measured with R2\* was associated with slight motor improvement in the early start group after 6 month of treatment with DFP. These results persisted in the delayed start group and ever after cessation of the treatment. During the pilot study one case of agranulocytosis and two cases of neutropenia were resolved after two weeks of DFP cessation.

Further chelating studies in PD should be based in the administration of several dosages of DFP in order to ascertain the clinical and radiological benefit as well as the side effects related to the iron chelators.

## 1.7. Summary of aims and group investigations

*Assessment of iron accumulation in Parkinson's disease using high-pass filtered imaging (chapter 3):*

- a. To evaluate differences in iron accumulation in deep brain nuclei in PD groups of varying motor severity at baseline and after 18 months' time.
- b. To investigate the association between motor symptomatology and iron load in a large cross-sectional and longitudinal PD cohort.

*Assessment of nigral depigmentation in Parkinson's disease using Neuromelanin-weighted imaging (chapter 4):*

- a. To assess nigral pigmentation differences between PD and HC groups at baseline and after 20 months' time.
- b. To investigate the clinical relationship of nigral pigmentation load with disease duration, clinical laterality, motor severity and motor phenotypes in a large cross-sectional and longitudinal PD cohort.

*Assessment of nigrostriatal pathways with multimodal imaging assessment using Neuromelanin-weighted MR imaging and  $^{11}\text{C}$ -PE2I PET in Parkinson's disease (Chapter 5):*

- a. To assess the regional pattern of neuromelanin signal intensity loss within the SNc between PD and HC groups.
- b. To describe the relationship between regional subdivision of nigral-pigmented load with Neuromelanin-weighted MR imaging and striatal dopamine transporter measured with  $^{11}\text{C}$ -PE2I in a moderate-stage PD cohort.

*Iron chelation with deferiprone as therapeutic target in Parkinson's disease (Chapter 6):*

- a. To evaluate changes in brain iron content by T2\* MR imaging as monitoring tool of chelation with deferiprone.
- b. To assess the drug safety in PD.
- c. To assess subjects' clinical status as characterised by PD disability using UPDRS III, mood and quality of life scales.
- d. To explore iron homeostasis and peripheral inflammation markers during chelation in PD.

## 2. Subjects and Methodology

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### 2.1. Subjects

#### 2.1.1. Parkinson's disease magnetic repository (PAMIR) study

The PaMIR (Parkinson's disease magnetic repository) is a multi-centre imaging study that aims translating recent advances of 3 Tesla MRI into clinically useful biomarkers in Parkinson's disease.

The main hypotheses of this project is that multimodal MRI at 3 Tesla in PD will support the diagnosis of PD in early stages, predicting the clinical subtypes and disease severity as well allowing assessment of disease progression.

This multi-centre study was conducted at Imperial College London, University of Nottingham, University of Newcastle and Glasgow NHS Hospitals. Research Ethical Approval from PaMIR study was obtained on 23/04/2014 and authorised for Professor Dorothee P Auer, PAMIR Chief Investigator. Furthermore Imperial College R&D approval was obtained on 19/09/2014 authorising Professor Paola Piccini as local principal investigator.

Patients were recruited from specialist movement disorder clinics at Hammersmith Hospital, Imperial College Healthcare NHS trust and London and National Hospital for Neurology and Neurosurgery, University College London Hospitals NHS Foundation Trust.

At the time of this thesis submission a total of 75 subjects (55 PD and 20 HC) were recruited at baseline and 18 months later. This study is still on going in the other three UK centres.

The inclusion criteria for PD patients are subjects with the diagnosis of PD based in the UK Brain Bank criteria made within the preceding three years with age of less of 90

years and willing to provide informed consent. PD volunteers were allowed to join the study after starting antiparkinsonian regime. Healthy subjects with similar age and gender than the PD group will be also included.

The exclusion criteria of PaMIR study include:

- Patient with severe comorbid illness.
- Patients with atypical parkinsonism, such as progressive supranuclear palsy or multiple system atrophy.
- Symmetrical lower body parkinsonism due to cortical and/or subcortical cerebrovascular disease
- Normal functional imaging of the presynaptic dopamine system
- Participants with any contraindication to MRI like intra-ocular metallic foreign bodies, intracranial aneurysm clips, cardiac pacemakers and defibrillators, cochlear implants or MR-incompatible metal implant or tattoos.
- Patients with significant head tremor
- Claustrophobia
- Pregnant women

### **2.1.2. The TransEurtudy**

The TransEuro multi-centre study aims to demonstrate that replacement of dopaminergic foetal cells in PD can be clinically efficacious in the absence of troublesome off-state motor complications, the so-called graft-induced dyskinesias, in patients with mild/moderate PD. The treatment rational is based in the development of safe methodology using allo-transplants of dopaminergic neuroblasts obtained from foetal ventral mesencephalic tissue in patients with PD with minimal side effects.

The TransEuro imaging study ('An imaging study to assess structural and functional connectivity in a longitudinal study of patients with Parkinson's disease', 10/H0805/73) obtained the ethical approval on 10/01/2012 being authorised for Professor Paola Piccini as local principal investigator. In the present thesis only pre-transplanted subjects were included. As part of the TransEuro study a neuroimaging protocol including MR and PET were included pre and post intervention.

The recruitment of PD subjects were recruited from specialist movement disorder clinics from three UK centres: Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge; National Hospital for Neurology and Neurosurgery, University College London Hospitals NHS Foundation Trust and Charing Cross Hospital, Imperial College Healthcare NHS Trust, London and from one centre in Sweden, Skåne University Hospital, Lund.

The inclusion criteria for the TransEuro study are subjects with the diagnosis of PD based in the UK Brain Bank criteria with disease duration between 2 and 13 years, with less of 68 years and Hoehn and Yahr scale less of 2.5 in "on" medication state. PD subjects should not have significant levodopa induced dyskinesias (AIMS less of 2).

Additional exclusion criteria of TransEuro not included in the PaMIR study are listed as follows:

- Clinical symptoms or early cognitive impairment such as Mini-Mental State Examination (MMSE) score of less than 26 or inability in copying interlocking pentagons.
- Patients with severe medical or psychiatric conditions.
- Patients undergoing concomitant regimes with neuroleptics and cholinesterase inhibitors.
- Moderate to severe levodopa-induced dyskinesia (AIMS >2)
- Previous history of neurosurgery, organ transplantation or cell therapy.
- Any contraindication of immunosuppression or anticoagulation.
- Previous neurosurgery, cell therapy or organ transplantation.

### **2.1.3. A pilot clinical trial with the iron chelator Deferiprone in Parkinson's disease**

This pilot double-blind, randomised, non-crossed over placebo controlled clinical trial aims to assess whether six months of treatment with clinical chelator Deferiprone is well tolerated by patients with PD as well as evaluating whether this treatment removes iron from brain regions affected in PD and is associated with clinical benefit.

This clinical trial was conducted at Imperial College London between October 2011 and October 2013. The present study (“A pilot clinical trial with the iron chelator Deferiprone in Parkinson’s disease”, 11/SC/0101; EudraCT 2011-001148-31) obtained ethical approval on 11/05/11 authorised for Professor David T Dexter as chief investigator and Professor Paola Piccini as co-investigator.

Patients were originally identified from specialist movement disorder clinics from two centres: Hammersmith and Charing Cross Hospitals, Imperial College Healthcare NHS trust.

The inclusion criteria for the present study are subjects with the diagnosis of PD based in the UK Brain Bank criteria with disease duration of less of 5 years with stable response to standard anti-Parkinsonian medication for greater than 6 weeks.

The exclusion criteria include:

- Patients with other neurological conditions, psychiatric disorders such as major depression.
- History of diabetes, renal, liver or haematological disorders.
- Patients receiving Rasagiline or Amantadine.
- Patients who are pregnant or breastfeeding.
- Patient immunocompromised with history of neutropenia, agranulocytosis or HIV.
- History of hypersensitivity with deferiprone.

## **2.2. Clinical assessments.**

Clinical motor examination of PD patients was performed using UPDRS part III motor scale (Goetz *et al.* 2008) For the PaMIR and TransEuro studies, motor examination was performed in “off-medication” state of 24 hours withdrawal of antiparkinsonian

medication. For the pilot clinical trial with deferiprone in PD motor examination was undertaken in “on-medication state”.

UPDRS part III motor scores were subdivided into clinical symptoms including bradykinesia (sums of items 2, 4-9 and 14), rigidity (item 3), tremor (sum of items 15-18) and axial symptoms (sum of items 1, 9-13). In addition a non-computerised motor tap test was also administered in the TransEuro study, which patients were instructed to produce as many alternating button presses as possible within 30 seconds.

Additional clinical evaluations were undertaken such as modified Hoehn and Yahr scale (Goetz *et al.* 2004) and disease duration measured in years.

Furthermore non-motor examinations included in this thesis are:

- Depression questionnaires; Montgomery-Åsberg scales (Montgomery and Asberg 1979)
- PDQ 39 questionnaires (quality of life) (Jenkinson *et al.* 1997)

The total equivalent levodopa daily dosage (LEDD) was calculated according to Tomlison (Tomlison 2010) formula.

### **2.3.Magnetic resonance imaging**

Magnetic resonance imaging (MRI) is a non-invasive imaging technique based in the principle of magnetic resonance using radiofrequency waves to study of tissue structure and function not requiring ionizing radiation. Radio waves are magnetic fields that oscillate in time with a determined frequency defined by number of oscillations per second (measured as Hertz) and amplitude.

The resonance phenomenon is explained by the rotation, the so-called spin, and precession of the hydrogen nuclei (protons) (*Figure 2.1*). Spin is a fundamental property of nuclei making them magnetic along the rotational axis. In addition there is a north rotation in a conical motion defined as precession.

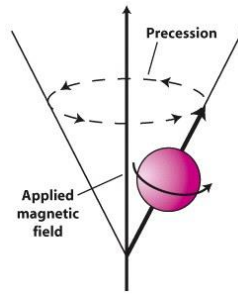


*Figure 2.1: Magnetic properties (rotation and precession) of the  $^1\text{H}$  proton nucleus when magnetic field is applied.*

To further understand precession frequency the so-called Larmour equation describes the connection between magnetic field ( $B_0$ ) and the resonance frequency:

$$f = \gamma B_0$$

In this equation, frequency ( $f$ ) depends to the gyromagnetic ratio ( $\gamma$ ) and proportional to the magnetic field ( $B_0$ )

### 2.3.1. $T_1$ and $T_2$

At equilibrium the total net magnetization vector follows the direction of the magnetic field ( $B_0$ ) and it is known as equilibrium magnetization ( $M_0$ ). The time constant, which determines how longitudinal magnetization ( $M_z$ ) returns to its equilibrium is called spin lattice relaxation time ( $T_1$ ):

$$M_z = M_0 (1 - e^{-t/T_1})$$

The time constant which expresses the recurrence to equilibrium of the transverse magnetization,  $M_{XY}$ , is called the spin-spin relaxation time,  $T_2$ :

$$M_{XY} = M_{XY_0} e^{-t/T_2}$$

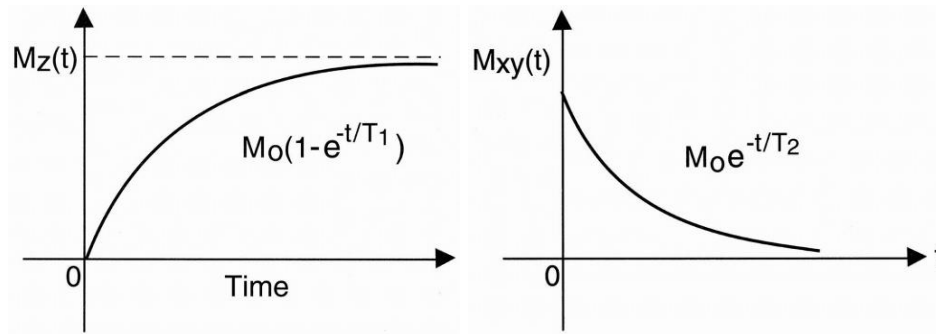


**Figure 2.2:** Diagram showing spin lattice relaxation ( $T_1$ , left) and spin-spin relaxation ( $T_2$ , right) times.

In summary,  $T_1$  is the time to reduce the difference between the longitudinal magnetization ( $M_z$ ) and its equilibrium value by a factor of  $e$ ; whereas  $T_2$  the time to reduce the transverse magnetization ( $M_{xy}$ ) by a factor of  $e$ .

### 2.3.2. Relaxometry

Relaxation ( $T_2^*$ ) is defined by molecular interactions of the  $T_2$  magnetic effect and variations in the magnetic field due to tissue inhomogeneities, being defined by the next formula:

$$1/T_2^* = 1/T_2 + 1/T_{2\text{inhomogeneities}}$$

Transverse relaxation ( $T_2^*$ ) is obtained by angling the longitudinal relaxation ( $T_1^*$ ) into transverse plane by using a pulse of radiofrequency.  $T_2^*$  precipitates irreversible dephasing of the transverse magnetization. In addition there is a reversible field dephasing effect originated by the local inhomogeneities.

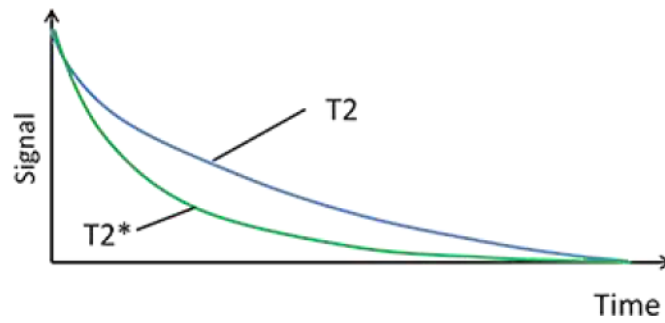


**Figure 2.3:** Diagram showing the T2 (blue) and T2\* relaxation (green) decay curves

The speed of relaxation is based on the firmness of the tissue or inhomogeneities that limit the water free movements originating a reversible field dephasing effect. Local inhomogeneities could be considered as paramagnetic and diamagnetic such as iron and calcium respectively.

Magnetic field inhomogeneities could be macroscopic (changes inter voxels) as air-tissue interfaces and microscopic (changes within voxels) like iron accumulation.

T2\* is seen with gradient-echo imaging (GRE) as transverse relaxation caused by magnetic field inhomogeneities is eliminated by the 180 degrees pulse at spin-echo imaging. T2\*-based GRE sequences are more sensitive to T2\* decay by changing parameters by using long echo time (TE) and repetition time (TR) and short flip angle. GRE sequences are specifically sensitive to iron-induced local field inhomogeneities, thus T2\* (or  $R2^* = 1/T2^*$ ) are often used to evaluate iron deposition.

This technique however presents several limitations. T2 is highly influenced by water content of brain tissue water thus limiting the value of iron-related structural pathology (Haacke *et al.* 2005). On the other hand for smaller amounts of iron there might be little noticeable effect on T2\* hence underestimating the total iron content.

T2\* has been used in a number of studies in order to quantify the iron accumulation in PD (see chapter 1). In this thesis I will undertake the analysis of iron deposition of deep brain grey nuclei with T2\* imaging in order to ascertain the clinical effect of iron chelation with deferiprone (chapter 6).

### 2.3.3. Susceptibility weighted imaging

Susceptibility weighted imaging (SWI) is a new contrast imaging that exploits the magnetic susceptibility in a tissue that differs from the susceptibility of neighbouring tissues. Magnetic susceptibility is defined as the magnetic response of a substance or tissue when an external radiofrequency pulse is applied. Magnetic susceptibility ( $M$ ) is defined as

$$M = \chi H$$

Where

$$H = B / \mu_0 - M$$

Where  $\chi$  is the magnetic susceptibility associated to  $M$  and  $H$ ,  $B$  is the induced magnet field and  $\mu_0$  the permeability in vacuum (Haacke *et al.* 2009).

SWI combines a phase and a magnitude image by applying a GRE with high magnetic field such as 3T. The phase sequences represent in the GRE image an average of magnetic fields protons that directly depend on the local susceptibility of the tissues.

Paramagnetism occurs as the result when tissues containing atoms with unpaired electrons are exposed to an external magnetic field. Diamagnetism is defined as magnetic field created by orbital motion of electrons opposite to an external magnetic field. Diamagnetism is weaker than the paramagnetic field. Paramagnetic substances, such as hemosiderin and ferritin, increase the magnetic field, resulting in a positive phase shift relative to the surrounding tissue, whereas diamagnetic substances, as calcium, lead to a negative phase shift.

Once the data are acquired, there are additional post-processing procedures that accentuate the signal intensity loss due to local susceptibility effects. These post-processing includes the creation of phase mask and the application of high-pass filter to remove low-signal components of the background field. This is often performed applying a 64x64 filter. Magnitude and high-pass filtered phase sequences are combined together

as final SWI sequence by multiplying the post-processed high-pass filtered phase image in to the original magnitude sequence (*figure 2.4*).

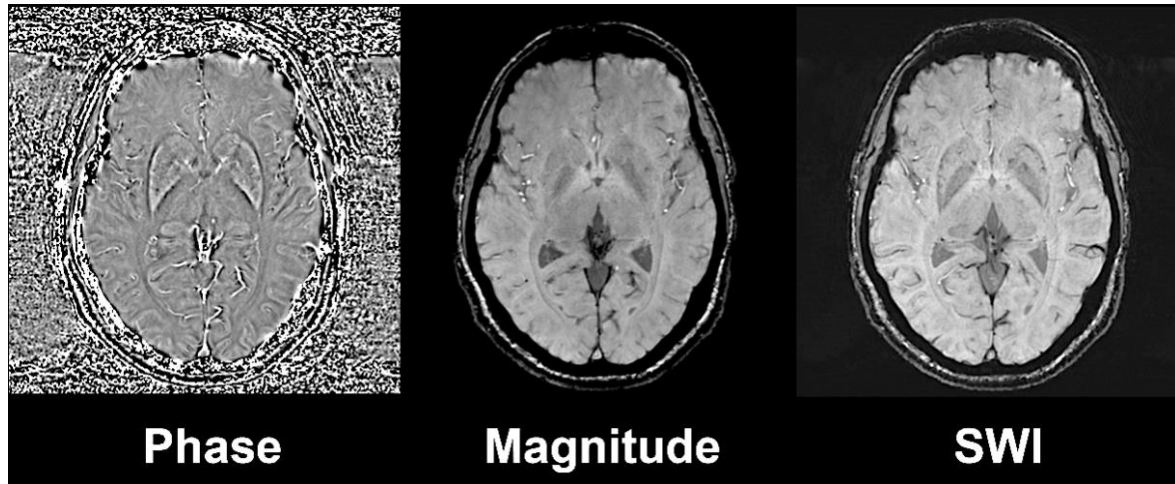


**Figure 2.4:** Susceptibility weighted image (left) is developed by the combination of high-pass filtered phase (left) and magnitude (central). Axial slices at the level of basal ganglia on a 3 Tesla Siemens Magnetom Trio system with 32-channel phased-array head coil.

Phase sequences present several limitations worth to be mentioned. First the difficulty in distinguishing paramagnetic and diamagnetic susceptibility with SWI techniques might be result of the convoluting effect of the dipole fields (Liu *et al.* 2015). In addition when iron content is too large, phase aliasing occurs and the lineal correlations may disappear, thus underestimating iron content (Haacke *et al.* 2005).

A number of studies have employed SWI to assess iron deposition in the deep brain grey nuclei of patients with PD by measuring the phase value from the high-pass filtered phase (see chapter 1). In this thesis, I will undertake the analysis of high-pass filtered phase imaging in a large cohort of PD patients and HC at baseline and follow-up visits in order to ascertain the motor associations of iron accumulation (chapter 3).

## 2.3.4. Analysis of iron-sensitive sequences and imaging protocols

### 2.3.4.1. Relaxometry imaging (T2\*)

T2\* imaging was used in order to evaluate the effect of iron chelation in several deep grey matter nuclei at three time points during 6 months of treatment (Chapter 6)

A 3D T1-weighted spoiled gradient echo volume of isotropic 1x1x1mm resolution was acquired at six echo times: 6.29, 13, 21, 29, 37, and 49ms in each TR of 56ms was acquired on a 3T Siemens Magnetom Verio system with 32-channel phased-array head coil (*figure 2.5*). A 240x256x160mm sagittal FOV and flip angle of 35° was used, with a parallel imaging factor of 2, requiring 10 minutes and 14 seconds.

A double-echo inversion recovery sequence (MP2RAGE) (Tanner *et al.* 2012) was also acquired with identical resolution and coverage at inversion times of 409ms and 1100ms in a 5s TR with a parallel imaging factor of 2, requiring 10 minutes and 57 seconds. This sequence generates two co-registered volumes: one with fluid nulled similar to the ADNI MPRAGE (Jack *et al.* 2008) and one with white matter nulled for subcortical visualization.

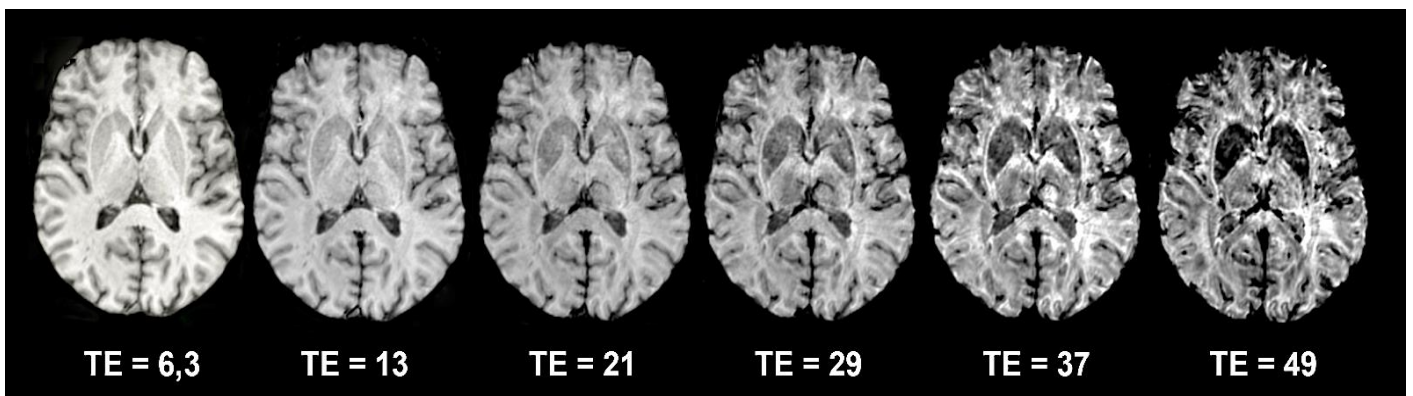


**Figure 2.5:** A demonstration of six T2\* images acquired at 6.3, 13, 21, 29, 37, and 49 milliseconds of echo time (TE). Axial slices at the level of basal ganglia on a 3 Tesla Siemens Magnetom Verio system with 32-channel phased-array head coil.

The signal decay in each voxel across the six-echo acquisition was fit to the non-linear signal equation:

$$S(TE) = S_0 \cdot e^{-\left(\frac{TE}{T2^*}\right)} + C$$

Where  $S_0$  represents the effective proton density, including partial T1 recovery, and the  $C$  term captures a rectified noise floor from magnitude imaging with possible low SNR in the final echo volume.

The first echo volume of the six echo acquisition was co-registered to the fluid-nulled volume from the first imaging visit using the FMRIB software library (FSL) FLIRT tool (FSL version 4.1.9). The resulting linear transformation matrix was applied to the T2\* volume. The first echo volume was also used for automatic segmentation using the FSL FIRST tool (FSL version 4.1.9) for automated delineation of the caudate, putamen, and globus pallidus.

All six echoes were automatically summed by the custom sequence software to result in a high SNR (Helms and Dechent 2009). This volume was used for the manual delineation of the substantia nigra (SN), red nucleus (RN), and the dentate nucleus (DN).

#### **2.3.4.2. High pass filtered phase imaging**

Phase imaging analysis were performed in order to investigate the iron-rich areas in the deep grey matter nuclei in a large cohort of PD and HC volunteers at baseline and follow-up visits (Chapter 3)

Susceptibility-weighted images were acquired on a 3T Siemens Magnetom Trio system with 32-channel phased-array head coil running a T2-weighted 3D gradient-echo sequence. SWI was acquired at 20 ms of echo time with TR of 28 ms. A 294\*320 matrix size with field of view of 230\*230mm and flip angle of 15° was used with bandwidth=120 Hz/Px and GRAPPA acceleration factor of 2, requiring 6 minutes.

72 slices of 1.9mm thickness and slice gap of 20% were obtained in an interleaved order parallel to the anterior-posterior commissural line in the left-hand reference system. A modified SWI protocol was added with 88 slices of 0.9 mm thickness with a 20% slice gap were also acquired.

Phase images were reconstructed automatically on the Siemens workstation (Syngo MR B17 software, SWI version 1), which included application of a 64\*64 high-pass filter to remove low spatial frequency effects. Each high-pass filtered phase image was rigidly registered to the 3D T<sub>1</sub>-weighted volume.

High-pass filtered imaging was used for manual delineation of regions of interest (ROI) with SPIN software (Signal Processing in Nuclear Magnetic Resonance; MRI Institute, Detroit, USA). Two slices were selected for each ROI analysis where well-defined border for the structures were not affected by sinus artefact. ROIs are first drawn in the high-pass filtered images and later copied to the magnitude images in order to guarantee their anatomical location.

ROIs were hand drawn on a single axial slice and included head of the caudate nucleus, putamen and globus pallidus (*figure 2.6*). The SN was delineated on the third axial slice ventral to the most dorsal aspect of the red nucleus. For images acquired under the modified SWI protocol, the sixth axial slice ventral to the most dorsal aspect of the red nucleus was used. The dentate nucleus (DN) was delineated following the corrugated wall adjacent to the 4<sup>th</sup> ventricle and the middle cerebello-pontine peduncle.

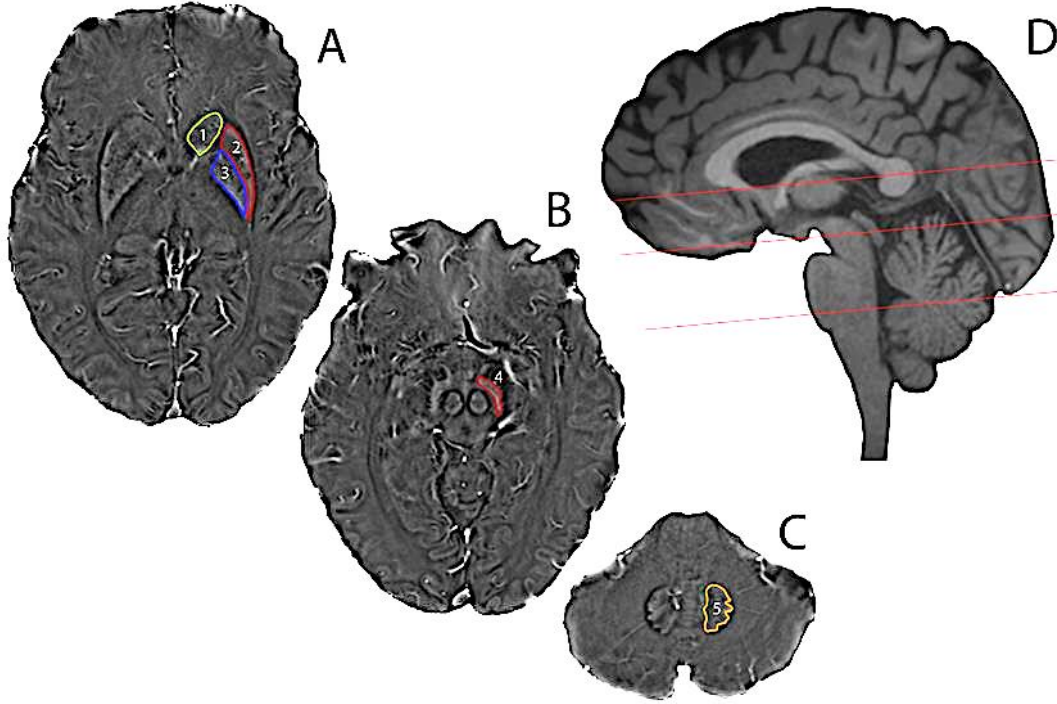


**Figure 2.6:** Regions of interest delineated on the axial plane of high-pass filtered phase images. **A:** Basal ganglia level includes head of caudate nucleus (1), putamen (2) and globus pallidus (3). **B:** Substantia nigra (4) delineated at the middle level of the midbrain. **C:** Dentate nucleus (5) delineated by following the corrugated wall adjacent to the 4<sup>th</sup> ventricle and the middle cerebello-pontine peduncle. **D:** Sagittal midline view of a 3D T1-weighted MPRAGE illustrating axial planes of ROI delineation.

Mean phase-shift values, number of voxels, standard deviations and regional areas were extracted for each ROI unilaterally before averaging to obtain bilateral data.

Phase-shift values were converted to radians according to the formula provided for the Siemens left-handed system:

$$Radians = \frac{(SPU - 2048) \pi}{2048}$$

Where SPU means Siemens phase units.

### **2.3.5. Neuromelanin-weighted MRI imaging**

NM-weighted MRI sequence is based on a T1 weighted sequence Turbo Spin Echo (TSE) that provides notable contrast between SNc and LC with the surrounding tissue. Magnetization transfer (MT) pulse is included in this sequence to establish the contrast between NM-containing regions and the surrounding. MT contrast is defined as the result of the interaction between protons associated with macromolecules such as iron and water protons thus enhancing paramagnetic contrast (Trujillo *et al.* 2016).

T1 shortening effects of the NM-MRI sequence are associated to the NM iron content in the form of Fe (III) and ferritin. On the other hand MT effect also enhances the paramagnetic contrast improving the NM visualization.

Enochs and colleagues (Enochs *et al.* 1997) were the first to quantitate the binding of metals to synthetic melanin in vitro, and to test whether such binding by natural melanin can be detected in cultured melanoma cells in vivo with MR imaging. A direct comparison between post-mortem NM-content neurons of SNc and the NM-MRI signal intensity showed a closed relationship that was not influenced by iron accumulation (Kitao *et al.* 2013).

Several studies have employed NM-MR imaging to evaluate nigral depigmentation of patients with PD (see chapter 1). In this thesis, I will use NM-MR imaging in a large cohort of PD patients and HC order to ascertain the clinical associations of nigral depigmentation (Chapter 4). In addition NM-MR imaging will be assessed in combination <sup>11</sup>C-PE2I PET imaging for further evaluation of nigrostriatal pathways in PD (chapter 5).

#### **2.3.5.1. Analysis of Neuromelanin-weighted MR imaging**

Neuromelanin-weighted T1 turbo spin-echo was acquired on a 3T Siemens Magnetom Trio system with 32-channel phased-array head coil. Neuromelanin-weighted MRI was acquired at 12 ms of echo time (echo train length of 4 ms and echo spacing of 11.5ms)

with TR of 829ms. A 320\*320 matrix size with in-plane voxel size of 0.8mm with FoV of 256\*256mm and of flip angle of 123° was acquired with low specific absorption rate.

For the TSE, 12 slices of 2.5mm thickness and slice gap of 0.25mm were acquired parallel to the AC-PC line with coverage of the whole midbrain and upper pons. Scans lasted 6 minutes and patients were instructed to remain as still as possible for the duration.

In the present thesis two basic approaches for Neuromelanin-weighted MR imaging analysis were performed: determination of hyperintense NM-rich volumes and SNc regional contrast ratios. For the first approach mesencephalic regions of interest (ROI) were first defined on two contiguous axial slices of the T1-weighted TSE MR image on which the SNc was visible at the level of the red nucleus and the inferior colliculus using Analyze11.0 (Biomedical Imaging Resource, Mayo Clinic) (*figure 2.7*). Each neuromelanin-weighted MR image was rigidly registered to the 3D MPRAGE.

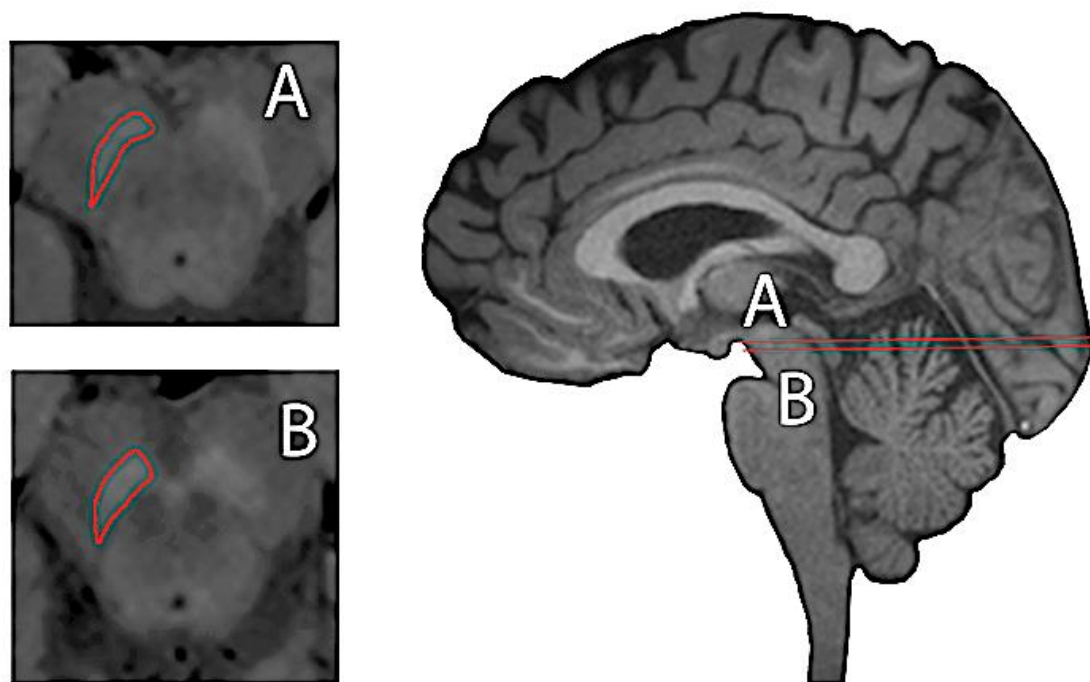


**Figure 2.7:** Regions of interest delineated on the axial plane of Neuromelanin-weighted MR imaging (left) and sagittal midline plane of a 3D T1-weighted MPRAGE illustrating axial planes of ROI delineation; **A and B:** Substantia nigra pars compacta (4) delineated at the middle level of the midbrain.

In addition, ROI delineation of the background areas included cerebral peduncles and midbrain tegmentum (*Figure 2.8*).

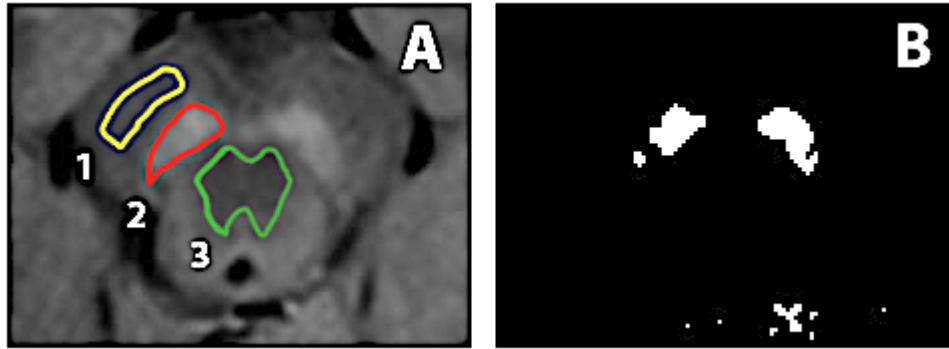


*Figure 2.8: Axial midbrain slices of a healthy control in a Neuromelanin-weighted MR image (A) indicating delineation of substantia nigra pars compacta (red) and background areas including cerebral peduncles (green) and midbrain tegmentum (yellow). B: The resulting pixels of the SNc after the application of threshold (mean background signal plus three standard deviations of the background signal), indicates the hyperintense SN area considered as Neuromelanin-rich areas.*

The signal intensity obtained from background area was averaged to further obtain the mean background signal intensity and the standard deviation (SD). According to the Schwarz et al (Schwarz *et al.* 2011, Schwarz *et al.* 2017) threshold signal intensity of SN is calculated as follow:

$$\text{Threshold}_{\text{NM}} = \text{Mean}_{\text{BG}} + 3 \text{ SD}_{\text{BG}}$$

The resulting threshold SNc areas will be then multiplied by the scan parameters (x, y, z) in order to obtain the hyperintense NM-rich volumes as measured by  $\text{mm}^3$ .

For the second quantitative evaluation of NM-weighted MRI scans, a study-specific template was first created using grey and white matter MPAGE segmentations from both PD and HC participants through exponentiated Lie algebra (DARTEL) in SPM12. Software defaults were modified to enable template and MPAGE-to-template warp field generation in native voxel size ( $1\text{mm}^3$ ). NM-weighted T1 images were co-registered to the corresponding MPAGE and DARTEL warp fields applied in one step to normalise

all images to common space. The neuromelanin template could then be generated through voxel-wise averaging (mean) over the entire study cohort.

Mesencephalic regions of interest (ROI) for the SNc were manually defined on five contiguous axial slices of the neuromelanin template on which the neuromelanin-related hyperintensity of the SNc was visible, at the level of the red nucleus and the inferior colliculus, in FSL. The volume of the SNc on each axial slice was divided into two sections representing the dorsal and ventral tiers, in accordance with post-mortem histological investigations on the striatal afferences of the SNc reported by Haber et al. (Haber 2014) (*Figure 2.9*). The decussation of the superior cerebral peduncle (CP) was also defined on the same axial slices as a reference region.

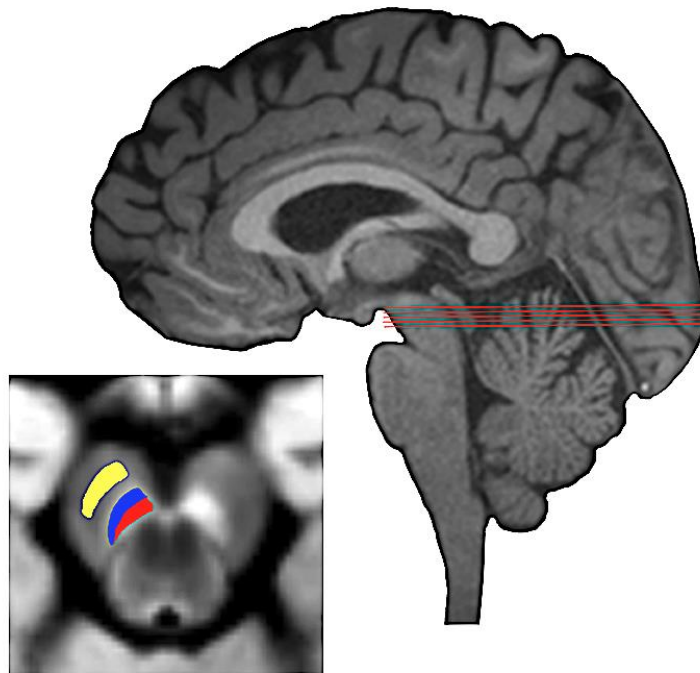


**Figure 2.9:** Regions of interest delineated on the axial of the neuromelanin template image (left) and sagittal midline plane of a 3D T1-weighted MPRAGE (right) illustrating axial planes of ROI delineation. SNc was delineated in five contiguous neuromelanin template on which the neuromelanin-related hyperintensity of the SNc was visible. SNc subdivision in ventral (blue) and dorsal tiers (red) were performed according to Haber et al (Haber et al. 2000, Haber 2014) along with cerebral peduncles (yellow).

Contrast ratios (CR) were calculated in accordance with Sasaki et al (Sasaki *et al.* 2006) using the formula (SNc-CP)/CP for dorsal (SNc-CR<sub>dor</sub>) and ventral (SNc-CR<sub>ven</sub>) ROIs separately.

$$\text{SNc\_CR} = \frac{\text{MeanSN} - \text{MeanCP}}{\text{MeanCP}}$$

## **2.4. Positron Emission Tomography**

### **2.4.1. Positron Emission Tomography basics.**

Positron Emission Tomography (PET) is a nuclear medicine technique that provides quantitative images of in vivo physiological and biochemical processes (Phelps 1977, Phelps 2000). This imaging modality is based on the detection of annihilation coincidence photons following the injection of positron-emitting radionuclides, the so-called tracers. These tracers are used to investigate different biologic processes or functions.

The unstable nucleus of radionuclide decays via positron emission and the resulting positrons annihilate on contact with electrons after travelling a short distance within the tissue (less of 1 mm). The annihilation produces two gamma particles converted into electromagnetic radiation in the form of 511 keV photons that travel in opposite directions that may be detected by the detectors collinearly aligned in the surrounding of the subject. The PET camera includes multiple rings of linked detectors consisting in scintillation crystals hence when two detection events from the same annihilation occur in a specific time window is considered “coincidence” (*Figure 2.10*).

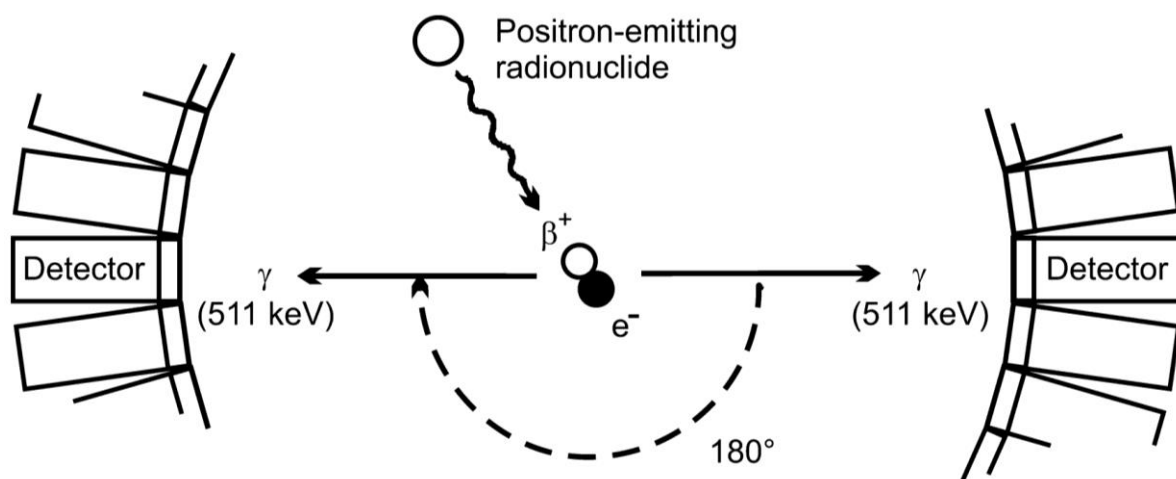


Figure 2.10: Schematic description of  $\beta^+$  annihilation and coincidence detection in positron emission tomography scanner. Figure extracted from (Verel et al. 2005)

Every coincidence event are stored in arrays as raw data set and reconstructed to projections through the patient (axial, sagittal and coronal planes). Coincidence events are assigned to a line of response (LOR) joining the two relevant detectors, thus positional information from the detected radiation without a physical collimator is known as electronic collimation (Figure 2.11).

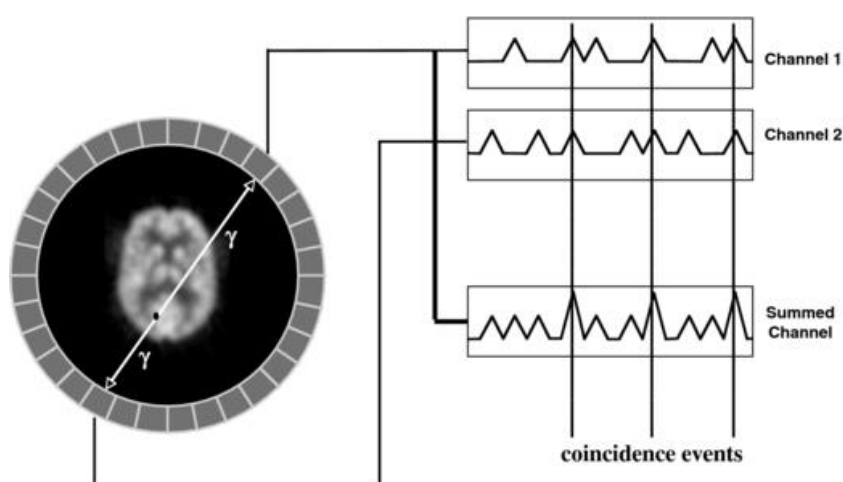


Figure 2.11: Coincidence detection in the PET camera. . Image taken from Introduction to PET Physics, <http://www.depts.washington.edu>

There are three major types of events that can cause coincidence events: true, scattered and random. A true coincidence occurs when both of the gamma rays are detected by detectors in coincidence without any other event detected within the coincidence-time window. A scattered coincidence occurs when one or two of the 511 keV annihilation photons decay scatter in the object being scanned resulting in reduction of spatial resolution and image contrast as well as wrong estimations of the isotope concentrations. Random coincidence occurs when two photons not originated from the same annihilation are detected within the same coincidence time window resulting in isotopic overestimation and additional statistical errors.

The coincidence events generated in the 3D PET camera are acquired to generate cross-sectional raw data of the isotopic activity distribution along the LOR. Corrections for scatter and random effects are applied prior to the reconstruction of images. Iterative and statistical reconstruction algorithms are commonly the method of choice.

The most used radionuclides in PET are  $^{15}\text{O}$ ,  $^{13}\text{N}$ ,  $^{11}\text{C}$  and  $^{18}\text{F}$  (half-lives 2 min, 10 min, 20 min and 110 min respectively). After intravenous administration, the radiotracer is monitored in order to obtain kinetic and structural information as distribution of the receptors in the brain or estimation of blood flow.

#### **2.4.2. The dopamine transporter**

The dopamine transporter (DAT) protein regulates the synaptic dopamine concentration by high-affinity synaptic reuptake (see review in (Piccini 2003)). DAT is widely distributed in axonal membranes and in less proportion in somatodendritic regions, thus regulating extracellular dopamine levels and helping dopamine clearance respectively (Hersch *et al.* 1997). DAT protein is highly expressed in striatal dopaminergic neurons with the highest levels in striatum and in lesser degree in amygdala, hypothalamus, SN and VTA.

### 2.4.3. $^{11}\text{C}$ -PE2I as PET tracer

The study of function and density of DAT has been widely performed in neurodegenerative disorders as Parkinson's disease with in vivo PET studies. The development of DAT radioligands for PET and SPECT imaging have used for the in vivo study of dopaminergic systems (see review in (Piccini 2003)). DAT radioligands traditionally used for clinical purposes such as  $^{123}\text{I}$ - $\beta$ -CIT and  $^{123}\text{I}$ -F-CIT have also affinity for non-dopaminergic receptors such as SERT (serotonergic transporter) and NET (norepinephrine transporter), which make difficult the dopaminergic study in PD (Neumeyer *et al.* 1991).

$^{11}\text{C}$ -PE2I (N-(3-iodoprop-2E-enyl)-2 $\beta$ -carbomethoxy-3 $\beta$ -(4-methyl-phenyl)nortropine) ( $^{11}\text{C}$ -PE2I) is a high-affinity radioligand which binds selectively and reversibly to the DAT in vivo (Halldin *et al.* 2003) (Figure 2.12).  $^{11}\text{C}$ -PE2I is a high selective DAT radioligand with optimal kinetic properties for accurate quantification of striatal DAT availability.  $^{11}\text{C}$ -PE2I has been shown to have greater DAT/SERT and DAT/NET selectivity than  $^{123}\text{I}$ - $\beta$ -CIT (Emond *et al.* 1997) (figure 2.12).

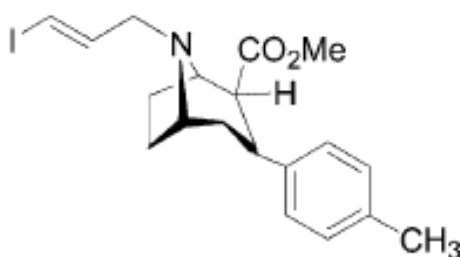


Figure 2.12: Structural formula of  $^{11}\text{C}$ -PE2I. Image extracted from (Halldin *et al.* 2003)

In this thesis I evaluated the nigrostriatal pathways in PD by combining striatal  $^{11}\text{C}$ -PE2I and nigral NM-MR sensitive imaging. PET methods will be explained in detail in chapter 5.

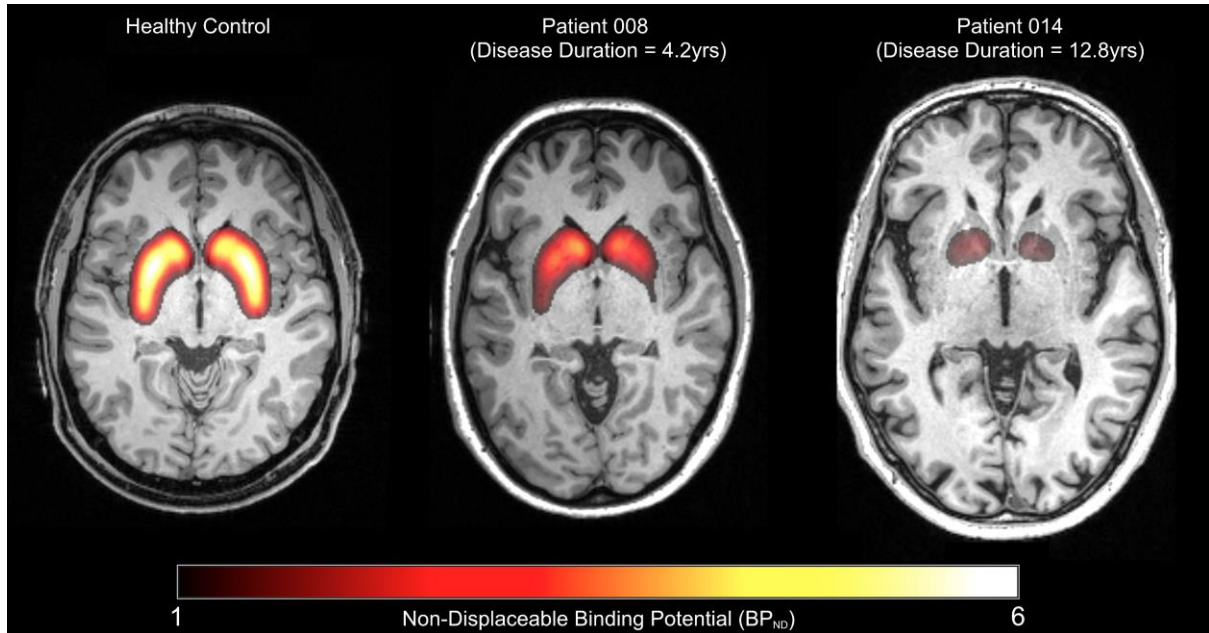


Figure 2.13: Striatal dopamine transporter assessed with  $^{11}\text{C}$ -PE2I positron emission tomography in a healthy control subject (left), a PD volunteer with right hemiparkinsonism and disease duration of 4.2 years (middle) and an advanced-stage PD subject with disease duration of 12.8 years (right). Asymmetric loss of the uptake of the tracer is more pronounced in the contralateral posterior putamen to the most clinical affected side (central), whereas bilateral marked reduction in the putamen and in lesser degree in the caudate is consistent with clinically advanced-stage PD (right). Images represent  $^{11}\text{C}$ -PE2I ADD images overlaid onto the structural MP<sub>RAGE</sub><sub>REG</sub> at the level of the anterior commissure. The present images are courtesy of Mr Nick P Lao-Kaim as part of his PhD.

# 3. Studies of iron-related susceptibility imaging in Parkinson's disease

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## 3.1.Introduction

Iron plays an important role in PD neurodegeneration (see chapter 1). Post-mortem and *in vitro* studies have demonstrated links between iron accumulation and the cardinal pathological features of Parkinson's disease, the loss of dopamine neurons in the SNc (Dexter *et al.* 1987) and the presence of  $\alpha$ -synuclein-rich Lewy bodies (Hashimoto *et al.* 1999).

Free ferrous iron (II) acts as a catalyst in a reaction with hydrogen peroxide (Fenton reaction), producing highly toxic reactive oxygen species (ROS) and ferric iron (III) that leads to oxidative stress-related damage to cellular components including proteins, lipids and DNA (Dexter *et al.* 1991). To relieve potential toxicity, excess ferrous iron within deep brain nuclei is accumulated and transported into the core of ferritin molecules where it is oxidized and safely stored in high concentrations in ferric state (Haacke *et al.* 2005). Since ferritin is highly paramagnetic and indeed one of the only non-heme iron molecules present in sufficient quantity to cause local field inhomogeneities (Schenck and Zimmerman 2004, Haacke *et al.* 2005), magnetic resonance imaging (MRI) can be used to measure ferritin-bound ferric iron *in vivo*. Given that the biosynthetic rate of ferritin is modulated by the availability of free iron (Munro and Linder 1978, Casey *et al.* 1988), changes in ferritin-related MR signal are reflective of alterations in iron load.

*In vivo* iron quantification using MRI relaxometry ( $T2^*$ ,  $R2^*$ ) or Susceptibility-Weighted Imaging (SWI) consistently demonstrates increased SN iron levels in patients with PD relative to healthy controls (Antonini *et al.* 1993, Graham *et al.* 2000, Kosta *et al.* 2006, Martin *et al.* 2008, Wallis *et al.* 2008, Zhang *et al.* 2010, Jin *et al.* 2012, Wang *et al.* 2012, Bunzeck *et al.* 2013, Wu *et al.* 2014), while results pertaining to striatal regions such as caudate nucleus, putamen and globus pallidus appear highly heterogeneous (Antonini *et al.* 1993, Ryvlin *et al.* 1995, Graham *et al.* 2000, Kosta *et al.* 2006, Martin *et*

*et al.* 2008, Wallis *et al.* 2008, Zhang *et al.* 2010, Jin *et al.* 2012, Wang *et al.* 2012). Several studies observed relationships between increased nigral iron accumulation, disease duration and motor severity (Martin *et al.* 2008, Wallis *et al.* 2008, Zhang *et al.* 2010, Wu *et al.* 2014) though to our knowledge there are currently no cross-sectional studies that evaluate whether significant changes in iron accumulation exist between different stages of disease burden in mesencephalic or striatal nuclei. In addition, it remains unclear as to the effect of iron accumulation on differing motor symptoms, with the two studies to investigate tremor and akinetic/rigid predominance yielding conflicting results (Jin *et al.* 2012, Bunzeck *et al.* 2013)

Nevertheless these findings have not been reliably extended to longitudinal settings. Some relaxometry investigations report strong associations between changes in nigral iron content and changes in global motor severity (Wieler *et al.* 2015) while others either fail to demonstrate this effect (Rossi *et al.* 2014) or report marginal significance (Ulla *et al.* 2013, Hopes *et al.* 2016) which, although alluding to the presence of such a relationship, does not offer strong support for the utility of relaxometry as an imaging marker for disease staging. Akin to cross-sectional data, longitudinal assessments have also yielded variable results pertaining to mineralization within striatal nuclei and other extra-nigral regions such as the Put, GP and caudate nucleus (CN) (Ulla *et al.* 2013, Rossi *et al.* 2014, Wieler *et al.* 2015, Hopes *et al.* 2016). Because relaxometry metrics like T2\* and R2\* are known to be affected by tissue water content, field inhomogeneities such as those arising from air-tissue interfaces and other local structural changes affecting relaxation times (Haacke *et al.* 2004, Haacke *et al.* 2005) it is possible that this inconsistency could be explained by technical constraints

## **3.2. Cross-sectional high-pass filtered susceptibility imaging in Parkinson's disease**

In this chapter, I have undertaken the analysis of high-pass filtered phase regional analysis of deep grey matter nuclei in a large cohort of 70 PD volunteers as well as 20 age and gender matched HC. Regional iron quantification in the PD cohort was assessed to explore their clinical associations with motor severity ("off-medication UPDRS III), disease duration and clinical laterality. Further subdivision of PD groups according to UDPRS III scale adjusted by age and regional areas was performed to ascertain the behaviour of regional iron deposition.

The aims of the present subchapter are:

- To evaluate differences in iron accumulation in deep brain nuclei in PD groups of varying motor severity
- To investigate the association between motor symptomatology and iron load in a large cross-sectional PD cohort.

The hypotheses of this subchapter are:

- Iron accumulation in SN and striatum in PD is significantly higher than HC group
- Nigral iron deposition is directly associated with UDPRS III and bradykinesia-rigidity symptoms.

### **3.2.1. Subjects and methods**

#### **3.2.1.1. Study subjects**

Seventy non-demented mild-moderate stage PD patients and twenty age-gender-matched healthy volunteers (HC) were included in this study. Patients were recruited as part of the PaMIR and TransEuro multi-centre studies.

Diagnosis of PD was performed by movement disorder specialists according to the PDUK Brain Bank Criteria (Hughes *et al.* 1992).

To further exclude atypical parkinsonisms (i.e. Progressive Supranuclear Palsy, Multisystem Atrophy or Corticobasal Syndrome), patients with pyramidal signs (hyperreflexia, Babinski's sign), supranuclear gaze palsy, severe loss of postural reflexes, early falls, neck dystonia (anterocollis and retrocollis), cerebellar signs, early severe autonomic dysfunction (orthostatic hypotension), early severe cognitive dysfunction and lack of response to dopaminergic regime were not included in the present study. Additional exclusion criteria include concomitant vascular load, repeated head injury, neuroleptic treatment at the onset of motor symptoms, neuroimaging findings of communicating hydrocephalus and history of encephalitis.

Data were collected carried out in accordance with the Declaration of Helsinki, after approval from the National Research Ethics Service Committee. All participants gave written informed consent before participation (see chapter 2).

### **3.2.1.2. Motor assessments**

Motor symptomatology was measured using the Unified Parkinson's Disease Rating Scale, part III (UPDRS III). Patients were instructed to withdraw from medication 24 hours prior to assessment. Off-medication UPDRS III scores were then subdivided into tremor (sum of items 15-18) and bradykinesia-rigidity subscores (sum of items 2-9 and 14). Levodopa-equivalent daily dose (LEDD) was calculated.

### **3.2.1.3. Susceptibility-Weighted Imaging**

Susceptibility-weighted images were acquired on a 3T Siemens Magnetom Trio system with 32-channel phased-array head coil running a T2-weighted 3D gradient-echo sequence (SWI: TR = 28ms, TE = 20ms, Flip Angle = 15°, bandwidth = 120 Hz/Px, matrix size = 294\*320, FoV = 230\*230mm, GRAPPA acceleration factor = 2). 72 slices of 1.9 mm thickness and slice gap of 20% were obtained in an interleaved order parallel

to the anterior-posterior commissural line in the left-hand reference system. A small number of participants (4PD, 7HC) underwent a modified SWI protocol in which 88 slices of 0.9mm thickness with a 20% slice gap were acquired. Phase images were reconstructed automatically on the Siemens workstation (Syngo MR B17 software, SWI version 1), which included application of a 64\*64 high-pass filter to remove low spatial frequency effects (See Chapter 2).

#### **3.2.1.4. Regions of Interest Analysis**

Manual ROI delineation was performed on both PD and HC groups using high-pass filtered phase images using SPIN (Signal Processing in Nuclear Magnetic Resonance; MRI institute, Detroit, Michigan). All study subjects were anonymised prior to delineation, thus blinding group and disease severity. ROIs were hand drawn on two axial slices and included head of the caudate nucleus, putamen and globus pallidus (see figure 9, chapter 2).

The SN was delineated on the 3<sup>rd</sup> axial slice ventral to the most dorsal aspect of the red nucleus, where there was demonstrated the highest phase shift value indicating high iron content within the nuclei. For images acquired under the modified SWI protocol, the 6<sup>th</sup> axial slice ventral to the most dorsal aspect of the red nucleus was used (*Figure 3.1*). The dentate nucleus (DN) was delineated following the corrugated wall adjacent to the 4<sup>th</sup> ventricle and the middle cerebello-pontine peduncle. Subjects with microvascular lesions or physiological calcifications in the GP were not included in the analysis.

Independent samples t-tests showed no significant differences in SN radians between the two SWI protocols for either group ( $p>0.05$ ). Subjects with microvascular lesions or physiological calcifications in the globus pallidus were not included in the analysis.

Mean phase-shift values, number of voxels, standard deviations and regional areas were extracted for each ROI unilaterally before averaging to obtain bilateral data. Phase-shift values were converted to radians according to the formula provided for the Siemens left-handed system:

$$\text{Radians} = (\phi - 2048) \pi / 2048$$

Where  $\phi$  means Siemens phase units.

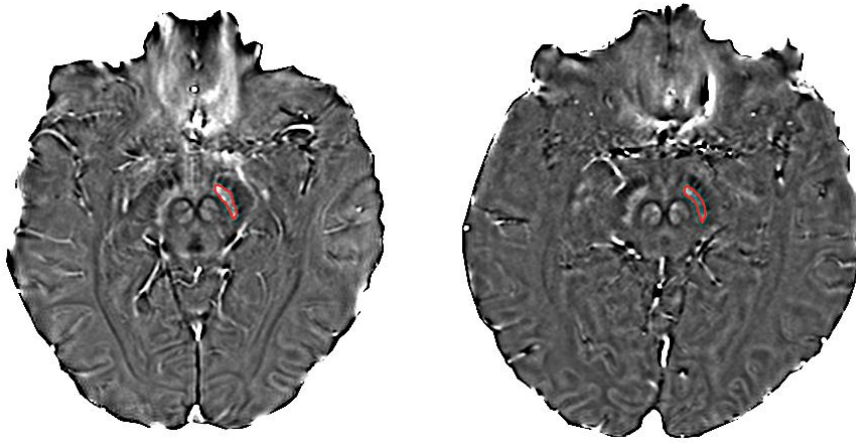


**Figure 3.1:** Axial slices of two different subjects comparing SN delineation between the main (slice thickness = 1.9, number of slices = 72) and modified (slice thickness = 0.9, number of slices = 88) SWI protocols. For the main SWI protocol, delineation was conducted on the 3<sup>rd</sup> axial slice ventral to the most dorsal aspect of the red nucleus (left). For the modified SWI protocol, delineation was conducted on the 6<sup>th</sup> axial slice ventral to the most dorsal aspect of the red nucleus (right).

### 3.2.1.5. Statistical Analysis

All statistical analyses were performed using SPSS statistical software (version 22.0 SPSS Inc., Chicago, Illinois).

Group demographics and bilateral radians for each region were compared between PD and HC using independent t-tests. ROIs showing a significant difference were considered for analyses of covariance (ANCOVA) in which PD patients, subdivided into 3 disease

severity groups using UPDRS III tertile calculations (Table 1) were compared to the HC group, adjusting for age and regional area and followed with post-hoc Bonferroni-adjusted pairwise comparisons.

To assess hemispheric iron load differences in the PD group, radians for clinically most/least affected sides, as indicated by UPDRS III laterality items, were compared for each region using independent t-tests.

Pearson's correlation coefficient was used to assess relationships between each ROI and measures of disease severity (disease duration, UPDRS III total, tremor and bradykinesia/rigidity).

Correlations were considered significant only if  $p < 0.05$  following Benjamini-Hochberg false discovery rate (FDR) adjustment.

### **3.2.2. Results**

#### **3.2.2.1. Demographics**

Clinical and demographic data are summarized in *Table 3.1*. There were no significant differences in age ( $p = 0.347$ ) between PD and HC. Although the male-to-female ratio was higher in the PD group ( $p = 0.039$ ), no significant gender differences in iron accumulation were found.

As expected, PD subgroups exhibited significant differences in motor severity, disease duration, LEDD and Hoehn and Yahr scale (*Table 3.1*). There were no significant differences between subgroups for age or gender.

|                          | HC           | PD            |              | Statistic           | <i>p</i>         |
|--------------------------|--------------|---------------|--------------|---------------------|------------------|
| Volunteers               | 20           | 70            |              | NA                  | NA               |
| Age (years)              | 53.11 ± 11.7 | 55.78 ± 7.22  |              | $t(23,18)= 3.38^a$  | 0.347            |
| Gender (m:f)             | 10:10        | 52:18         |              | $\chi^2(1)= 4.28^b$ | 0.039            |
| Disease duration (years) | NA           | 5.39 ± 2.45   |              | NA                  | NA               |
| UPDRS III                | NA           | 31.47 ± 11.56 |              | NA                  | NA               |
| Hoehn and Yahr scale     | NA           | 1.87 ± 0.47   |              | NA                  | NA               |
| LEDD                     | NA           | 563 ± 344     |              | NA                  | NA               |
| PD SUBGROUPS             |              |               |              |                     |                  |
|                          | PD group 1   | PD group 2    | PD group 3   | Statistic           | <i>p</i>         |
| Volunteers               | 23           | 22            | 25           | NA                  | NA               |
| Age (years)              | 55.78 ± 7.76 | 54.66 ± 5.08  | 57.26 ± 8.51 | $F(2,67)= 0.694^c$  | 0.558            |
| Gender (m:f)             | 15:8         | 15:7          | 22:3         | $\chi^2(2)= 7.74^b$ | 0.052            |
| Disease duration (years) | 4.33 ± 1.97  | 6.19 ± 2.29   | 5.62 ± 2.71  | $F(2,67)= 3.77^c$   | 0.028            |
| UPDRS III                | 19.3 ± 3.11  | 29.27 ± 3.13  | 44.60 ± 6.42 | $F(2,67)= 213.9^c$  | <b>&lt;0.001</b> |
| Hoehn and Yahr scale     | 1.63 ± 0.48  | 1.86 ± 0.46   | 2.12 ± 0.33  | $F(2,67)= 7.07^c$   | 0.731            |
| LEDD                     | 411 ± 244    | 647 ± 431     | 640 ± 313    | $F(2,67)= 3.11^c$   | 0.828            |

**Table 3.1:** Demographic and clinical characteristics of PD patients and HC group at baseline (up) and PD subgroups divided into 3 disease severity groups using UPDRS III tertile calculations in PD group 1 (UPDRS III= 19.3 ± 3.11), PD group 2 (UPDRS III= 29.27 ± 3.13) and PD group 3 (UPDRS III= 44.60 ± 6.42) (down). Values represent the mean ± SD. NA= not applicable; UPDRS III= Unified Parkinson's Disease Rating Scale Part III; LEDD= Levodopa equivalent daily dose; <sup>a</sup>= Indicates that differences were tested using independent t-tests; <sup>b</sup>= Indicates that differences were tested using Pearson's Chi-square test for independence; <sup>c</sup> = Indicates that differences were tested using one-way ANOVA; (df) = degrees of freedom.

### 3.2.2.2. Phase-shift analysis

No significant differences were found in the original delineated areas along ROIs between HC and PD groups ( $p > 0.05$ ) (Figure 3.2).

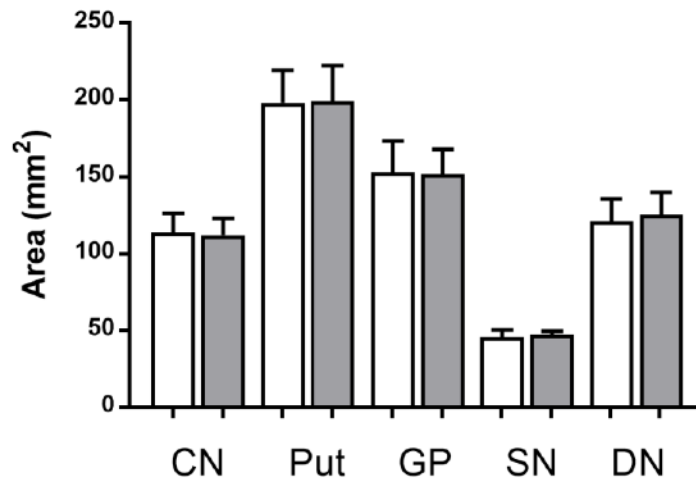


**Figure 3.2:** Bar graph showing the mean and standard deviation of bilateral average area values (in  $\text{mm}^2$ ) of PD (grey bars) and HC (white bars) in the caudate (CN), putamen (Put), globus pallidus (GP), substantia nigra (SN) and dentate nucleus (DN) at baseline. Comparison of regional radiances by analysis of independent  $t$  tests with post-hoc Bonferroni correction for multiple comparisons showed significant differences between area values of HC and PD groups.

Shapiro-Wilk's test revealed that putaminal and pallidal radiances were not normally distributed ( $p < 0.05$ ). Square root transformations were therefore applied to the putamen and logarithmic transformation to globus pallidus. The PD group showed significantly higher radiances in the GP ( $p < 0.01$ ), Put, SN and DN ( $p < 0.001$ ), as compared to HC (Figure 3.3). No significant difference was found in the caudate nucleus.

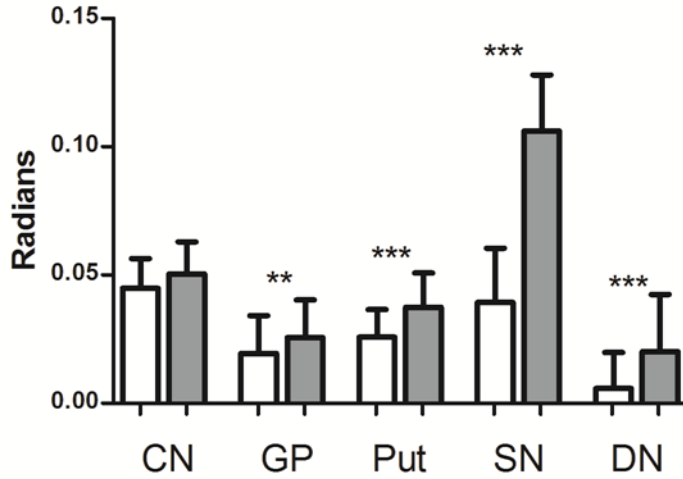


**Figure 3.3:** Bar graph showing the mean and standard deviation of bilateral average phase values (in radians) of PD (grey bars) and HC (white bars) in the caudate (CN), putamen (Put), globus pallidus (GP), substantia nigra (SN) and dentate nucleus (DN) at baseline. Comparison of regional radians by analysis of independent *t* tests with post-hoc Bonferroni correction for multiple comparisons showed significant increase in the SN ( $p < 0.001$ ), Put ( $p < 0.001$ ), DN ( $p < 0.001$ ) and GP ( $p < 0.001$ ) in PD when compared to HC group. \*\*= $p < 0.01$ ; \*\*\*= $p < 0.001$ .

ANCOVA including HC and PD subgroups indicated main effects of motor severity in the putamen ( $F(3,82) = 6.346$ ,  $p < 0.001$ ), globus pallidus ( $F(3,82) = 7.998$ ,  $p < 0.001$ ), SN ( $F(3,82) = 65.008$ ,  $p < 0.001$ ) and DN ( $F(3,82) = 4.221$ ,  $p = 0.008$ ) (Table 3.2). Post-hoc Bonferroni-adjusted pairwise comparisons showed that radians in all three PD subgroups were significantly higher than HC in the GP ( $p = 0.025$ ,  $p < 0.001$  and  $p = 0.001$  respectively), SN ( $p < 0.001$ ,  $p < 0.001$  and  $p < 0.001$  respectively) and DN ( $p = 0.01$ ,  $p < 0.01$  and  $p < 0.001$  respectively) (Table 2). Putaminal radians were significantly higher in PD subgroups 1 and 2 ( $p = 0.006$ ,  $p < 0.001$ ) as compared to HC. Pairwise comparisons between the PD subgroups revealed significance only in the SN, that is, PD group 1 showed significantly lower radians as compared to PD groups 2 ( $p = 0.049$ ) and 3 ( $p = 0.001$ ). Although PD group 3 showed the highest radians in the SN, no statistically significant difference was found when compared with PD group 2 ( $p = 0.236$ ) (Figure 3.4).

|                        |            | Mean $\pm$ SD      | Controls<br>(N= 20) |  | PD group 2<br>(N= 22) | PD group 3<br>(N= 25) | ANCOVA                         |
|------------------------|------------|--------------------|---------------------|--|-----------------------|-----------------------|--------------------------------|
| <b>Put<sup>a</sup></b> | Controls   | 0.130 $\pm$ 0.052  |                     |  |                       |                       | $F(3,82)= 6.346$<br>$p= 0.001$ |
|                        | PD group 1 | 0.183 $\pm$ 0.035  |                     |  |                       |                       |                                |
|                        | PD group 2 | 0.195 $\pm$ 0.036  |                     |  |                       |                       |                                |
|                        | PD group 3 | 0.172 $\pm$ 0.054  |                     |  |                       |                       |                                |
| <b>GP<sup>b</sup></b>  | Controls   | -1.621 $\pm$ 0.176 |                     |  |                       |                       | $F(3,82)= 7.998$<br>$p<0.001$  |
|                        | PD group 1 | -1.488 $\pm$ 0.153 |                     |  |                       |                       |                                |
|                        | PD group 2 | -1.413 $\pm$ 0.123 |                     |  |                       |                       |                                |
|                        | PD group 3 | -1.441 $\pm$ 0.143 |                     |  |                       |                       |                                |
| <b>SN</b>              | Controls   | 0.037 $\pm$ 0.021  |                     |  |                       |                       | $F(3,82)=65.008$<br>$p<0.001$  |
|                        | PD group 1 | 0.094 $\pm$ 0.017  |                     |  |                       |                       |                                |
|                        | PD group 2 | 0.111 $\pm$ 0.021  |                     |  |                       |                       |                                |
|                        | PD group 3 | 0.117 $\pm$ 0.023  |                     |  |                       |                       |                                |
| <b>DN</b>              | Controls   | -0.001 $\pm$ 0.005 |                     |  |                       |                       | $F(3,82)= 4.221$<br>$p= 0.008$ |
|                        | PD group 1 | 0.018 $\pm$ 0.005  |                     |  |                       |                       |                                |
|                        | PD group 2 | 0.018 $\pm$ 0.004  |                     |  |                       |                       |                                |
|                        | PD group 3 | 0.021 $\pm$ 0.005  |                     |  |                       |                       |                                |

**Table 3.2:** Analysis of covariance matrices according to UPDRS III in PD group 1 (UPDRS III=19.3  $\pm$  3.11), PD group 2 (UPDRS III=29.27  $\pm$  3.13) and PD group 3 = 44.60  $\pm$  6.42) as well as HC group adjusted for age and regional area. Coloured boxes indicate the significance level of post-hoc Bonferroni-corrected pairwise comparisons:  $p>0.05$  (blue),  $p<0.05$  (yellow),  $p<0.01$  (orange),  $p<0.001$  (red). Put= Putamen; GP=Globus Pallidus; SN= Substantia Nigra; DN=Dentate Nucleus; <sup>a</sup>=Square-root transformation; <sup>b</sup>=Logarithmic transformation.

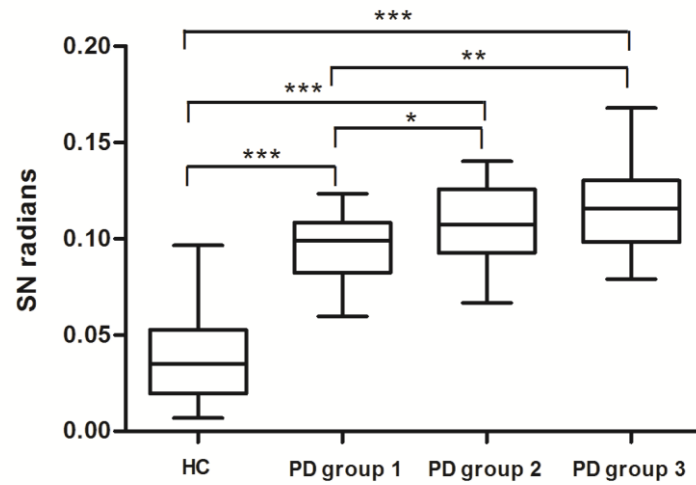


**Figure 3.4:** Box and whisker plot displaying the SN radians of PD subgroups divided according to UDPRS III in PD group 1 (UDPRS III=  $19.3 \pm 3.11$ ), PD group 2 (UDPRS III=  $29.27 \pm 3.13$ ) and PD group 3=  $44.60 \pm 6.42$ ) as well as HC group. Results of post-hoc Bonferroni-adjusted pairwise comparison following significant one-way ANCOVA are adjusted by age and regional delineated area. \*\*\*=  $p < 0.001$ ; \*\*=  $p < 0.01$ ; \*=  $p < 0.05$ .

### 3.2.2.3. Analysis of clinical laterality

The SN contralateral to the clinically most affected side showed higher radians ( $p < 0.001$ ) compared to the least affected side. No other regional significant results were found (Figure 3.5).

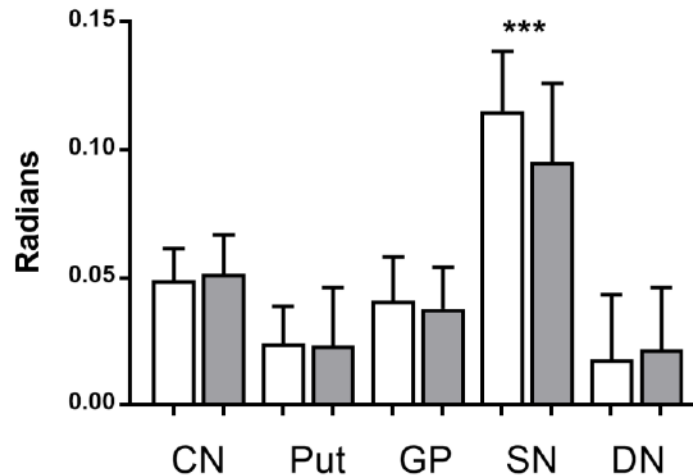


**Figure 3.5:** Bar graph showing the mean and standard deviation of bilateral average phase values (in radians) of PD indicating most (white bars) and least affected (white bars) contralateral to the symptomatic clinical sides in the in caudate (CN), putamen (Put), globus pallidus (GP), substantia nigra (SN) and dentate nucleus (DN) at baseline. Comparison of regional radians by analysis of independent *t* tests with post-hoc Bonferroni correction for multiple comparisons showed significant increase in the SN ( $p < 0.001$ ) in the most affected when compared to the least affected. \*\*\*= $p < 0.001$ .

#### 3.2.2.4. Clinical correlations

Pearson's correlation analysis revealed significant positive correlations between SN radians and total UPDRS III ( $r = 0.420$ ,  $p < 0.001$ ) and bradykinesia-rigidity subscores ( $r = 0.407$ ,  $p = 0.001$ ) but not with tremor ( $r = 0.219$ ,  $p = 0.071$ ) (Figure 3.6). Modest correlation between SN radians and disease duration ( $r = 0.242$ ,  $p = 0.044$ ) did not survived Benjamini-Hochberg FDR correction for multiple comparisons. No correlations were found with Hoehn and Yahr scale and total LEDD.

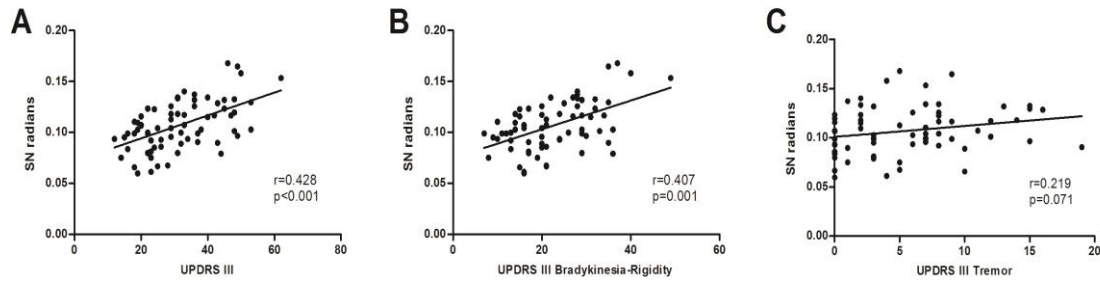


**Figure 3.6:** Scatterplots and Pearson's correlations showing significant positive relationships between SN radians and UPDRS III (left) and bradykinesia-rigidity scores (central) as well as no correlations with tremor scores (right).

Within the HC group, SN radians positively correlated with age ( $r=0.533$ ,  $p=0.016$ ).

### 3.2.2.5. Validation of phase-shift data

To validate whether phase-shift data provide a quantitative measure of iron, Pearson's correlations were conducted between deep brain grey matter nuclei radians from the HC group and distribution of non-heme iron at autopsy, as reported by Hallgren and Sourander (1958) (Hallgren and Sourander 1958).

After exclusion of CN as an outlier, I found a positive correlation between radians and non-heme iron (mg/100 g fresh weight) ( $r= 0.874$ ,  $p= 0.01$ ) (Figure 3.5). I hypothesised that the appearance of the CN as an outlier was related to a regional discordance; while Hallgren and Sourander (1958) reported non-heme values for the whole caudate, in this study I originally reported values specifically for the caudate head only, which I observed had the highest iron values within the whole nucleus. I therefore also analysed the body of the caudate (CNb) and re-ran the correlation, substituting CN for CNb. Again, we found a significant correlation between radians and non-heme iron (mg/100 g fresh weight) ( $r= 0.624$ ,  $p= 0.034$ ).

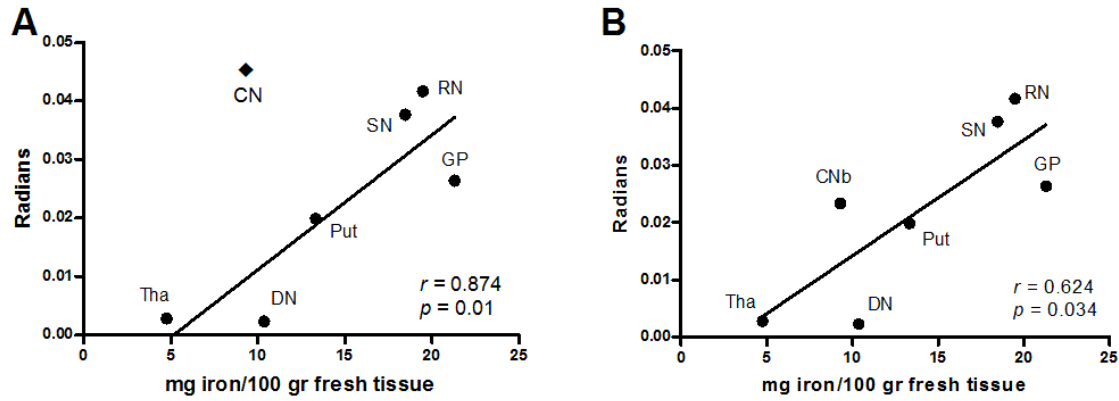


**Figure 3.7:** Pearson's correlations between phase-shift radians of deep brain grey matter nuclei from the healthy control group ( $n = 20$ ) and the distribution of non-heme iron at autopsy (mg iron/100 gr fresh tissue), as reported by Hallgren and Sourander (1958). In addition to the putamen (Put), globus pallidus (GP), Substantia Nigra (SN) and head of the caudate nucleus (CN) (Figure 3.5A) and body of CN (Figure 3.5B), radians were also derived from the thalamus (Tha), red nucleus (RN) and dentate nucleus (DN).

### 3.2.3. Discussion

The present subchapter evaluated regional iron deposition in deep brain nuclei, using high-pass filtered phase images to quantify average phase-shift (radians) in a large cohort of PD patients as well as age-matched healthy controls.

High-pass filtered phase shifts correspond linearly to regions with increased iron deposition in post-mortem studies when measured with synchrotron X-ray fluorescence (Hopp *et al* 2013). The relationship between phase shifts and iron is higher in grey matter nuclei than in white matter, which is accordance to the total iron deposition more than the water content (Hallgren and Sourander 1958) (see chapter 2).

Higher levels of iron, as measured using radians, were found in the SN of PD patients relative to the HC group. Further, when PD groups were subdivided according to UPDRS-III score, the SN showed a stratified distribution in which iron accumulation increased with motor severity. The SN is particularly vulnerable to abnormal increases in

iron. NM, which under normal conditions acts as a neuroprotective iron chelator, is released into the extracellular space as a result of nigral cell death. Extracellular NM then releases ferric iron, which in turn promotes oxidative stress, microglial activation and further neuronal death (Zecca *et al.* 2001). Indeed, increased nigral iron has been largely demonstrated in imaging studies using both SWI and relaxometry techniques (Antonini *et al.* 1993, Graham *et al.* 2000, Kosta *et al.* 2006, Martin *et al.* 2008, Wallis *et al.* 2008, Zhang *et al.* 2010, Jin *et al.* 2012, Wang *et al.* 2012, Bunzeck *et al.* 2013, Wu *et al.* 2014). Our design provides further evidence that this effect is not limited to increases within individuals but that significant changes can be seen between patients at different stages of disease. I note that although there was an increase in iron from moderate to high PD groups, this was not significant, suggesting that the rate of accumulation may decrease as the disease progresses. These findings cannot be explained by age differences, which is well known to correlate with nigral iron levels (Pfefferbaum *et al.* 2009) nor between-patient nigral size, since both were added as covariates in our model.

Increased iron deposition was also found in the putamen and globus pallidus, though no difference was found in the caudate nucleus. Results relating to striatal mineralisation have been highly variable, with several studies reporting increases (Antonini *et al.* 1993, Kosta *et al.* 2006, Wallis *et al.* 2008, Wang *et al.* 2012), reductions (Ryvlin *et al.* 1995, Graham *et al.* 2000) or normal levels (Martin *et al.* 2008, Zhang *et al.* 2010, Jin *et al.* 2012) in PD. One possible explanation for this heterogeneity could be due to the presence of calcifications in the striatum, which may cause SWI and relaxometry techniques to underestimate iron levels. Recently however, Ulla *et al.* (Ulla *et al.* 2013) demonstrated in an early PD cohort, that whilst there was a significant increase in R2\* signal in the caudal putamen between baseline and three year follow-up as compared to healthy volunteers, the rostral putamen remained at normal levels. Thus, the heterogeneity of results within the literature may be attributable to a rostro-caudal gradient of mineralisation, which in turn may be dependent on disease duration. In addition, the present data, though non-significant, show a trend for an initial increase of iron accumulation from low to moderate PD motor severity followed by a decrease from moderate to high motor severity in both the putamen and globus pallidus. Interestingly, this trend is in accordance with Ryvlin *et al.* (Ryvlin *et al.* 1995) who suggested that mineralization in these regions inversely correlate with disease duration when the duration of illness is above ten years. In contrast to the SN, it is clear that the mechanism behind striatal iron accumulation is

not yet understood. Further longitudinal studies with patients followed-up to the point of moderate to high motor severity are required to more accurately characterise the trajectory of striatal mineralisation in PD.

SN iron has previously been shown to strongly correlate with disease duration and motor severity (Martin *et al.* 2008, Wallis *et al.* 2008, Zhang *et al.* 2010, Wu *et al.* 2014). Here, I add that this relationship may be driven by bradykinesia/rigidity symptomatology, since although a strong relationship was found between these measures no association with tremor was identified. Bradykinesia and rigidity symptoms result as a direct consequence of dopaminergic nigral neurodegeneration (Vingerhoets *et al.* 1997), which is associated with regional iron accumulation (Dexter *et al.* 1987). In contrast, tremor does not only reflect dopaminergic nigrostriatal degeneration but may also be associated with serotonergic and noradrenergic pathology (Loane *et al.* 2013). Indeed, a recent work of our lab found that dopaminergic presynaptic terminal availability as well as storage capacity in the putamen and caudate nucleus were negatively associated with bradykinesia and rigidity scores, but not with tremor (Li *et al.* 2017). Bunzeck *et al.* found a significant association between striatal iron accumulation and tremor symptomatology, suggesting that putaminal iron may be used as a predictor for tremor dominance in early PD (Bunzeck *et al.* 2013). However, their measures were collected in the “on” medication state whilst the present assessments were conducted in the “off” medication state in order to avoid the confounding effect of variable clinical benefit with respect to tremor. Thus the present results suggest that iron levels in the SN may be a feasible biomarker of bradykinesia and rigidity progression in PD.

Previous studies measuring iron deposition with high-pass filtered phase imaging in PD (Zhang *et al.* 2009, Zhang *et al.* 2010, Jin *et al.* 2012) showed similar pattern of iron accumulation in the deep grey matter nuclei in PD than the present study. Two of the previous studies used “right-hand” system (Zhang *et al.* 2009, Jin *et al.* 2012) hence the presence of negative values pertaining to iron accumulation. “Left-hand” system defines the 0 intensity on the phase image as  $n$  and the intensity of 4095 as  $-n$  and “right-hand” system the contrary ( $-n$  to  $n$ ). Paramagnetic substances like iron would appear bright in left-hand systems and dark in the right-hand systems.

In conclusion, in the present subchapter I demonstrated that the degree of increased iron accumulation in PD is stratified according to disease severity in the SN but not in striatal subregions and that, nigral iron is associated with the severity of bradykinesia and rigidity rather than tremor.

### **3.3. Longitudinal high-pass filtered susceptibility imaging in Parkinson's disease**

In this subchapter I have evaluated changes in iron accumulation in deep grey matter nuclei including the SN, striatum and dentate nucleus over the course of 18 months using high-pass filtered phase imaging in 42 early-moderate PD patients as well as 6 age and gender matched HC. Second, I have explored whether regional iron deposition progresses with respect to separable motor symptoms, regardless of phenotypic grouping.

The aims of the present subchapter are:

- To evaluate the temporal changes in deep brain nuclei iron accumulation in PD groups of varying motor severity after 18 months' time.
- To investigate the association between changes in motor symptomatology and alterations over time of iron deposition in a large longitudinal PD cohort.

The hypotheses of this subchapter are:

- Longitudinal iron accumulation in SN and striatum in PD group is significantly increased when compared to HC.
- Changes in nigral iron deposition will be associated with changes in motor severity and bradykinesia-rigidity symptoms.

### **3.3.1. Subjects and methods**

#### **3.3.1.1. Study subjects**

Forty-two non-demented PD patients and six age and gender matched HC included in Chapter 3.1 were assessed at baseline and after  $18.45 \pm 2.4$  months.

Diagnosis of PD was performed by movement disorder specialists according to Parkinson's UK Brain Bank Criteria (Hughes 1992), excluding atypical parkinsonism, concomitant vascular load, history of cognitive impairment, psychiatric disorders and any factors that would preclude MRI scanning.

#### **3.3.1.2. Motor examination**

Neurological examination including UPDRS III was performed by in the practically defined off-medication state during baseline and follow-up scanning visits.

The UPDRS-III was used to calculate total motor severity and further subdivided into tremor (items 15-18) and bradykinesia-rigidity (items 2-9 and 14) subscores. The difference ( $\Delta$ ) in UPDRS-III, tremor and bradykinesia-rigidity subscores between baseline and follow-up visits were calculated.

Levodopa equivalent daily dosage (LEDD) for each participant was calculated and shown in Table 6.

All PD patients were ranked according to the change in disease severity ( $\Delta$  total UPDRS III) between baseline and follow-up and divided into 3 equal groups ( $n=14$ ) representing low ( $PD_{low}$ ), medium ( $PD_{med}$ ) and high ( $PD_{high}$ ) degrees of progression.

### **3.3.1.3. Susceptibility imaging weighted**

SWI protocol was previously described in Chapter 3.1. 24 PD subjects underwent the SWI protocol with 72 slices of 1.9 mm thickness and slice gap of 20% obtained in an interleaved order parallel to the anterior-posterior commissural line in the left-hand reference system. An additional 18 PD participants underwent a modified SWI protocol in which 88 slices of 0.9 mm thickness with a 20% slice gap were acquired

Phase images were reconstructed automatically on the Siemens workstation (Syngo MR B17 software, SWI version 1, Siemens, Erlangen, Germany), which included application of a 64\*64 high-pass filter to remove low spatial frequency effects.

### **3.3.1.4. Region of interest analysis.**

ROI analysis was detailed in chapter 3.1.

### **3.3.1.5. Statistical analysis**

Statistical analyses were conducted using SPSS statistical software (version 22.0 SPSS Inc., Chicago, IL, USA)

Demographic data were compared using paired *t*-tests, one-way ANOVA and Chi-square test for independence, as appropriate.

Shapiro-Wilk test was used to ascertain the normality distribution of regional iron measurements. The change in bilateral regional radians between baseline and follow-up for all PD patients was assessed using paired *t*-tests. Bilateral ROIs showing a significant effect were considered for one-way analyses of covariance (ANCOVA) to test the difference in  $\Delta$ radians (follow-up – baseline) between PD subgroups (PD<sub>low</sub>, PD<sub>med</sub>, PD<sub>high</sub>) representing the degree of motor progression over time, adjusting for regional area and age and followed with post-hoc Bonferroni-adjusted pairwise comparisons.

To assess longitudinal differences in iron load between clinically most and least affected sides, as indicated by UPDRS-III laterality items,  $\Delta$ radians for each region were compared across hemispheres for all patients (n =42) using paired *t*-tests.

Spearman's correlation coefficient was used to assess the relationship between  $\Delta$ radians in each region bilaterally and changes in motor severity measurements ( $\Delta$ UPDRS III,  $\Delta$ tremor and  $\Delta$ bradykinesia-rigidity).

Results were corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) and considered significant at  $p < 0.05$ .

### **3.3.2. Results**

#### **3.3.2.1. Demographics**

The mean time difference between baseline and follow-up imaging was  $18.45 \pm 2.4$  months. For all PD patients (n= 42), there was a significant increase in UPDRS III, Hoehn & Yahr stage and LEDD at follow-up as compared to baseline.

As expected, there was a significant difference between PD<sub>low</sub>, PD<sub>med</sub> and PD<sub>high</sub> subgroups in terms of  $\Delta$ UPDRS III score. Interestingly there was an important gap between PD<sub>med</sub> and PD<sub>high</sub> subgroups'  $\Delta$ UPDRS III scores, even though no atypical parkinsonisms (PSP, MSA) were included in this study. In addition, no subgroup differences were detected for age, gender,  $\Delta$ disease duration,  $\Delta$ Hoehn & Yahr or  $\Delta$ LEDD (*Table 3.3*).

| PD PATIENTS              |                   |                   |                    |                                     |                  |
|--------------------------|-------------------|-------------------|--------------------|-------------------------------------|------------------|
|                          | Baseline          | Follow-Up         |                    | Statistic                           | <i>p</i>         |
| Volunteers               | 42                | 42                |                    | NA                                  | NA               |
| Age (years)              | 57.79 ± 7.81      | 59.41 ± 7.82      |                    | <i>t</i> (41)= 4.49 <sup>a</sup>    | <0.001           |
| Gender (m:f)             | 32:10             | 32:10             |                    | NA                                  | NA               |
| Disease duration (years) | 4.69 ± 2.23       | 6.24 ± 2.24       |                    | <i>t</i> (41)= 10.71 <sup>a</sup>   | <0.001           |
| UPDRS III                | 29.27 ± 0.84      | 37.71 ± 10.28     |                    | <i>t</i> (41)= 7.59 <sup>a</sup>    | <0.001           |
| Hoehn and Yahr scale     | 1.91 ± 0.38       | 2.08 ± 0.29       |                    | <i>t</i> (41)= 2.95 <sup>a</sup>    | 0.005            |
| LEDD                     | 473 ± 321         | 610 ± 326         |                    | <i>t</i> (41)= 7.40 <sup>a</sup>    | <0.001           |
| PD SUBGROUPS             |                   |                   |                    |                                     |                  |
|                          | PD <sub>low</sub> | PD <sub>med</sub> | PD <sub>high</sub> | Statistic                           | <i>p</i>         |
| Volunteers               | 14                | 14                | 14                 | NA                                  | NA               |
| Age (years)              | 59.46 ± 8.84      | 50.72 ± 7.82      | 60.41 ± 7.59       | <i>F</i> (2,39)= 0.29 <sup>b</sup>  | 0.745            |
| Gender (m:f)             | 3:11              | 3:11              | 4:10               | $\chi^2$ (2)= 0.17 <sup>c</sup>     | 0.549            |
| Δ Disease duration       | 1.59 ± 0.31       | 1.57 ± 0.11       | 1.59 ± 0.19        | <i>F</i> (2,39)= 0.05 <sup>b</sup>  | 0.948            |
| Δ UPDRS III              | 0.26 ± 2.58       | 6.92 ± 1.33       | 14.14 ± 3.46       | <i>F</i> (2,39)= 98.77 <sup>b</sup> | <b>&lt;0.001</b> |
| Δ Hoehn and Yahr scale   | 0.01 ± 0.36       | 0.08 ± 0.29       | 0.12 ± 0.51        | <i>F</i> (2,39)= 0.32 <sup>b</sup>  | 0.731            |
| Δ LEDD                   | 96 ± 103          | 73 ± 134          | 99 ± 117           | <i>F</i> (2,39)= 0.19 <sup>b</sup>  | 0.828            |

**Table 3.3:** Demographic and clinical characteristics of all PD patients at baseline and 18.45 ± 2.4 months follow-up (up) and PD subgroups representing low (ΔUPDRS III= 0.26 ± 2.58), medium (ΔUPDRS III= 6.92 ± 1.33) and high (ΔUPDRSIII= 14.14 ± 3.46) degrees of motor severity progression between baseline and follow-up (down). Values represent the mean ± Standard deviation (SD). NA= not applicable; UPDRS III = Unified Parkinson's Disease Rating Scale Part III; LEDD= Levodopa equivalent daily dose; m:f = male:female; Δ= follow-up – baseline; PD= Parkinson's disease; <sup>a</sup>= Indicates that differences were tested using paired *t*-tests; <sup>b</sup>= Indicates that differences were tested using one-way ANOVA; <sup>c</sup>= Indicates that differences were tested using Pearson's Chi-square test for independence; (df) = degrees of freedom.

### 3.3.2.2. Regional phase analysis

Independent samples *t*-tests showed no significant differences in radians between the two SWI protocols for any of the bilateral ROIs ( $p>0.05$ ).

The analysis for the control group revealed no significant change after 18 months in any of the ROIs ( $p>0.05$ ). PD patients showed a significant increase in radians in the bilateral SN ( $p<0.001$ ) between the two scan points (annual increase of 12.93%). No significant longitudinal differences were found for the CN, Put or GP, however there was a trend for increased radians in the bilateral DN ( $p=0.067$ ) (Figure 3.8).

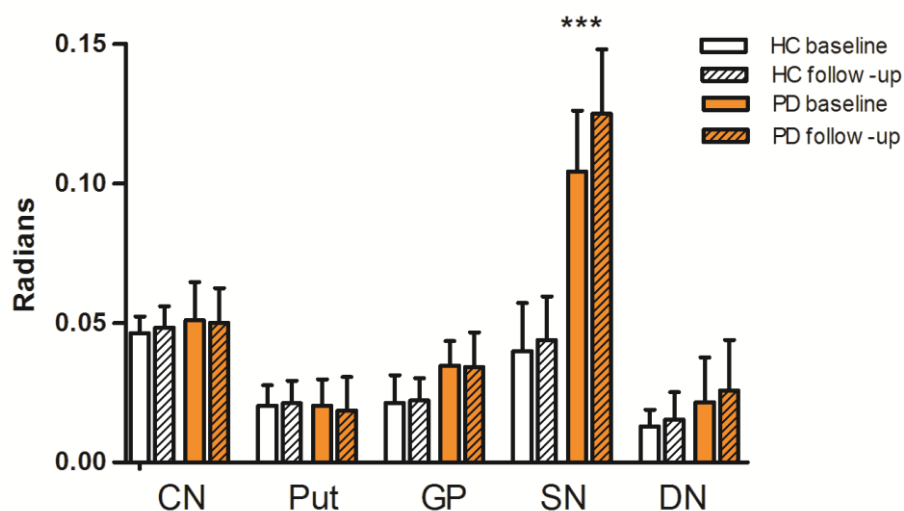


**Figure 3.8:** Bar graph showing the mean and standard deviation of the radians for bilateral caudate (CN), putamen (Put), globus pallidus (GP), substantia nigra (SN) and dentate nucleus (DN) at baseline (solid bars) and  $18.45 \pm 2.4$  follow-up (striped bars) for PD patients (orange bars,  $n=42$ ) and healthy control participants (white bars,  $n=6$ ). Comparison of regional radians by paired *t*-test showed a significant increase in the SN over time ( $p<0.001$ ) in the PD group by using paired *t*-tests between baseline and follow-up visits followed by post-hoc Bonferroni correction for multiple comparisons. \*\*\*= $p<0.001$ .

When PD subgroups (PD<sub>low</sub>, PD<sub>med</sub>, PD<sub>high</sub>) were entered into a one-way ANCOVA, there was a main effect of group in the bilateral SN ( $F(2,39)= 14.333, p<0.001$ ) after adjusting for regional area and age. Post-hoc Bonferroni-adjusted pairwise comparisons indicated that  $\Delta$ radians in this region were significantly greater for PD<sub>med</sub> ( $\Delta$ UPDRS III=  $6.93 \pm 1.33$ ) and PD<sub>high</sub> ( $\Delta$ UPDRS III=  $14.14 \pm 3.46$ ) as compared to PD<sub>low</sub> ( $\Delta$ UPDRS III=  $0.036 \pm 2.56$ ) ( $p<0.01$  and  $p<0.001$  respectively). No significant difference was found between PD<sub>med</sub> and PD<sub>high</sub> subgroups (Figure 3.9).

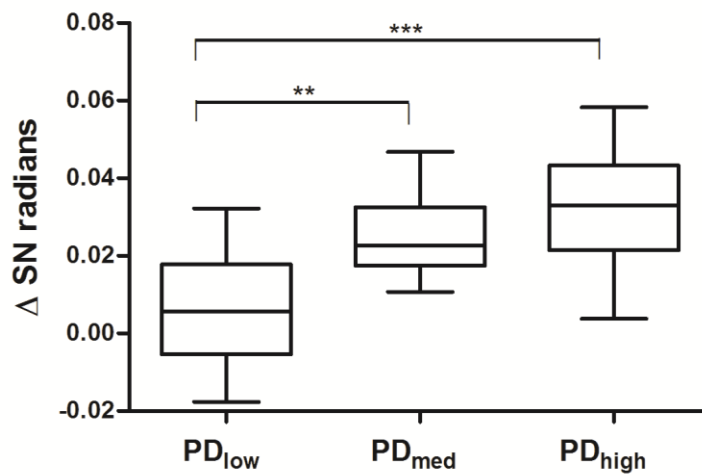


**Figure 3.9:** Box and whisker plot showing  $\Delta$ radians in the bilateral SN of PD<sub>low</sub> ( $\Delta$ UPDRS III=  $0.036 \pm 2.56$ ), PD<sub>med</sub> ( $\Delta$ UPDRS III=  $6.93 \pm 1.33$ ) and PD<sub>high</sub> ( $\Delta$ UPDRS III=  $14.14 \pm 3.46$ ) subgroups and results of post-hoc Bonferroni-adjusted pairwise comparisons following significant one-way ANCOVA (adjusted by age and regional area). \*\*\*=  $p < 0.001$ ; \*\* =  $p < 0.01$ ; \* =  $p < 0.05$ .

### 3.3.2.3. Analysis of clinical laterality

The SN on both the contralateral and ipsilateral hemispheres with respect to the clinically most affected side showed significant increases in radians between baseline and follow-up ( $p<0.001$ , annual increases of 13.05% and 12.81% respectively). Independent  $t$ -test revealed no significant difference in  $\Delta$ radians between the contralateral and ipsilateral SN. For CN, Put, GP and DN, no significant changes in radians were detected at follow-up as compared to

baseline for each hemisphere individually and there was no significant difference in  $\Delta$ radians between the clinically defined most and least affected sides.

### 3.3.2.4. Clinical correlations

Pearson's correlation showed a significant positive relationship between  $\Delta$ radians in the bilateral SN and  $\Delta$ UPDRS III ( $r = 0.650$ ,  $p < 0.001$ ) and  $\Delta$ bradykinesia-rigidity score ( $r = 0.523$ ,  $p = 0.001$ ). Furthermore  $\Delta$ radians in the bilateral DN were significantly correlated with  $\Delta$ tremor subscore ( $r = 0.464$ ,  $p = 0.007$ ) (Figure 3.10). No significant correlations were found between  $\Delta$ radians in the bilateral CN, Put or GP and  $\Delta$ UPDRS-III,  $\Delta$ bradykinesia-rigidity or  $\Delta$ tremor scores.

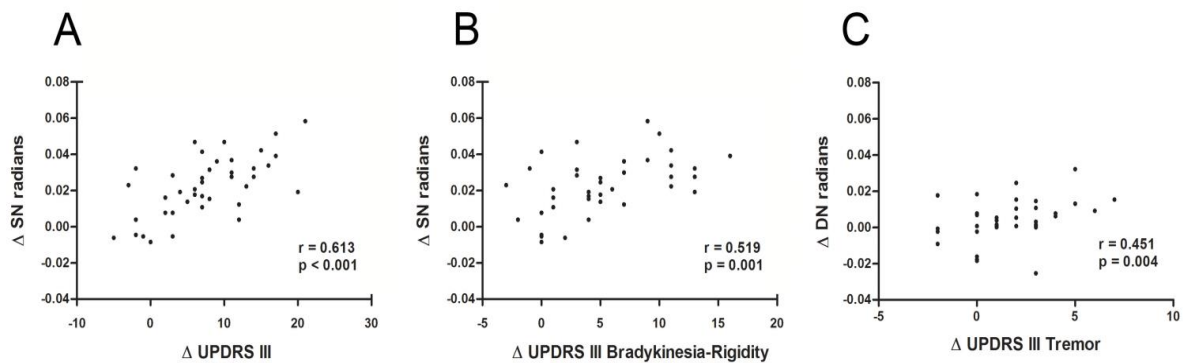


**Figure 3.10:** Scatterplots and Spearman's rho correlations showing significant positive relationships between  $\Delta$ radians in the bilateral SN and  $\Delta$ UPDRS-III (A) and  $\Delta$ bradykinesia-rigidity scores (B), as well as a significant positive correlation between  $\Delta$ radians in the bilateral DN and  $\Delta$ tremor scores (C).

### 3.3.3. Discussion

The present subchapter assessed changes in regional iron accumulation in deep grey matter nuclei of PD patients over the course of 18 months using high-pass filtered phase imaging. These observations demonstrate the presence of significant increases in iron deposition in the SN over time. The degree of this nigral mineralization over the study period was associated with changes in general motor severity and bradykinesia-rigidity, but not with tremor. Additionally, while the increase in iron accumulation in the DN did not reach statistical significance at follow-up, the change in iron levels in this region strongly correlated with changes in tremor subscore, but not with general motor severity or bradykinesia-rigidity. Collectively, these results imply that iron accumulation in PD is associated with the progression of separable motor symptoms in a regionally dependent manner.

Previous studies assessing follow-up changes of iron accumulation in PD performed with relaxometry techniques ( $R2^*$  and  $T2^*$ ) showed variable results pertaining longitudinal mineralisation in PD and its clinical associations (Ulla 2013, Rossi *et al.* 2014, Wieler 2014, Hopes *et al.* 2016). These observations may be explained at least in part by the presence of heterogeneity in the patient cohort and experimental design, for example age, disease severity or execution of the motor examination in an “on-medication” state. Significant iron increase in SN (Ulla 2013, Rossi *et al.* 2014, Hopes *et al.* 2016), Put (Ulla 2013, Hopes *et al.* 2016), GP (Rossi *et al.* 2014) and CN (Rossi *et al.* 2014, Hopes *et al.* 2016) were demonstrated in the previous longitudinal imaging cohorts. Furthermore, increased changes of nigral iron levels showed significant (Wieler 2014), moderate (Ulla 2013, Hopes *et al.* 2016) or no correlation (Rossi *et al.* 2014) with temporal changes in motor severity. First Ulla *et al.* (Ulla 2013) reported longitudinal increased iron nigral and putaminal levels after 36 months in PD patients in a 1.5T MRI system. The authors found moderate nigral follow-up iron changes with motor severity however this finding offers no clinical validation as motor examination was performed in “on medication-state”. Additional significant follow-up changes in striatal nuclei including GP, CN and SN were showed in a PD cohort in 2 year follow-up with no clinical longitudinal associations (Rossi *et al.* 2014). A recent study (Wieler 2014) showed no changes in regional iron accumulation in PD patients in a 36 months follow-

up, highlighting the large variance in nigral mineralization over time. In the former case, however, a significant correlation was showed between longitudinal changes in nigral iron deposition and motor severity. In a large cohort of 35 early-stage PD patients (Hopes *et al.* 2016) significant changes of iron levels were showed in Put, CN and SN after 2 year follow-up as well as moderate correlation of temporal nigral iron changes with motor severity. In the present subchapter a large baseline and follow-up sample size of PD subjects (n =42) was examined in practically defined off-medication state for both visits, allowing us to avoid inconsistencies brought about by differing LEDD and medicinal response found in previous longitudinal studies. Significant temporal changes in nigral iron deposition over 18 months' time using high-pass filtered phase imaging could reflect lesser technical biased than relaxometry studies.

Significant correlations between nigral iron accumulation and changes in motor severity as assessed with UPDRS-III were demonstrated in the present study. In addition, ANCOVA further showed that patients with larger increases in motor severity over 18 months displayed greater increases in iron accumulation as opposed to those whose disease did not progress. The present design confirms that nigral iron deposition shows an ascendant stratified distribution with regards with changes in motor severity. These results might be explained by the oxidative vulnerability of the SN by iron deposition and its relationship with dopaminergic degeneration (Dexter *et al.* 1987) suggesting longitudinal nigral deposition as a reliable biomarker of disease progression in PD.

Moreover, I have shown that while there is a relationship between nigral mineralization and bradykinesia and rigidity, there is no relationship with the symptom tremor. These results are also in accordance with previous data from the cross-sectional study (chapter 3.1). Indeed bradykinesia and rigidity are directly associated with dopaminergic nigral degeneration, as assessed with  $^{18}\text{F}$ -DOPA PET studies (Vingerhoets *et al.* 1997). Interestingly, a recent original work of our lab recently found that longitudinal changes in striatal dopamine transporter density as measured using  $^{11}\text{C}$ -PE2I as well as presynaptic dopamine synthesis capacity were negatively associated with temporal changes in bradykinesia-rigidity scores, but not with tremor (Li *et al.* 2017). To the best of my knowledge no other longitudinal MRI studies of iron in PD have shown temporal motor associations with longitudinal regional iron deposition patterns.

Furthermore, a significant positive correlation was found between changes in iron accumulation in the DN after 18 months with tremor progression. In the previous cross-sectional study I found higher DN iron load in PD subjects when compared with HC, however no motor associations were displayed. DN is directly implicated in the cerebello-dentato-thalamic pathway, which is involved in postural and rest tremor genesis (Helmich *et al.* 2012). It has been hypothesized that basal ganglia dysfunction might trigger tremor symptoms and cerebellar impairment would manipulate tremor amplitude. Several imaging studies have implicated the cerebellum as having a causal role in tremor in PD (Mure *et al.* 2011, Ma *et al.* 2015), however symptom-specific outcome of cerebellar mineralization is yet to be ascertained. Previous attempts to evaluate tremor in PD with relaxometry showed a significant association with putamenal iron accumulation in a cross-sectional study (Bunzeck *et al.* 2013), though again, the results were obtained using motor severity assessments conducted in the “on-medication” state using analysis of subtype ratios, thus such firm conclusions are severely limited, particularly since it is not expected that iron load would change proportionally with motor severity between “on” and “off” states. In addition, higher DN iron levels were shown in an essential tremor cohort when compared to HC (Novellino *et al.* 2013). Although no further associations were found with tremor clinical scales, this study suggests increased iron accumulation in DN plays a role in tremor genesis.

Recent therapeutic attempts targeting iron accumulation in PD with iron chelators as deferiprone have shown promising clinical outcomes (Devos *et al.* 2014) (see chapter 6). Deferiprone is a membrane-permeant bidentate chelator with neuroprotective properties as demonstrated in animal models in PD (Dexter *et al.* 2011). Two randomized clinical trials with deferiprone have recently been undertaken in PD assessing iron chelation with relaxometry techniques (T2\* or R2\*). Devos *et al.* (Devos *et al.* 2014) demonstrated minimal reduction of UPDRS-III motor scale after 12 months of treatment with deferiprone associated with reduction of R2\* values in SN. The authors suggested removing iron from SN might offer clinical motor benefit in PD. Furthermore iron chelation of extranigral nuclei such as DN and caudate nucleus was associated with no change in motor severity during 6 months of treatment (see chapter 6). As multi-centre studies using iron chelators are needed in order to ascertain whether these drugs have neuroprotective/antioxidant effects in PD, high-pass filtered phase techniques might

constitute reliable imaging markers for monitoring the pathological progress of iron chelation *in vivo*.

In conclusion the results of the present subchapter provide evidence that specific pattern of regional iron deposition in SN is associated with temporal changes in disease severity and bradykinesia-rigidity and in DN with tremor.

### **3.4. Limitations and future directions**

There are some limitations in the present chapter. Cross-sectional and longitudinal PD cohorts assessed in the chapter were fairly extensive and comprehensive. Nevertheless the inclusion of bigger cohorts of age and gender matched HC groups in longitudinal subchapter is required in order to ascertain the alterations over time in iron deposition in healthy ageing.

Second, I analyzed two slices with the highest iron-content in order to avoid background artifacts. Although no ROI size differences were found between PD and HC, this method may not reflect the iron content of the entirety of each nucleus. It is important to remark that after manual delineation of the each nucleus of the high-pass filtered map, the next step should be coregistration to the MPRAGE sequence by affine registration in order to reduce variability introduced by manual segmentation and other registration processes.

Third, although it is well documented that the pars compacta (SNc) and pars reticulata (SNr) present differential levels of mineralization, I analyzed the SN without further segmentation in chapters 3 and 6. Post-mortem studies including those immunostaining for calbindin D<sub>28K</sub> (Damier *et al.* 1999) have provided valuable information on the definition of nigral compartments based on dopamine cell-independent anatomical landmarks. However, translation to MRI segmentation is not straightforward due to technical considerations (acquisition volume, slice orientation and thickness) and imperfect correspondence between anatomically defined nigral distribution and that observed on T2-weighted images (Oikawa *et al.* 2002, Sasaki *et al.* 2008). Nevertheless, attempts have been made to manually separate the nigra albeit with significant methodological variation (Martin *et al.* 2008, Ulla *et al.* 2013), highlighting disagreement on best localization procedures. Given the inability of phase and relaxometry images to resolve intra-nigral boundaries, I opted to delineate the SN as a whole

in order to maintain accuracy of the measurements, though I acknowledge that this was at the cost of specificity and conclusiveness regarding to the level of mineralization for each subregion and their relationship to PD pathology. Higher MR field strength and the use of quantitative susceptibility mapping (Barbosa *et al.* 2015) which can further reduce phase image artefacts may provide greater spatial validity with respect to iron and improve the accuracy with which nigral compartments can be interrogated.

Fourth, it has not been yet documented differential levels of iron accumulation in accordance to the ventromedial degree of neurodegeneration showed within the SNc (Fearnley and Lees 1991). In this chapter I analyzed the entire SN without segmenting SN medial and ventral tiers whilst it was not segmented the SNc and SNr. However, recent works assessing the loss of nigrosome 1 in the lateroventral areas of the SNc (Schwarz *et al* 2013) using high-pass filtered imaging could give light to the aforementioned iron nigral gradient as it is translated in increased iron deposition. To further develop the previous point a multi-center imaging study conducted at University of Nottingham and Imperial College London, the so-called PaMIR study, is been currently developing an MRI multi-sequence protocol including NM-MR imaging and QSM within the Nigrosome 1 in patients with suspected PD.

## 4. Studies of Neuromelanin-weighted magnetic imaging in Parkinson's disease

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### 4.1. Introduction

Loss of neuromelanin-containing dopaminergic neurons of SNc is considered the cardinal neuropathological finding along with the presence of alpha-synuclein aggregates in PD (Fearnley and Lees 1991) (see chapter 1). In vivo quantification of pigment load with Neuromelanin-weighted MR imaging has invariably displayed the presence of reduced pigmentation in PD when compared HC (Sasaki *et al.* 2006, Kashiara *et al.* 2011, Schwarz *et al.* 2011, Matsuura *et al.* 2013, Ogisu *et al.* 2013, Ohtsuka *et al.* 2013, Castellanos *et al.* 2015, Reimao *et al.* 2015, Fabbri *et al.* 2017). This constant finding could be considered along with increased nigral iron deposition as robust imaging diagnostic biomarkers in PD.

Several studies reported associations between decreased nigral pigment load with disease duration (Kashiara *et al.* 2011, Schwarz *et al.* 2011, Matsuura *et al.* 2013), Hoehn and Yahr scale (Kashiara *et al.* 2011, Matsuura *et al.* 2013, Fabbri *et al.* 2017) and total UPDRS scale (Schwarz *et al.* 2011). Interestingly, no significant motor relationships were observed in the aforementioned studies, which was probably due to inclusion of “on-medication” clinical examinations.

To my knowledge, there are no cross-sectional studies that evaluate whether significant changes in nigral pigmentation are present in different stages of disease duration or whether are associated with classical parkinsonian motor symptoms.

Furthermore the previous findings have not been consistently replicated in longitudinal studies. To date only one study using Neuromelanin-weighted MRI (Matsuura *et al.* 2016) evaluated the temporal changes of nigral depigmentation in PD. The authors

reported strong associations between changes in nigral pigment content and changes in disease duration and, in less degree, with Hoehn and Yahr scale. This study however presents several caveats that are worth to mention. First, the PD cohort size could be considered as smaller when compared to previous cross-sectional studies, besides no HC group was included. In addition, associations with motor severity were not described in the present study, probably due to inclusion of different time points of the baseline and follow-up scans, varying from one to four years.

## **4.2. Cross-sectional study of Neuromelanin-weighted magnetic imaging in Parkinson's disease**

In this subchapter, I have undertaken volumetric analysis of Neuromelanin of the SNc (SNc-NM) by using Neuromelanin-weighted MR in a large early-moderate stage PD cohort of 66 volunteers with 20 age and gender matched HC. Volumetric analysis of SNc-NM in the PD cohort were used to further explore their clinical associations with disease duration, clinical laterality, motor severity as assessed with UPDRS III “off-medication state” and motor symptomatology. According to the literature, reduction in pigment load is associated with disease duration (Kordower *et al.* 2013) hence further subdivision of PD groups according with their disease duration was performed adjusted by age and regional area in order to ascertain the pattern of neuromelanin intensity loss in PD.

The aims of this subchapter are:

- To assess nigral pigmentation differences between PD and HC groups.
- To assess the clinical relationship of nigral pigmentation load with disease duration, clinical laterality, motor severity and motor phenotypes in the PD cohort.

The hypotheses of this subchapter are:

- PD patients will show reduced nigral pigmentation when compared to HC.
- Reduced neuromelanin-related volumes will correlate with disease duration and motor severity as assessed with UPDRS III.

- The most symptomatic side of SNc will display reduced neuromelanin-related volumes when compared with least symptomatic side.

#### **4.2.1. Subjects and methods**

##### **4.2.1.1. Study subjects**

Sixty-six early-moderate stage PD patients and twenty age and gender-matched HC were included in this study. PD patients were recruited from three UK centers (Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge; National Hospital for Neurology and Neurosurgery, University College London Hospitals NHS Foundation Trust and Charing Cross Hospital, Imperial College Healthcare NHS Trust, London) and one center from Sweden (Skåne University Hospital, University Hospital, Lund, Sweden) as part of the PaMIR (Parkinson's disease Magnetic Imaging Repository) and TransEuro projects.

Diagnosis of PD was performed as explained chapter 3. Patients were instructed to withdraw from anti-PD medications 24 hours prior to scanning. Off-medication UPDRS-III scores were then subdivided into tremor (sum of items 15–18) and bradykinesia-rigidity subscores (sum of items 2–9 and 14). Levodopa- equivalent daily dose (LEDD) was calculated.

All PD patients were ranked according to their disease severity (years) divided into 3 equal groups ( $n=22$ ) representing low ( $PD_{low}$ ), medium ( $PD_{med}$ ) and high ( $PD_{high}$ ) disease duration.

##### **4.2.1.2. Neuromelanin-weighted magnetic imaging**

MRI scans were acquired on a 3T Siemens Magnetom Trio system with 32-channel head coil and consisted of neuromelanin-sensitive T1-weighted turbo spin-echo (TSE: TR = 829ms, TE = 12ms, flip angle =  $123^\circ$ ; echo train length = 4, echo spacing = 11.5ms, low specific absorption rate, FoV = 256\*256mm; matrix size = 320\*320, in-plane voxel size = 0.8mm) sequences. For the TSE, 12 slices of 2.5 mm thickness and slice gap of 0.25

mm were acquired parallel to the AC-PC line with coverage of the whole midbrain and upper pons. Scans lasted 332 seconds and patients were instructed to remain as still as possible for the duration. (See chapter 2)

#### **4.2.1.3. Region of interest analysis**

For quantitative evaluation of the Neuromelanin-weighted MRI, the ROIS were measured in three slices of the midbrain where SNc hyperintensity was defined. Bilateral manual delineation of SN and background included cerebral peduncles and midbrain tegmentum was performed on Analyze software version 11 (Mayo Clinic, USA) (see figure 10, Chapter 2). All study subjects were anonymised prior to delineation, thus blinding group and disease severity.

Signal of intensity as background expressed in mean and standard deviation was averaged to further calculate the calculated to define the threshold to define Neuromelanin-containing voxels. In the present chapter, I used the previously published method (Schwarz *et al.* 2011, Schwarz *et al.* 2017) and applied the threshold of background signal intensity (cerebral peduncles and midbrain tegmentum) (*figures 2.6 and 2.7*) according to next formula:

$$\text{SNc-NM threshold} = \text{Background}_{\text{Mean}} + 3 \text{ Background}_{\text{SD}}$$

Number of voxels, standard deviations and regional areas were extracted for each SN unilaterally before averaging obtaining bilateral data and then multiplied by 0.8x0.8x2.5 in order to compare the neuromelanin-related volumetric differences between groups.

Additional analysis of evaluation of nigral hyperintensity areas was performed by calculating contrast ratios, according to Sasaki et al (Sasaki et al., 2006) using the formula (SNc-CP)/CP. Contrast ratios of total SNc were calculated by averaging bilateral data.

#### 4.2.1.4. Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 22.0 SPSS Inc., Chicago, Illinois). Shapiro-Wilk test was used to ascertain the normality distribution of SNc-NM volumes.

Group demographics and bilateral SNc-NM volumes were compared between PD and HC groups using independent t-tests.

To assess clinical laterality of SNc-NM volumes within the PD group, nigral volumes of clinically most/least affected sides, as indicated by UPDRS III laterality items, were compared for each region using independent t tests.

Pearson's correlation coefficient was used to assess relationships between SNc-NM volumes and measures of disease severity (disease duration, UPDRS III total, tremor and bradykinesia/rigidity). Correlations were considered significant only for  $P < 0.05$  following Benjamini-Hochberg false discovery rate (FDR) adjustment.

Further analysis of covariance (ANCOVA) were performed subdividing PD subjects into three disease severity groups using disease duration tertile calculations (Table 2), along with the HC group, adjusting for age and regional area and followed with post hoc Bonferroni-adjusted pairwise comparisons

#### 4.2.2. Results.

##### 4.2.2.1. Demographics

Clinical and demographic data are summarized in *Table 4.1*. There were no significant differences in age ( $p= 0.347$ ) or gender ( $p= 0.059$ ) between PD and HC groups.

As expected, there was a significant difference between PD<sub>low</sub>, PD<sub>med</sub> and PD<sub>high</sub> subgroups in terms of disease duration, LEDD ( $p<0.001$ ) and Hoehn and Yahr scale ( $p<0.05$ ). No subgroup differences were detected for age, gender and UDPRS III scores.

|                          | HC            | PD            |               | Statistic                            | <i>p</i>         |
|--------------------------|---------------|---------------|---------------|--------------------------------------|------------------|
| Volunteers               | 20            | 66            |               | NA                                   | NA               |
| Age (years)              | 57.51 ± 9.93  | 58.83 ± 8.38  |               | <i>t</i> (23,16)= 0.53 <sup>a</sup>  | 0.477            |
| Gender (m:f)             | 10:10         | 48:18         |               | $\chi^2(1)= 1.99$ <sup>b</sup>       | 0.057            |
| Disease duration (years) | NA            | 5.24 ± 2.41   |               | NA                                   | NA               |
| UPDRS III                | NA            | 33.26 ± 11.77 |               | NA                                   | NA               |
| Hoehn and Yahr scale     | NA            | 1.89 ± 0.41   |               | NA                                   | NA               |
| LEDD                     | NA            | 578 ± 352     |               | NA                                   | NA               |
| PD SUBGROUPS             |               |               |               |                                      |                  |
|                          | PD group 1    | PD group 2    | PD group 3    | Statistic                            | <i>p</i>         |
| Volunteers               | 22            | 22            | 22            | NA                                   | NA               |
| Age (years)              | 60.41 ± 9.91  | 57.88 ± 7.53  | 58.16 ± 7.46  | <i>F</i> (2,63)= 0.54 <sup>a</sup>   | 0.658            |
| Gender (m:f)             | 15:7          | 16:6          | 16:6          | $\chi^2(2)= 0.15$ <sup>b</sup>       | 0.926            |
| Disease duration (years) | 2.63 ± 0.69   | 5.25 ± 0.58   | 7.99 ± 1.46   | <i>F</i> (2,63)= 159.41 <sup>c</sup> | <b>&lt;0.001</b> |
| UPDRS-III                | 28.22 ± 12.59 | 35.31 ± 9.84  | 36.55 ± 11.47 | <i>F</i> (2,63)= 2.86 <sup>c</sup>   | 0.065            |
| Hoehn and Yahr scale     | 1.68 ± 0.58   | 1.95 ± 0.21   | 2.08 ± 0.24   | <i>F</i> (2,63)= 4.01 <sup>c</sup>   | 0.023            |
| LEDD                     | 345 ± 283     | 628 ± 333     | 805 ± 282     | <i>F</i> (2,63)= 9.46 <sup>c</sup>   | <b>&lt;0.001</b> |

**Table 4.1:** Demographic and clinical characteristics of Parkinson's disease subjects and controls at baseline (up) and subgroups divided into 3 disease duration groups using tertile calculations in PD group 1 (disease duration= 2.63 ± 0.69 years), PD group 2 (disease duration= 5.25 ± 0.58 years) and PD group 3 (disease duration= 7.99 ± 1.46 years) (down). Values represent the mean ± standard deviation (SD). NA= not applicable; UPDRS III= Unified Parkinson's Disease Rating Scale Part III; LEDD= Levodopa equivalent daily dose; <sup>a</sup>= Indicates that differences were tested using independent *t*-tests; <sup>b</sup>= Indicates that differences were tested using Pearson's Chi-square test for independence; <sup>c</sup>= Indicates that differences were tested using one-way ANOVA; (df) = degrees of freedom).

#### 4.2.2.2. Neuromelanin volumetric analysis

No significant differences were found in the original delineated areas along ROIs between HC ( $50.75 \pm 7.81 \text{ mm}^2$ ) and PD groups ( $51.52 \pm 7.55 \text{ mm}^2$ ) ( $p > 0.05$ ).

The PD group ( $175.52 \pm 54.34$ ) showed significantly reduced SNc-NM volumes ( $p < 0.001$ ) when compared to HC ( $272.12 \pm 47.75$ ) (Figure 4.1). The PD patients displayed a 35.5% decrease of SNc-NM volumes compared to the control cohort, with an overlap of the individual ranges. In addition PD group ( $0.088 \pm 0.058$ ) displayed further reductions of SNc-NM contrast ratios ( $p < 0.001$ ) when compared to HC ( $0.117 \pm 0.062$ ), showing a 24.79% of decrease.

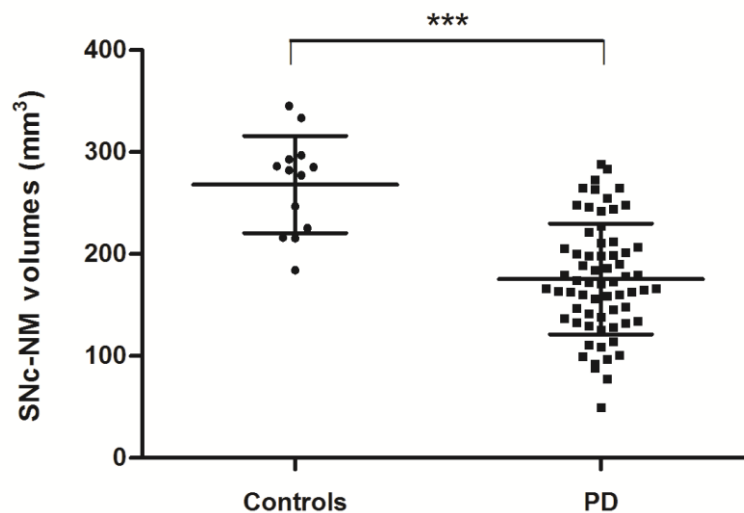


**Figure 4.1:** Scatter plots showing the mean and standard deviation of bilateral average of SNc-NM volumes (in  $\text{mm}^3$ ) of PD (square dots,  $n = 66$ ) and HC (round dots,  $n = 20$ ) groups. Comparison of SNc-NM volumes using analysis of independent  $t$ -tests showed significant reductions of SNc-NM (\*\*\*) ( $p < 0.001$ ) in PD when compared to HC group.

In order to explore the significant association between SNc-NM load and disease duration in PD further analysis of covariance (ANCOVA) of three equal PD subgroups divided with tertially calculations according to disease duration as well as HC group was performed adjusted by age and original delineated area (Table 4.1).

ANCOVA including HC and PD subgroups indicated main effects of disease duration in SNc-NM volumes ( $F(3,75)= 65.008$ ,  $p<0.001$ ) (Figure 4.2). Post hoc Bonferroni-adjusted pairwise comparisons showed that the SNc-NM volumes in all three PD subgroups were significantly reduced than HC ( $p<0.001$ ). Pairwise comparisons between the PD subgroups revealed PD group 3 showed significantly lower volumes compared to PD groups 1 ( $p<0.001$ ) and PD group 2 ( $p<0.01$ ).

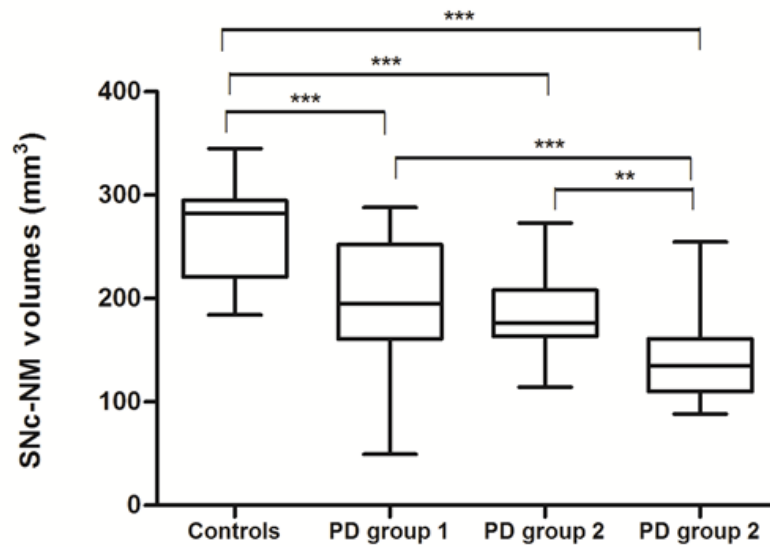


Figure 4.2: Box and whisker plot displaying the SN volumes of PD subgroups divided according to disease duration using tertile calculations in PD group 1 (disease duration=  $2.63 \pm 0.69$  years), PD group 2 (disease duration=  $5.25 \pm 0.58$  years) and PD group 3 (disease duration=  $7.99 \pm 1.46$  years) as well as HC group in addition to results of post-hoc Bonferroni-adjusted pairwise comparison following significant one-way ANCOVA (adjusted by age and regional area). \*\*\*=  $p<0.001$ ; \*\*=  $p<0.01$ ; \*=  $p<0.05$ .

#### 4.2.2.3. Analysis of clinical laterality

The SN contralateral to the clinically most affected side ( $156.11 \pm 55.62$ ) showed reduced SNc-NM volumes ( $p < 0.001$ ) compared to the least affected side ( $194.93 \pm 62.49$ ) (figure 4.3). In addition, SNc-NM contrast ratios of the most affected side ( $0.0083 \pm 0.021$ ) were lower than the least affected side ( $0.093 \pm 0.024$ ) ( $p = 0.024$ ).

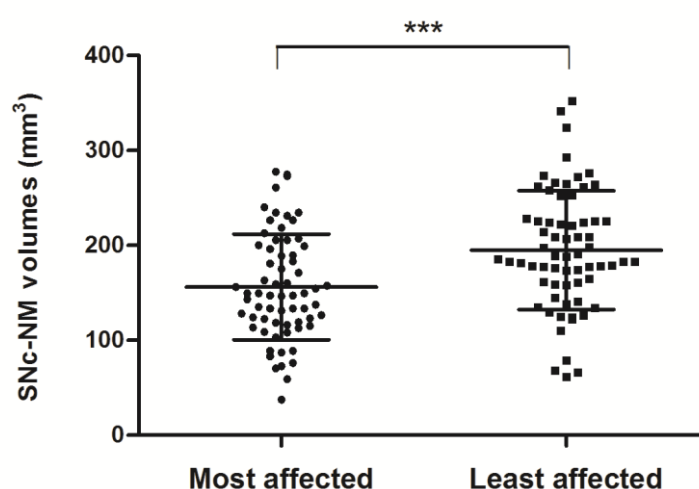


**Figure 4.3:** Scatter plots showing the mean and standard deviation of SNc-NM volumes (in mm<sup>3</sup>) of PD indicating most (round dots) and least affected (square dots) contralateral to the symptomatic clinical sides. Comparison of unilateral PD SNc-NM volumes using analysis of independent *t* tests showed significant reductions in the most affected (\*\*\*) in PD when compared to the least affected contralateral to the symptomatic side.

#### 4.2.2.4. Clinical correlations

Pearson correlation analyses revealed significant negative correlations between SNc-NM volumes and disease duration ( $r = -0.501$ ,  $p < 0.001$ ), total UPDRS III scale ( $r = -0.319$ ,  $p = 0.011$ ), Hoehn and Yahr scale ( $r = -0.351$ ,  $p = 0.004$ ) and LEDD ( $r = -0.399$ ,  $p = 0.001$ ) (Figure 4.4). No significant correlations were found with bradykinesia-rigidity or tremor subscores.

Spearman non-parametric correlations only showed negative correlations between SNc-NM contrast ratios with disease duration ( $r = -0.305$ ,  $p = 0.019$ ), but not with the rest of clinical scales.

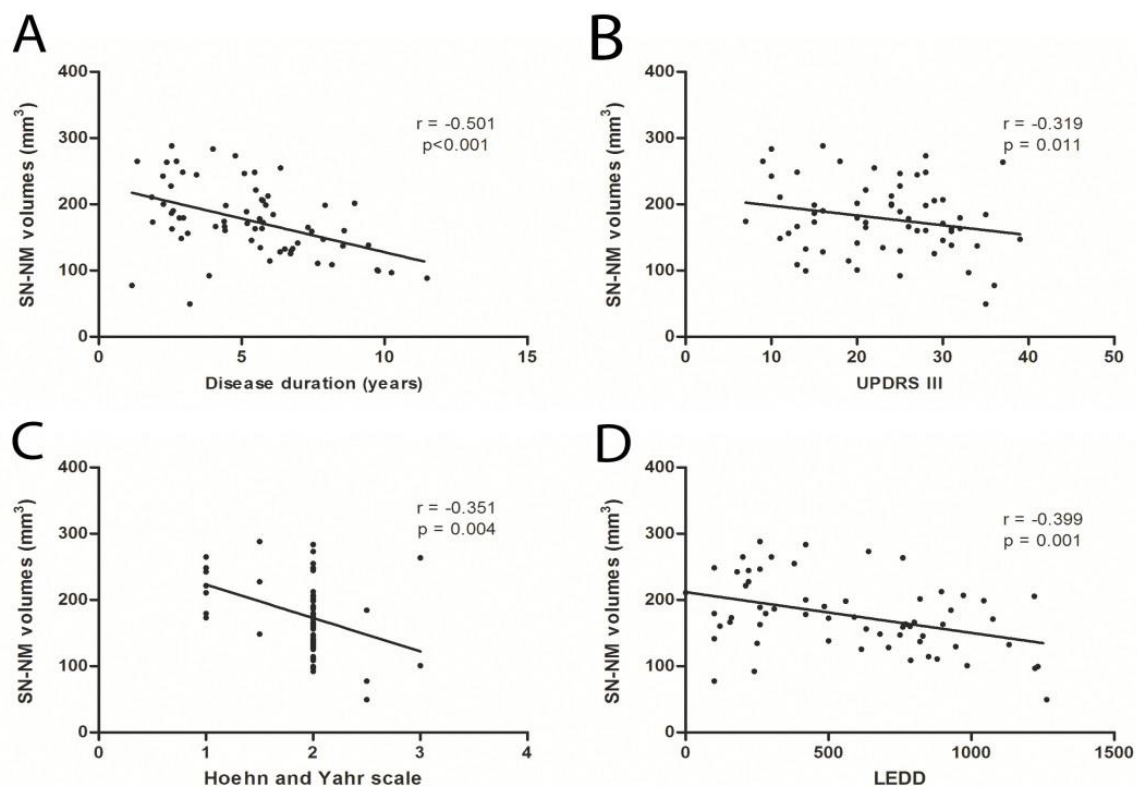


**Figure 4.4:** Scatterplots and Pearson's correlations showing negative relationships between SNc-NM volumes and disease duration (A, \*\*\* $p < 0.001$ ), UPDRS III (B, \* $p < 0.05$ ), Hoehn and Yahr scale (C, \*\* $p < 0.01$ ) and LEDD (D, \*\* $p = 0.001$ ).

#### 4.2.3. Discussion

This cross-sectional magnetic resonance subchapter assessed nigral pigment load using Neuromelanin-weighted MRI in a large cohort of PD patients as well as age-matched healthy controls. Lower nigral pigmentation was observed in PD patients when compared to HC group. The degree of nigral depigmentation was strongly associated with disease duration and in lesser degree with motor severity, Hoehn and Yahr stage and total LEDD.

Furthermore, when PD groups were subdivided according to disease duration, the nigral SNc-NM volumes showed a descendant stratified distribution. These findings cannot be explained by age differences, which are well known to correlate with nigral pigment load nor between-patient nigral size, since both were added as covariates in the present model. NM induces T1 shortening effects due to the paramagnetic effects of iron (Enochs *et al.* 1997). Previous T1 weighted studies were not able to distinguish NM contrast with surrounding tissue in midbrain. Later Sasaki *et al.* (Sasaki *et al.* 2006) demonstrated a correct visualization of NM-containing areas of SNc by using 2D FSE sequence with 3 T MRI. Furthermore several reports displayed a correct NM contrast by using FSE sequences with MT pulses by suppressing the brain background intensity (Sasaki *et al.* 2006, Kashihara *et al.* 2011, Schwarz *et al.* 2011, Matsuura *et al.* 2013, Ogisu *et al.* 2013, Ohtsuka *et al.* 2013, Castellanos *et al.* 2015, Reimao *et al.* 2015, Fabbri *et al.* 2017). This technique has been validated in a post-mortem study (Kitao *et al.* 2013) in which a direct association between post-mortem signal intensity in the SNc with quantity of NM-containing neurons was reported.

In the present cross-sectional study the PD cohort showed a 35% reduction of nigral pigment load when compared to HC group. These results are in line with previous neuropathological (Fearnley and Lees 1991, Greffard *et al.* 2006) and imaging studies (Sasaki *et al.* 2006, Kashihara *et al.* 2011, Schwarz *et al.* 2011, Matsuura *et al.* 2013, Ogisu *et al.* 2013, Ohtsuka *et al.* 2013, Castellanos *et al.* 2015, Reimao *et al.* 2015, Fabbri *et al.* 2017) studies. Furthermore the differences between the ipsilateral and contralateral SN-NM volumes in agreement with previous post-mortem (Kempster *et al.* 1989) and imaging (Castellanos *et al.* 2015) studies. Interestingly, significant differences pertaining clinical motor laterality with nigral depigmentation shows certain parallelism with nigral iron deposition (See chapter 3.1).

Reduced SNc-NM volumes have been previously reported to correlate with disease duration (Kashihara *et al.* 2011, Schwarz *et al.* 2011, Matsuura *et al.* 2013). In addition the present results are in line with the Fabbri *et al.* (Fabbri *et al.* 2017) who reported a specific pattern of duration-dependent volume loss in PD. In order to examine the aforementioned clinical associations the PD cohort was subdivided into three equal subgroups according to their respective disease duration adjusted by regional areas and age. SN depigmentation displayed an inverse stratified distribution being more marked in

later stages of disease duration, which suggest that nigral pigmentation loss accelerates as the disease progresses. These findings cannot be explained by age differences, which is well-known to correlate with nigral NM levels (Zucca *et al.* 2014) nor between-patient nigral size, since both were added as covariates in the present model.

In the present chapter, a moderate association was showed between reduced nigral neuromelanin-related volumes and motor severity as assessed with off-medication UDPRS III. This is to date the first study showing a significant relationship between nigral depigmentation and motor severity as previous studies described correlations with “on-medication” total UPDRS scale (Schwarz *et al.* 2011) or UPDRS II subscores of daily basis activities (Fabbri *et al.* 2017). Previous post-mortem studies evaluating nigral pigmented neuronal count showed direct correlations with UDPRS III (Greffard *et al.* 2006), thus suggesting this motor scale as good predictor of the neuronal loss. Surprisingly in the present study I did not find any correlations with bradykinesia-rigidity or tremor symptomatology. On the side of these observations, Xiang et al (Xiang *et al.* 2017) reported that nigral NM load was significantly more reduced in patients with akinetic-rigid phenotype in the medial parts of SNc when compared to tremor-predominant PD. In this study however, clinical findings were conducted with composite symptoms scores as the clinical severity index whilst the analysis of subtype ratio does not take into account the absolute severity of each motor symptom. Bradykinesia and rigidity symptoms have previously been reported as result of dopaminergic nigral degeneration (Vingerhoets *et al.* 1997), and they are associated with increased nigral iron accumulation (See chapter 3). The lack of associations with motor symptoms could be in part explained by the protective role of remaining intraneuronal NM deposition as compared with toxic iron accumulation in PD. In addition it is well known that the loss of pigmented markers lags behind the loss of dopaminergic markers in PD (Kordower *et al.* 2013) thus clinical associations might not be found at earlier stages of PD.

In the current chapter two methods of analysis the NM-hyperintensity were performed; the analysis of suprathresholded hyperintensity volumes and the contrast ratios of SNc regarding to the surrounding areas (cerebral peduncles). The latter analysis showed significant differences between HC and PD groups and marginal differences pertaining clinical laterality and correlations. Although I did not perform an analysis of inter and intra-rater reliability as Matsuura et al (2016) in order to compare both analysis methods,

the analysis of suprathresholded NM-SNc volumes seem to be a better approach to study the nigral depigmentation in PD.

In conclusion, this subchapter's results demonstrated that the degree of nigral pigmented load in PD presents an inverse stratification according to disease severity and besides nigral depigmentation is associated with disease severity but not with motor symptomatology.

#### **4.3. Longitudinal study of Neuromelanin-weighted magnetic imaging in Parkinson's disease.**

In this subchapter, I have undertaken a volumetric analysis of Neuromelanin of the SNc (SNc-NM) by using Neuromelanin-weighted MR in a cohort of 25 PD subjects as well as 7 age and gender matched healthy controls over the course of 20 months. Volumetric analysis of SNc-NM of SN in the PD cohort were used to further explore their clinical associations with clinical laterality, motor severity as assessed with UPDRS III "off-medication state" and motor symptomatology.

The aims of this subchapter are:

- To describe the temporal changes in nigral pigmentation in PD and HC groups.
- To describe the longitudinal associations of changes of nigral depigmentation with clinical laterality, motor severity and motor phenotypes in the PD cohort.

The hypotheses of this subchapter are:

- PD patients will show reduction of the nigral pigmentation over time when compared to HC.
- Changes of neuromelanin-related volumes will correlate with changes in motor severity as assessed with UPDRS III.

### **4.3.1. Subjects and methods.**

#### **4.3.1.1. Study subjects.**

Twenty-five non-demented PD patients with no other psychiatric or movement disorder and seven age and gender matched healthy volunteers were assessed at baseline and after  $19.92 \pm 3.42$  months.

Study recruitment was reported in detail in subchapter 4.1.

#### **4.3.1.2. Neuromelanin-weighted magnetic imaging**

NM-weighted MRI procedure was explained in subchapter 4.1.

#### **4.3.1.3. Region of interest analysis.**

ROI analysis was detailed in subchapter 4.1.

#### **4.3.1.4. Statistical analysis.**

All statistical analyses were performed using SPSS statistical software (version 22.0 SPSS Inc., Chicago, Illinois).

Group demographics and bilateral  $\Delta$ SNc-NM volumes were compared between PD and HC groups using paired t-test.

To assess longitudinal nigral depigmentation according to clinical laterality in the PD group,  $\Delta$ SNc-NM volumes for clinically most and least affected sides, as indicated by UPDRS-III laterality items, were compared using paired t-tests.

Spearman's rho correlation coefficient was used to assess relationships between  $\Delta$ SNc-NM volumes and changes of disease severity ( $\Delta$ UPDRS-III,  $\Delta$ tremor and  $\Delta$ bradykinesia  $\Delta$ rigidity),  $\Delta$ Hoehn and Yahr scale and  $\Delta$ LEDD. Results were corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) and considered significant at  $p < 0.05$ .

## 4.3.2. Results

### 4.3.2.1. Demographics

There were significant increases in UPDRS-III ( $p < 0.001$ ), Hoehn and Yahr ( $p < 0.01$ ) and LEDD ( $p < 0.001$ ) between consecutive scanning sessions (*Table 4.2*).

|                          | Baseline         | Follow-up        | Statistics       | p      |
|--------------------------|------------------|------------------|------------------|--------|
| Volunteers               | 25               | 25               |                  |        |
| Age                      | 58.07 $\pm$ 9.41 | 59.73 $\pm$ 8.78 | $t(23) = 2.57^a$ | 0.008  |
| Disease Duration (years) | 4.19 $\pm$ 2.33  | 5.85 $\pm$ 2.31  | $t(23) = 5.83^a$ | <0.001 |
| UPDRS III                | 28.7 $\pm$ 11.25 | 33.76 $\pm$ 9.44 | $t(23) = 5.68^a$ | <0.001 |
| Hoehn and Yahr           | 1.82 $\pm$ 0.49  | 2.08 $\pm$ 0.31  | $t(23) = 2.94^a$ | 0.007  |
| LEDD                     | 403 $\pm$ 335    | 551 $\pm$ 362    | $t(23) = 8.49^a$ | <0.001 |

**Table 4.2:** Demographic and clinical characteristics of Parkinson's disease volunteers at baseline and follow-up visits. Values represent mean  $\pm$  standard deviation (SD). UPDRS III= Unified Parkinson's Disease Rating Scale Part III; LEDD= Levodopa equivalent daily dose;

<sup>a</sup> = Indicates that differences were tested using *t* student for paired data; (DF) = with degrees of freedom.

#### 4.3.2.2. Neuromelanin volumetric analysis

The analysis for the control group revealed no significant change of SNc-NM volumes after 20 months (Visit 1:  $279.37 \pm 50.48$ ; Visit 2:  $267.51 \pm 52.71$ ,  $p > 0.05$ ) (Figure 4.5). In the PD group, patients showed a significant reduction of SNc-NM volumes (Visit 1:  $183.48 \pm 61.45$ ; Visit 2:  $144.01 \pm 50.81$ ,  $p < 0.001$ ) between the two scan points presenting an annual neuromelanin load decline of 14.21%.

In addition, no significant changes of SNc-NM contrast ratios were displayed in the control (Visit 1:  $0.112 \pm 0.034$ ; Visit 2:  $0.115 \pm 0.028$ ,  $p > 0.05$ ) and PD groups (Visit 1:  $0.087 \pm 0.027$ ; Visit 2:  $0.082 \pm 0.042$ ,  $p > 0.05$ ).

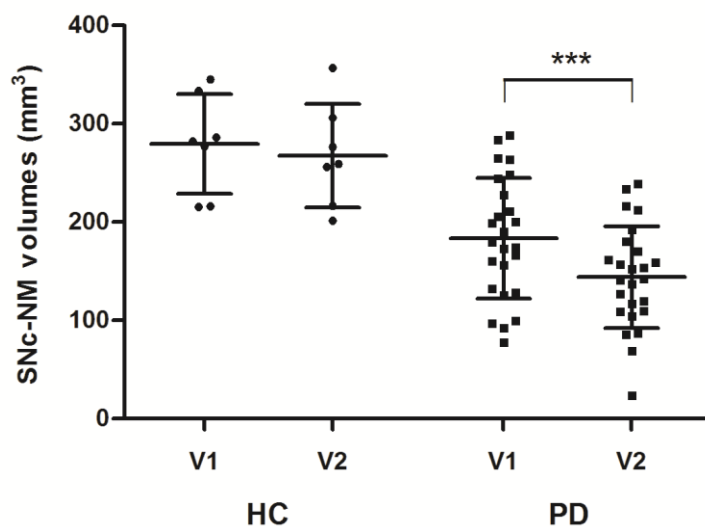


**Figure 4.5:** Scatter plots showing the mean and standard deviation of SNc-NM volumes (in mm<sup>3</sup>) of PD (square dots,  $n = 25$ ) and HC (round dots,  $n = 7$ ) groups at baseline (V1) and follow-up (V2) visits. Comparison of SNc-NM volumes by paired  $t$ -test showed a significant reduction over time ( $*** = p < 0.001$ ) in the PD group by using paired  $t$ -tests between baseline and follow-up visits followed by post-hoc Bonferroni correction for multiple comparisons.

#### 4.3.2.3. Analysis of clinical laterality

The SNc-NM volumes on contralateral and ipsilateral with respect to the clinically most affected side showed significant reduction of SN-NM load between baseline and follow-up ( $p < 0.001$ , 18.3% and 9.9% per annum respectively) (figure 4.6). No significant changes in SN-NM contrast ratios were found in the most and least affected sides contralateral to clinical laterality after 20 months.

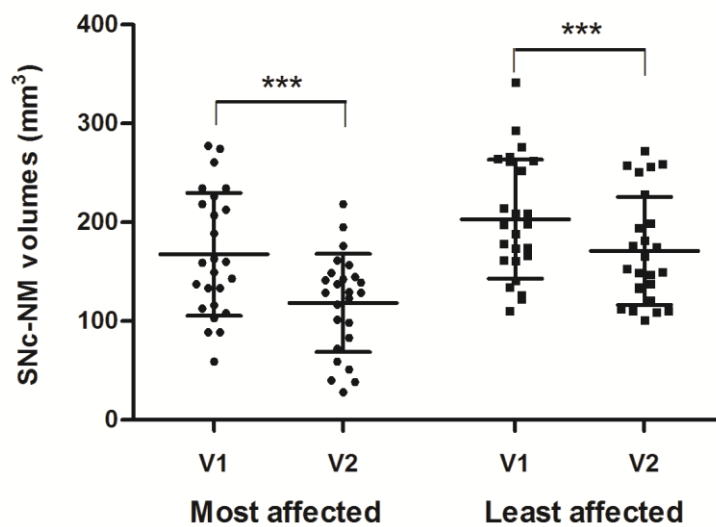


**Figure 4.6:** Scatter plots showing the mean and standard deviation of SNc-NM volumes (in mm<sup>3</sup>) of PD indicating most (round dots) and least affected (square dots) contralateral to the symptomatic clinical sides at baseline (V1= Visit 1) and follow-up (V2= Visit 2) visits. Comparison of SNc-NM volumes by paired *t*-test showed significant reductions over time in the most and least affected sides (\*\*\*=  $p < 0.001$  respectively) contralateral to the symptomatic sides.

#### 4.3.2.4. Clinical correlations

Spearman's rho correlation showed a significant relationship between  $\Delta$ SNc-NM volumes with  $\Delta$ UPDRS III ( $r = -0.435$ ,  $p = 0.040$ )  $\Delta$ Bradykinesia scores ( $r = -0.409$ ,  $p = 0.020$ ) (figure 4.7). Correlations between  $\Delta$ SNc-NM volumes and  $\Delta$ Hoehn and Yahr scale ( $r = -0.342$ ,  $p = 0.047$ ) did not survive the post-hoc FDR analysis for multiple

correlations. No significant correlations were found between  $\Delta$ SNC-NM volumes and  $\Delta$ rigidity,  $\Delta$ tremor,  $\Delta$ Hoehn and  $\Delta$ LEDD.

No significant correlations were found between  $\Delta$ SNC-NM contrast ratios and  $\Delta$ UPDRS III and other motor subscores.

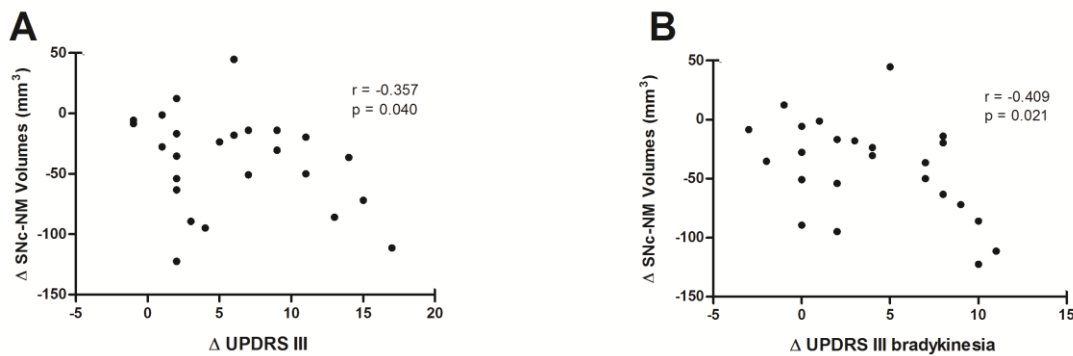


**Figure 4.7:** Scatterplots and Spearman's rho correlations showing significant negative relationships between  $\Delta$ SNC-NM volumes and  $\Delta$ UPDRS III (A,  $*p < 0.05$ ) and  $\Delta$ bradykinesia scores (B,  $*p < 0.05$ ).

### 4.3.3. Discussion

The present subchapter evaluated the changes in nigral SNc-NM volumes in of PD patients over the course of 20 months using Neuromelanin-MR imaging. The current observations demonstrate significant reductions of nigral pigmentation over time. The rate of nigral degeneration is more marked in the contralateral nigral side to the clinically most symptomatic side. The degree of nigral depigmentation over the study period was associated with mild changes total motor severity as assessed with UPDRS III and bradykinesia. Collectively, these results imply that the rate of nigral depigmentation in PD is associated with the progression of motor symptoms.

To date one study assessed the longitudinal changes of nigral pigmentation in PD with Neuromelanin-MR imaging (Matsuura *et al.* 2016). The authors described significant reduction of SNc-NM pixels over time (annual decline of 17.5%) as well as moderate negative relationship with disease duration and Hoehn and Yahr scales. This study however presents several design limitations such as the absence of HC group, small PD sample size (n= 14) and the absence of a fixed follow-up scanning frame, e.g. volunteers were re-scanned between one and five years. In addition no temporal clinical associations were described in the aforementioned study. Furthermore this study methodology is based on the quantification of SNc-NM pixels as the Neuromelanin-weighted MRI was acquired with 2D FSE protocol. In this chapter a significant annual decline of SN-NM volumes in the PD group of 14.22% is described. This significant temporal nigral depigmentation showed an uneven topographical distribution according to clinical laterality as it is more marked in the contralateral SN to the most symptomatic side. These results showed certain similarities with the previous follow-up iron study (See chapter 3.2), although no differences were observed in the temporal iron deposition pertaining to clinical laterality.

A recent longitudinal study assessing the motor progression of PD with dopamine transporter PET imaging  $^{11}\text{C}$ -PE2I PET (Li *et al.* 2017) reported an annual decline varying from 7.5% in caudate and 8.8 in posterior putamen. These results might reflect a different rate of degeneration of the nigrostriatal pathway with marked nigral pigmented degeneration when compared with dopaminergic striatal efferences (Kordower *et al.* 2013). A multimodal imaging assessment to further examine the nigrostriatal pathways using nigral Neuromelanin-weighted MR imaging and  $^{11}\text{C}$ -PE2I will be described in chapter 5.

To date this is the first study describing the associations between temporal nigral depigmentation as assessed with Neuromelanin-weighted MR imaging and changes in motor severity. Moderate but significant associations between changes in Neuromelanin-related volumes and UPDRS III and bradykinesia symptoms were observed. Bradykinesia is a motor symptom invariably present in PD and is directly related to nigral neurodegeneration in PD (Vingerhoets *et al.* 1997) when compared to tremoric symptoms which are mainly extra-nigral and non-dopaminergic in nature. Longitudinal associations

with bradykinesia have been previously described with iron accumulation (see chapter 3.2) and dopaminergic striatal decline (Vingerhoets *et al.* 1997, Li *et al.* 2017)

In conclusion the current results present evidence that temporal nigral depigmentation in PD as assessed with Neuromelanin-weighted MR imaging is associated with changes in motor severity and bradykinetic symptoms. Further longitudinal studies including larger sample size of PD and healthy controls are needed in order to validate the present results.

#### **4.4.Limitations and future directions**

There are some limitations in the present chapter. Similarly to the previous chapter, cross-sectional and longitudinal PD cohorts assessed in the present chapter were fairly extensive. However the inclusion of larger cohorts of age and gender matched HC groups in longitudinal subchapter would be ideal to further understand the nigral increase of pigment in healthy ageing as previously described (Zecca *et al* 2004, Fedorow *et al* 2000).

Second, I analyzed two slices of SNc where the NM contrast were present in both HC and PD groups, however it may not reflect the pigment content of the entire nucleus. Although no ROI size differences were found between PD and control group, the original delineated areas were included as covariant in the further analysis of the covariance in order to assess group differences. As previously it is important to remark that after manual delineation of the each nucleus of the NM-weighted MRI sequence, the next step should be coregistration to the MPRAGE sequence by affine registration in order to reduce variability introduced by manual segmentation and other registration processes.

Third, although neuromelanin loss in PD subjects have been quantified across imaging different imaging protocols and platforms (Schwarz *et al.* 2017) with high diagnostic accuracy, this technique applies relatively low spatial resolution with particularly regard to the slice direction hence resulting in partial volume effects as well as insufficient coverage, especially in lower segmentation areas including LC.

The need of multimodal neuroimaging studies in PD is remarkably important for the correct understanding of pathophysiological mechanisms and clinical monitoring of PD.

The combination of different imaging measures such DTI and neuromelanin-weighted MR imaging in PD has showed heterogeneous results (Langley *et al.* 2015, Langley *et al.* 2016). Indeed multimodal imaging protocols combining quantitative susceptibility mapping (QSM) and neuromelanin-weighted MR imaging in cross-sectional and longitudinal settings would be needed in order to ascertain the interplay between iron deposition and pigmentation load as previously attempted in post-mortem studies (Lee *et al.* 2016). The latter multimodal protocol is currently been performed in the PaMIR study at Imperial College London and University of Nottingham.

# 5. Brain iron chelation with deferiprone in Parkinson's disease

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## 5.1.Introduction

Increased iron deposition in the SNc in PD has been confirmed from post mortem studies (Dexter *et al.* 1987, Dexter *et al.* 1989), magnetic resonance imaging (MRI), and transcranial ultrasound studies (Gotz *et al.* 2004, Walter 2010, Han *et al.* 2013). Additionally, electron probe x-ray microanalysis has confirmed that individual SNc dopaminergic neurons in PD have raised iron levels (Oakley *et al.* 2008) as well as an increased labile iron pool (Kaur *et al.* 2009). Low molecular weight iron can cause oxidative damage to proteins, lipids and DNA by generating reactive oxygen species (ROS), processes for which there is evidence in the PD brain (Dexter *et al.* 1986, Dexter *et al.* 1994). ROS activity will be exacerbated by decreased anti-oxidant levels, e.g. reduced glutathione, in the PD brain (Sian *et al.* 1994) while alpha-synuclein aggregation is promoted by the excess iron (Ostrerova-Golts *et al.* 2000, Mandel *et al.* 2004). Therefore the removal of such iron from the SN may be beneficial in retarding the progression of PD.

Previous animal studies have shown that iron chelators are able to cross the blood brain barrier and are able to remove excess iron from various brain regions in models of brain iron overload (Ward *et al.* 1995, Sohn *et al.* 2008, Dexter *et al.* 2011). In early studies, we demonstrated that iron chelators such as desferrioxamine, deferasirox and deferiprone, currently in clinical use for treating peripheral iron overload in thalassemia major, significantly attenuated SNc dopaminergic neuronal and striatal dopamine loss in the 6-hydroxydopamine PD model, whilst reducing hydroxyl radical formation (Dexter *et al.* 2011).

The potential benefit of using an iron chelator in a neurodegenerative condition, in the absence of systemic iron overload, was exemplified in a clinical trial utilizing deferiprone in Friedreich Ataxia (FA). Cardiomyopathy, as well as neurodegeneration in the dorsal

root ganglia and the dentate nucleus are prominent features in FA, and may be related to the dysregulation of iron and the resultant excess in regionalized intracellular labile iron. Deferiprone treatment, with 20 to 30 mg/kg/day, divided into 2 daily oral doses, for up to 6 months, in 9 FA patients, reduced dentate nucleus iron content, as assessed by relaxometry MRI, whilst concomitantly reducing neuropathy and ataxic gait in some patients (Boddaert *et al.* 2007).

The aims the present chapter were:

- To evaluate changes in brain iron content by T2\* MRI.
- To assess the drug safety in PD.
- To assess subjects' clinical status as characterised by PD disability using UPDRS III, mood and quality of life scales.
- To explore iron homeostasis and peripheral inflammation markers during chelation.

The hypotheses of this study were:

- Selective iron removal in deep grey nuclei including SN would be monitored by T2\* MRI.
- Changes in iron accumulation assessed with T2\* will be associated with reduction in motor severity as assessed with UPDRS III.

## **5.2. Patients and methods**

### **5.2.1. Study subjects**

In the present chapter 22 non-demented and non-depressed early-stage PD subjects were studied prospectively. Participants were excluded if they had insulin-dependent diabetes mellitus, renal or liver disease, or were immunocompromised as revealed by neutropenia (see chapter 2). Written informed consent was obtained from patients on their initial screening visit before enrolment.

The PD patients were recruited and randomly selected to receive placebo or 20 or 30 mg/kg/day deferiprone (80 mg/ml deferiprone solution or excipient matched placebo

provided by ApoPharma Inc., Toronto, ON, Canada) which was divided into 2 daily oral doses, morning and evening, and administered for 6 months (*table 5.1*).

|     | MI of oral solution<br>to obtain<br>20mg/kg/day | MI of oral solution<br>to obtain<br>30mg/kg/day |
|-----|-------------------------------------------------|-------------------------------------------------|
| 50  | 12.5                                            | 18.75                                           |
| 60  | 15                                              | 22.5                                            |
| 70  | 17.5                                            | 26.25                                           |
| 80  | 20                                              | 30                                              |
| 90  | 22.5                                            | 33.75                                           |
| 100 | 25                                              | 37.5                                            |

**Table 5.1:** Table showing recommended volume of oral solution to obtain 20 or 30mg/kg/day according to body weight dispensed by Hammersmith Hospital Pharmacy (Imperial College NHS Trust) and administered by Apotex Europe BV according to the study randomization.

Randomisation was carried out by the Hammersmith Hospital Pharmacy and the randomization scheme was generated using the web site Randomization.com (<http://www.randomization.com>). The code for the randomisation was only disclosed at the conclusion of the trial or if the patient experienced unexpected adverse side effects. Patients, care providers and those assessing clinical outcomes were blinded to the intervention given.

From the original group of 22 patients, 19 completed the 6-month course of deferiprone. The analysis of the study was performed on the subjects that remained in (*Figure 5.1*).

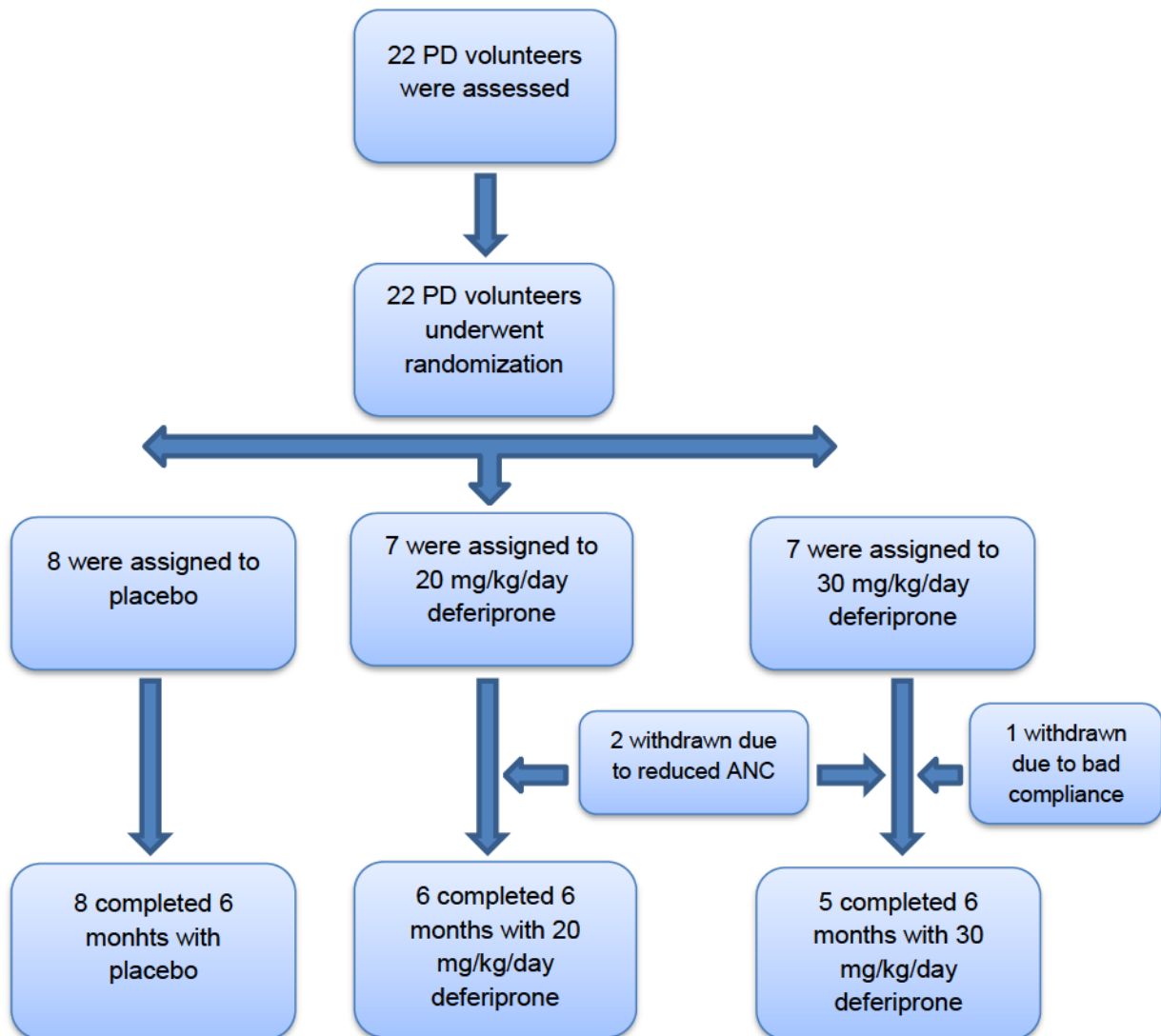


**Figure 5.1:** Flow diagram showing the random assignments of the two doses of deferiprone, 20 ( $n= 7$ ) or 30 mg/kg ( $n= 7$ ) and placebo ( $n= 8$ ) to the 22 Parkinson's disease patients. From the original recruitment sample, 19 subjects completed the 6 months drug study, 2 subjects withdrew due to reduced plasmatic neutrophil levels and one subject was excluded due to incorrect therapeutic compliance.

### **5.2.2. Neurological examination**

PD patients were clinically assessed at baseline, 2 months, 4 months and 6 months for PD severity using MDS-UPDRS part 3 whilst on-medication. Anti-Parkinsonian medication dosages were maintained at a constant level for each individual throughout the study.

In addition, subjects were assessed with Mini-mental State of Folstein, Montgomery-Åsberg DRS and PDQ39 questionnaires (*Table 5.3*).

### **5.2.3. Blood and urine analysis**

Blood specimens were provided by each patient weekly for haematological assessment to ensure that there were no adverse effects, particularly neutropenia. Agranulocytosis occurs in 1-2% of beta-thalassemia patients, who receive much higher doses of deferiprone, 75-100 mg/kg/day. Biochemical assessments were made on a monthly basis to assess various markers of iron metabolism, immunoglobulin status, hepatic and renal function, as well as levels of deferiprone and deferiprone 3-*O*-glucuronide to ensure patient compliance. Spot urine samples were collected weekly to assess iron excretion.

Inductively coupled plasma mass spectrometry (Thermo XII, Massachusetts, USA), was used for the analysis of urinary iron. Urine samples were diluted in 1% nitric acid to which the internal standard gallium had been added. A calibration curve was prepared in a similar fashion and internal quality controls were included in all analytical runs. Creatinine was analysed by a standard biochemical method and results were expressed as nmol Fe/mmol creatinine.

### **5.2.4. Quantification of deferiprone and deferiprone glucuronide**

Deferiprone and its main metabolite, deferiprone glucuronide, were assessed in the plasma to monitor patient compliance. Perchloric acid (200 µL, 0.4 M) was added to plasma samples (200 µL) to precipitate plasma proteins. After brief vortexing, followed by 10 min incubation on ice, the samples were centrifuged (35,000 x g) for 15 min at

4°C. The supernatant (10 µL) was injected onto the High-performance liquid chromatography (HPLC) column and analysed by UV detection.

#### **5.2.5. T2\* region of interest analysis**

The imaging protocol using T2\* conducted in the current chapter was set independently to the SWI protocol of chapter 3 (See chapter 2, Subjects and Methodology).

T2\* relaxometry characteristics and ROI analysis as detailed in chapter 2

#### **5.2.6. Summary of investigations.**

The baseline and follow-up assessments of the present chapter are indicated in *table 5.2* are listed below:

- Neurological examination: UDPRS III, MMSE, Montgomery-Åsberg depression scale and PDQ-39 questionnaire) at baseline, 2<sup>nd</sup>, 4<sup>th</sup> and 6<sup>th</sup> month of treatment.
- MRI scan with T2\* sequence (at baseline, 3<sup>rd</sup> and 6<sup>th</sup> month of treatment).
- Routine medical assessment including ECG, blood pressure, full blood count (haemoglobin, haematocrit, white cell count and platelets) and liver profiles (alanine aminotransferase, alkaline phosphatase, bilirubin and total protein) were performed on a weekly basis.
- Plasmatic (ferritin, transferrin saturation and free iron) and urine iron metabolism performed a monthly basis.
- Deferiprone metabolism with analysis of un-conjugated and conjugated forms of deferiprone by high performance liquid chromatography (HPLC) method performed at 1<sup>st</sup>, 3<sup>rd</sup> and 6<sup>th</sup> months of treatment.
- Cytokines determination such as tumour necrosis factor alpha (TNFα) and interleukin 6 (IL6) performed on a monthly basis.

|                          | Months during treatment |   |   |   |   |   |   |
|--------------------------|-------------------------|---|---|---|---|---|---|
|                          | Pre-treatment           | 1 | 2 | 3 | 4 | 5 | 6 |
| MRI-T2*                  | X                       |   |   | X |   |   | X |
| History, physical exam   | X                       |   |   |   |   |   |   |
| ECG                      | X                       |   |   |   |   |   |   |
| Neurological assessments | X                       |   | X |   | X |   | X |
| Blood sample monitoring  | X                       | X | X | X | X | X | X |
| Urine iron measurement   | X                       | X | X | X | X | X | X |
| Deferiprone metabolism   |                         | X |   | X |   |   | X |
| Cytokines                | X                       | X | X | X | X | X | X |
| Informed consent         | X                       |   |   |   |   |   |   |

**Table 5.2:** Table indicating the summary of investigations, treatment and clinical assessments at baseline and during 6 months of treatment. MRI-T2\*= Magnetic resonance relaxometry imaging; ECG= electrocardiogram; Pre-treatment = screening visit.

### 5.2.7. Statistical Analysis

All statistical analyses were performed using SPSS statistical software (version 21 SPSS Inc., Chicago, Illinois) and SAS 9.3.

Demographic data was compared between PD subgroups according to their therapeutic randomizations using independent t-tests and Chi-square test for independence.

The statistical methodology used in the analysis was mixed analysis of variance applied to longitudinal data. This method takes into account the correlation between the time points (screening visit, third and sixth months) within the different brain regions of interest. Estimated values from T2\* values means were analysed using Pairwise comparisons with post-hoc Bonferroni correction using T2\* as dependent variable. The threshold for statistical significance was set to  $p < 0.05$ .

To assess differences between PD subgroups according to their therapeutic randomization and haematological/urinary parameters (plasmatic levels of deferiprone, deferiprone glucuronide concentration, full blood count and iron metabolism parameters), a mixed analysis of variance (ANOVA) was conducted with group (placebo, 20 and 30 mg/kg/day deferiprone) as the between-subjects factor and haematological/urinary parameters as the within-subjects factor

Correlation analysis between IL-6, TNF-alpha and ferritin with T2\* regional values were performed with non-parametric Spearman tests with post hoc Benjamini-Hochberg corrections for multiple correlations.

## **5.3.Results**

### **5.3.1. Demographics**

Demographics of enrolled patients are summarized in Table 5.3. There were no significant differences in age ( $p = 0.414$ ), disease duration ( $p = 0.521$ ), UPDRS-III ( $p = 0.879$ ), Montgomery-Åsberg Depression Rating Scale ( $p = 0.388$ ), MMSE ( $p = 0.450$ ), PDQ 39 of quality of life ( $p = 0.689$ ) or LEDD ( $p = 0.399$ ) within the three PD subgroups according to their therapeutic randomization.

|                          | Placebo          | 20 mg/kg/day<br>Deferiprone | 30 mg/kg/day<br>Deferiprone | Stats                | p     |
|--------------------------|------------------|-----------------------------|-----------------------------|----------------------|-------|
| Number                   | 8                | 7                           | 7                           | NA                   | NA    |
| Gender (m:f)             | 3:5              | 4:3                         | 2:5                         | $\chi^2(2) = 1.72^a$ | 0.414 |
| Age (years)              | $64.38 \pm 3.23$ | $68.57 \pm 2.17$            | $62.85 \pm 2.74$            | $F(2,19) = 1.0^b$    | 0.384 |
| Disease duration (years) | $3.54 \pm 0.34$  | $2.82 \pm 0.69$             | $3.02 \pm 2.69$             | $F(2,19) = 0.67^b$   | 0.521 |
| UPDRS III                | $11.38 \pm 1.29$ | $11.43 \pm 1.31$            | $10.57 \pm 1.34$            | $F(2,19) = 0.13^b$   | 0.879 |
| MADRS                    | $8.25 \pm 1.78$  | $7.14 \pm 1.49$             | $5.14 \pm 1.37$             | $F(2,19) = 0.99^b$   | 0.388 |
| MMSE                     | $29.25 \pm 0.49$ | $29.42 \pm 0.29$            | $28.57 \pm 0.61$            | $F(2,19) = 0.83^b$   | 0.450 |
| PDQ 39                   | $23 \pm 4.03$    | $31.42 \pm 10.35$           | $24.28 \pm 6.29$            | $F(2,19) = 0.38^b$   | 0.689 |
| LEDD                     | $284 \pm 55$     | $395 \pm 57$                | $432 \pm 118$               | $F(2,19) = 0.96^b$   | 0.399 |

**Table 5.3:** Demographic and clinical characteristics of PD subgroups divided into 3 therapeutic arms in Placebo (n= 8), 20 mg/kg/day deferiprone (n= 7) and 30 mg/kg/day deferiprone (n= 7) groups. Values represent the mean  $\pm$  standard error mean (SEM). NA= Not applicable; UPDRS III= Unified Parkinson's Disease Rating Scale Par-III; MADRS= Montgomery-Åsberg Depression Rating Scale; MMSE= Mini-Mental State Examination of Folstein; PDQ 39= Parkinson's disease questionnaire of quality of life; LEDD= Levodopa equivalent daily dose; <sup>a</sup>= Indicates that differences were tested using Pearson's Chi-square test for independence; <sup>b</sup> = Indicates that differences were tested using one-way ANOVA; (df) = degrees of freedom.

### **5.3.2. Clinical Parameters**

#### **5.3.2.1.Side effects of deferiprone therapy**

Deferiprone therapy was well tolerated by PD subjects with only minor side effects being reported. Seven subjects reported an exacerbation of pre-existing muscular/joint pain, while another 3 patients experienced mild gastrointestinal upset during the first months of the study, all of which resolved without any clinical intervention. In one patient, we detected an increase of liver enzymes after 4 weeks of deferiprone administration that returned to normal values after a 7-day break from deferiprone therapy. In two PD subjects administered deferiprone, a reduction in neutrophil cell numbers was observed warranting drug withdrawal. In the first subject the neutrophil decline was very rapid and severe, with zero cells being detected. In the second subject there was a slower decline in neutrophil numbers and the subject was withdrawn from the drug prior to the neutrophil numbers reaching critical levels. These subjects were monitored subsequently and their neutrophil numbers returned quickly to within normal values after deferiprone withdrawal.

#### **5.3.2.2.Neurological assessment**

In the placebo-administered and 20mg/kg /day deferiprone PD groups the UPDRS-III scores increased over the 6 months' treatment period which indicated a slight worsening of motor disability, however this did not reach significance. Conversely, there was a trend for a decrease in motor-UPDRS scores over the 6-month treatment period in the 30 mg/kg/day deferiprone treatment groups compared to their baseline value prior to drug treatment, indicative of improvement, but this did not reach significance (*Figure 5.2*). A similar but more pronounced trend was observed with the PDQ-39 scores, with the placebo and 20mg/kg/day PD groups showing strong trends for worsening quality of life whilst the 30mg/kg/day deferiprone PD group showed a slight improvement over 6 months but this did not reach statistical significance (data not shown). There were no changes in the clinical rating obtained from the minimal State of Folstein or the Montgomery-Åsberg depression scale within the placebo or deferiprone drug treated groups throughout the study (data not shown).

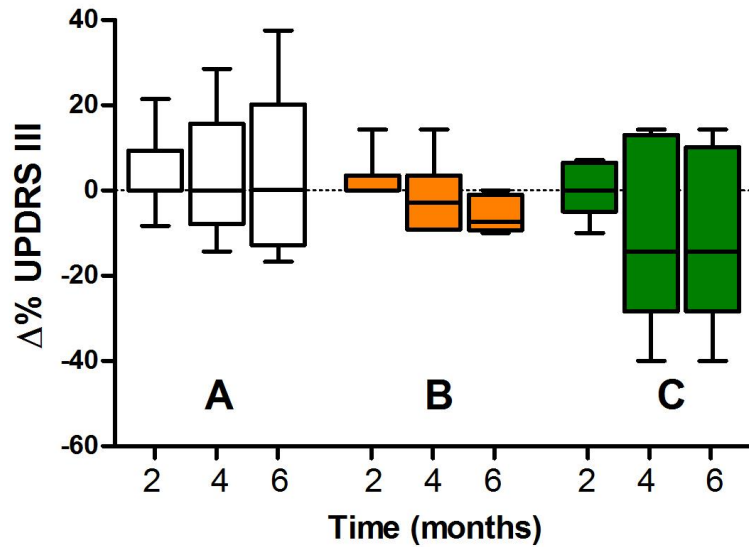


**Figure 5.2:** Box and whisker plot displaying the percentage change in the median UPRDS III motor scale of PD patients receiving placebo (A, empty bars,  $n=8$ ), 20 (B, orange bars,  $n=6$ ) or 30 (C, green bars,  $n=5$ ) mg/kg/deferiprone for 2, 4 and 6 months of treatment. Values are presented in box plots with whiskers representing the minimum and maximum values.

### 5.3.3. Regional T2\* analysis

Administration of 20 or 30 mg/kg/day deferiprone resulted in a significant time-dependent decrease in iron concentrations in both the dentate and caudate nuclei. An increase in the mean T2\* MRI values was evident in the dentate nucleus: the values increased significantly from  $29.2 \pm 0.8$  to  $30.6 \pm 0.9$  and from  $27.2 \pm 1.6$  to  $29.9 \pm 1.1$  ( $p < 0.01$ ) after six months of 20mg/kg and 30mg/kg/day deferiprone administration, respectively, while no changes were evident in the placebo group (Figure 5.3 A). Similarly, in the caudate nucleus: T2\* values increased significantly in both treatment groups from  $38.9 \pm 1.5$  to  $41.1 \pm 3.4$  ( $p < 0.01$ ) in the 20 mg/kg/day group and from  $40.8 \pm 1.7$  to  $44.6 \pm 1.6$  ( $p < 0.01$ ) in the 30 mg/kg/day group after 6 months of therapy (Figure 5.3 B).

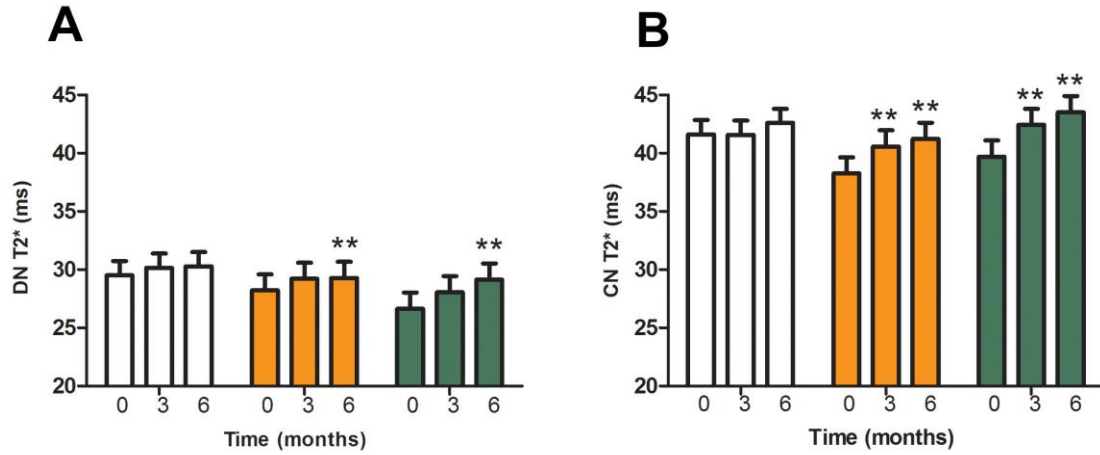


**Figure 5.3:** Bar graph showing the mean T2\* values (in milliseconds) in the dentate nucleus (A) and caudate nucleus (B) of subjects receiving placebo (open bars, n= 8), 20 mg/kg/day (orange bars, n= 6) or 30 mg/kg/day (green bars, n= 5) of deferiprone at baseline, 3 and 6 months of treatment. Values are represented as mean  $\pm$  standard error mean (SEM). Pairwise comparisons of regional T2\* values displayed a decrease of iron deposition in dentate (A) and caudate nuclei (B) after 6 months of treatment ( $p < 0.01$ ) in subjects receiving 20 mg/kg/day and 30 mg/kg/day deferiprone. \*\*=  $p < 0.01$ .

Across each treatment group there were no significant changes in T2\* mean values for SNc, red nucleus, putamen or globus pallidus in PD patients receiving either 20 mg/kg/day or 30 mg/kg/day over the period of the study (Table 5.4). However, we did observe a decrease in SNc iron concentration with deferiprone therapy in three patients, two of whom were hypoferritinemic by the end of the study.

|                     | Months | SN           | RN           | DN              | CN              | Put          | GP           |
|---------------------|--------|--------------|--------------|-----------------|-----------------|--------------|--------------|
| <i>Placebo</i>      | 0      | 28.09 ± 1.52 | 27.37 ± 0.82 | 30.51 ± 0.59    | 43.92 ± 1.22    | 37.26 ± 1.18 | 27.41 ± 1.39 |
|                     | 3      | 29.36 ± 1.84 | 28.76 ± 1.06 | 30.54 ± 0.86    | 43.65 ± 1.62    | 35.76 ± 0.90 | 26.25 ± 0.58 |
|                     | 6      | 28.22 ± 1.81 | 28.58 ± 1.23 | 30.74 ± 0.65    | 45.98 ± 2.08    | 36.55 ± 0.86 | 26.43 ± 0.87 |
| <i>20 mg/kg/day</i> | 0      | 26.41 ± 1.25 | 28.10 ± 1.19 | 29.14 ± 0.84    | 38.86 ± 1.53    | 33.99 ± 1.80 | 25.55 ± 1.23 |
|                     | 3      | 27.05 ± 1.23 | 26.86 ± 1.56 | 30.15 ± 0.49    | 40.29 ± 1.74 ** | 33.54 ± 1.27 | 25.43 ± 1.08 |
|                     | 6      | 27.68 ± 1.06 | 27.78 ± 1.05 | 30.59 ± 0.87 ** | 41.05 ± 3.43 ** | 33.94 ± 1.75 | 25.89 ± 0.96 |
| <i>30 mg/kg/day</i> | 0      | 23.89 ± 2.24 | 27.21 ± 1.56 | 27.16 ± 1.56    | 40.75 ± 1.68    | 33.67 ± 2.14 | 25.72 ± 1.76 |
|                     | 3      | 23.74 ± 1.84 | 26.99 ± 1.04 | 29.04 ± 1.82    | 44.69 ± 1.93 ** | 37.24 ± 2.31 | 24.97 ± 1.14 |
|                     | 6      | 24.57 ± 1.69 | 27.55 ± 1.12 | 29.86 ± 1.10 ** | 44.55 ± 1.57 ** | 38.19 ± 2.04 | 26.26 ± 1.33 |

**Table 5.4:** Table displaying mean T2\* values of six brain deep grey matter nuclei in PD patients analysed at 0, 3 and 6 months after administration of either 20 mg/kg/day or 30 mg/kg/day deferiprone or receiving placebo. Values are represented as mean ± standard error mean (SEM). Comparison of regional T2\* values (in milliseconds) with post-hoc Pairwise comparisons showed an decrease of iron deposition in dentate (A) and caudate nuclei (B) after 6 months of treatment ( $p < 0.01$ ) in subjects receiving 20 mg/kg/day and 30 mg/kg/day deferiprone. SN= Substantia nigra; RN= Red nucleus; DN= Dentate nucleus; CN= Caudate nucleus; Put= Putamen; GP= Globus pallidus; \*\*=  $p < 0.01$ .

### 5.3.4. Blood parameters

#### 5.3.4.1. Plasma levels of deferiprone and deferiprone glucuronidate

PD patients who received the higher dose of the chelator, 30mg/kg/day, showed a significantly higher concentration of both deferiprone and deferiprone glucuronidate than those administered 20 mg/kg/day ( $p<0.05$ ), confirming that the patients had conformed to the instructions of the clinical trial (Figure 6.4).

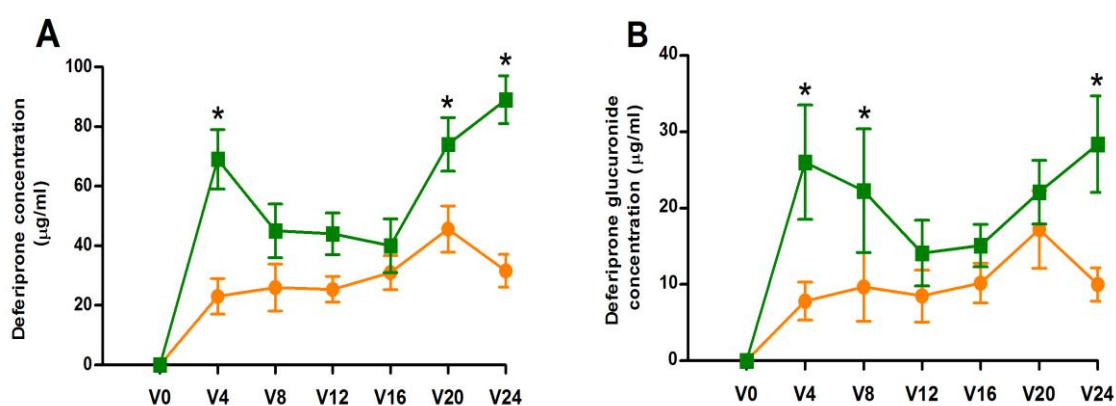


**Figure 5.4:** Steady state plasmatic concentrations of deferiprone (A) and deferiprone glucuronidate (B) as measured in µg/ml of subjects receiving 20 mg/kg/day (orange circles,  $n= 6$ ) or 30 mg/kg/day (green squares,  $n= 5$ ) of deferiprone at baseline, 1, 2, 3, 4, 5 and 6 months of treatment. Values are represented as mean  $\pm$  standard error mean (SEM). Pairwise comparisons of deferiprone and glucuronidate concentrations were increased in subjects receiving 30 mg/kg/day than 20 mg/kg/day deferiprone. V0= Screening visit; V4= Month 1; V8= Month 2; V12= Month 3; V16= Month 4; V20= Month 5; V24= Month 6; \*=  $p<0.05$ .

### 5.3.4.2. Haematological and biochemical parameters

Haemoglobin, transferrin and serum iron levels, transferrin saturation, and blood cell counts did not differ between placebo administered and deferiprone-treated patient groups during the 6-month period of the clinical study (Table 5.5).

|                          | Months | Hb (10 <sup>9</sup> /L) | WBC (g/L)   | ANC (10 <sup>9</sup> /L) | Platelets (10 <sup>9</sup> /L) | Ferritin (ng/ml) | Iron (nmol/L) | TS (%)   |
|--------------------------|--------|-------------------------|-------------|--------------------------|--------------------------------|------------------|---------------|----------|
| Placebo                  | 0      | 141 ± 0.59              | 6.23 ± 0.41 | 3.95 ± 0.33              | 223 ± 18                       | 100 ± 27         | 14 ± 1.2      | 25 ± 2.7 |
|                          | 2      | 137 ± 0.61              | 5.58 ± 0.33 | 3.33 ± 0.20              | 222 ± 15                       | 77 ± 19          | 16 ± 1.4      | 25 ± 2.6 |
|                          | 4      | 137 ± 0.63              | 5.98 ± 0.41 | 3.36 ± 0.31              | 219 ± 15                       | 82 ± 21          | 15 ± 1.8      | 27 ± 4.1 |
|                          | 6      | 137 ± 0.55              | 5.78 ± 0.29 | 3.37 ± 0.13              | 220 ± 12                       | 88 ± 21          | 17 ± 1.4      | 25 ± 2.9 |
| 20 mg/kg/day Deferiprone | 0      | 136 ± 0.67              | 6.70 ± 0.81 | 4.08 ± 0.66              | 222 ± 20                       | 189 ± 62         | 21 ± 2.1      | 32 ± 5.5 |
|                          | 2      | 132 ± 0.81              | 6.78 ± 0.62 | 4.30 ± 0.59              | 237 ± 22                       | 187 ± 62         | 22 ± 1.9      | 36 ± 5.4 |
|                          | 4      | 134 ± 0.63              | 6.93 ± 0.97 | 4.48 ± 0.78              | 215 ± 21                       | 165 ± 59         | 18 ± 2.2      | 30 ± 2.4 |
|                          | 6      | 135 ± 0.63              | 6.61 ± 0.62 | 4.30 ± 0.56              | 224 ± 23                       | 141 ± 47*        | 18 ± 1.1      | 33 ± 6.6 |
| 30 mg/kg/day Deferiprone | 0      | 141 ± 0.48              | 6.73 ± 0.66 | 4.38 ± 0.38              | 249 ± 17                       | 132 ± 37         | 17 ± 1.9      | 24 ± 2.2 |
|                          | 2      | 134 ± 0.41              | 6.38 ± 0.42 | 4.26 ± 0.20              | 229 ± 14                       | 136 ± 50         | 21 ± 2.5      | 28 ± 1.9 |
|                          | 4      | 140 ± 0.27              | 5.63 ± 0.33 | 3.70 ± 0.23              | 217 ± 14                       | 115 ± 41**       | 20 ± 1.9      | 30 ± 1.7 |
|                          | 6      | 138 ± 0.32              | 6.05 ± 0.64 | 4.20 ± 0.53              | 223 ± 17                       | 107 ± 38**       | 19 ± 1.2      | 28 ± 2.9 |

**Table 5.5:** Table showing haematological and iron status measurements expressed as medians +/- SEM of Parkinson's disease patients receiving 20 mg/kg/day (n= 6) or 30 (n= 5) mg/kg/day deferiprone or placebo (n= 8) for 6 months. Values are represented as mean ± standard error mean (SEM). Hb= Haemoglobin; WBC= White blood count; ANC= Absolute neutrophil count; TS= Transferrin saturation. \*\*=  $p < 0.01$ ; \*=  $p < 0.5$ .

Ferritin concentrations in the PD patient groups administered 20 or 30 mg/kg/day deferiprone significantly declined over the 6-month period, compared to the PD placebo group, which remained comparable to baseline values (*Figure 6.5*).

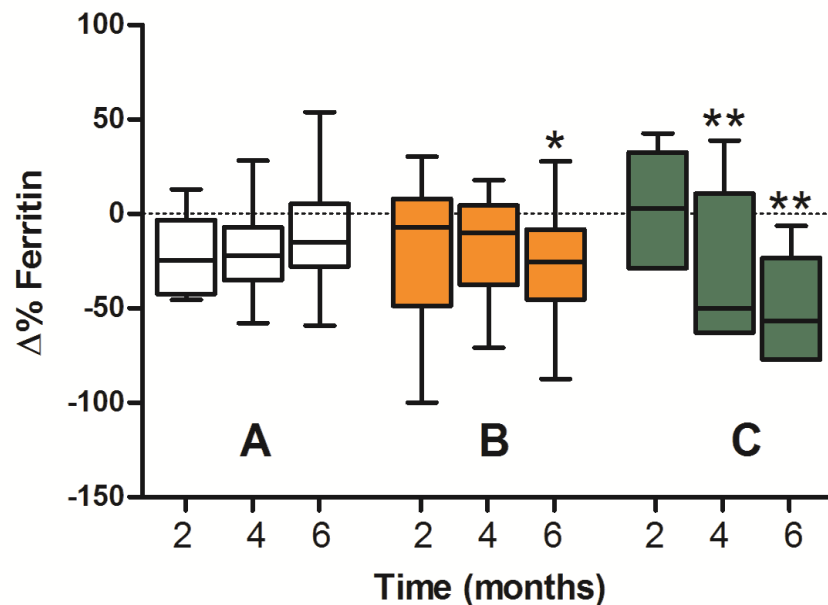


**Figure 5.5:** Bar graph showing the mean percentage change in the median plasmatic ferritin values of Parkinson's disease patients receiving placebo (A, empty columns,  $n=8$ ), 20 mg/kg/day (B, orange columns,  $n=6$ ) or 30 mg/kg/day (C, green columns,  $n=5$ ) deferiprone for 2, 4 and 6 months of treatment. Values are presented in box plots with whiskers representing the minimum and maximum values. Post-hoc Pairwise comparisons showed significant reduction of plasmatic ferritin in subjects receiving 20 mg/kg/day (A) and 30 mg/kg/day deferiprone (B) after 6 months of treatment ( $p<0.05$  and  $p<0.01$  respectively). \*\*= $p<0.01$ ; \*= $p<0.05$ .

In two subjects where the initial ferritin value was  $<100\text{ng/ml}$ , it was noteworthy that there was a greater decrease in brain iron in the dentate and caudate nuclei, as well as the SNc, as assessed by T2\* MRI, after iron chelation (*Figure 5.6.A*) when compared to other PD patients where the plasma ferritin level commenced at values  $>100\text{ng/ml}$  (*Figure 5.6.B*).

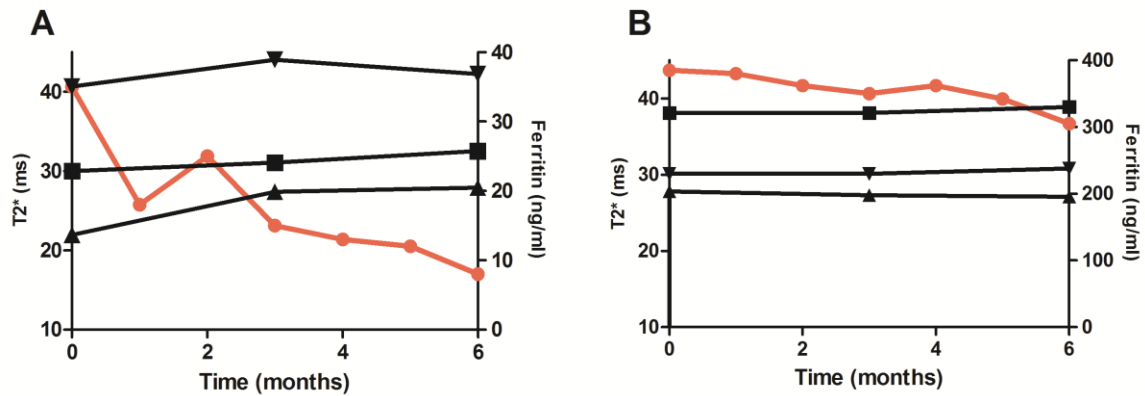


**Figure 5.6:** Examples of changes in ferritin levels and T2\* values in two PD patients receiving chelation therapy during 6 months with baseline plasmatic ferritin level <100ng/ml (A) and >300 ng/ml (B). Ferritin values are denoted by red circles, T2\* values (in milliseconds) in the caudate nucleus were represented by upright triangles, dentate nucleus values by downward triangles and substantia nigra by squares.

### 5.3.5. Urinary iron content

There was significant increase in the median value of urinary iron values in PD patients over the six month period who received deferiprone therapy, 20mg/kg/day and 30mg/kg/day;  $132 \pm 104$  ( $p < 0.01$ ) and  $172 \pm 160$  nmol Fe/mmol creatinine respectively, compared to  $23.1 \pm 4.8$  nmol Fe/mmol creatinine in the placebo group ( $p < 0.001$ ) (data not shown).

### 5.3.6. Plasma Inflammatory markers

Plasmatic concentrations of IL-6 and TNF-alpha were analysed at monthly intervals during the 6-month clinical trial period. Whilst there were no changes in IL-6 and TNF-alpha concentration with time in either the placebo or deferiprone treated PD groups, there was a positive correlation between the plasma levels of IL-6 and ferritin at baseline (Figure 5.7). No further correlations were found between IL-6, TNF alpha, ferritin and regional T2\* values at baseline and follow up visits.

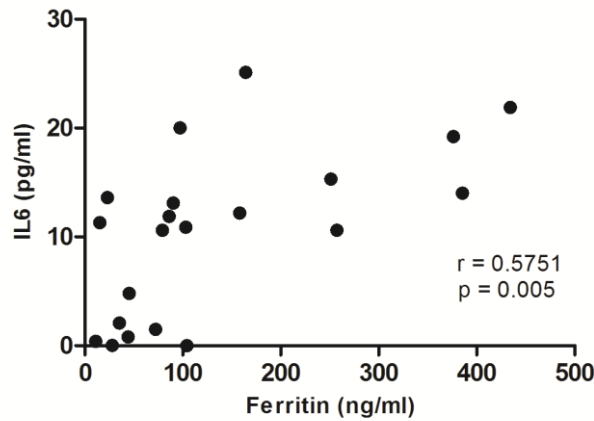


**Figure 5.7:** Scatterplots and Spearman's rho correlation showing significant positive relationships between plasmatic IL-6 (pg/ml) and ferritin (ng/ml) baseline determinations in PD subjects included in the study.

#### 5.4.Discussion

In the present chapter iron chelator deferiprone clearly demonstrated that over the 6 months' treatment period there was gradual iron removal from specific brain regions, namely the caudate nucleus and dentate nucleus, as assessed by T2\* MRI, in the majority of the PD patients while only three subjects showed alterations in the T2\* MRI values for SNc. Deferiprone therapy was well tolerated by the PD patients, with a few patients experiencing mild side effects, while a decline in white cell counts warranting drug withdrawal was evident in 2 PD subjects. Deferiprone administration did not adversely affect PD severity, mood, cognitive function or quality of life or peripheral iron status. Furthermore, serum ferritin levels appear to be a surrogate marker to identify which patients might respond optimally to iron chelation by deferiprone. Additionally, plasma ferritin levels correlated with IL-6 concentrations, implicating a link between peripheral inflammation and iron metabolism in PD.

The present MRI findings of a decrease in the iron content of the dentate nucleus correlate with the clinical trial in FA patients carried by Boddaert and colleagues (Boddaert *et al.* 2007) who also utilised 20 or 30mg/kg/day doses of deferiprone over 6 months. Furthermore, Velasco-Sanchez et al (Velasco-Sanchez *et al.* 2011) administered 20 mg/ kg/day deferiprone in addition to the antioxidant idebenone, to FA patients and

showed an increase in T2\* values in dentate nucleus after 11 months. Nevertheless, we did not find any association between tremor subscores and dentate iron accumulation.

Interestingly short-term deferiprone treatment was only observed to remove iron from the SNc in three patients receiving deferiprone. This could possibly be explained by the fact that iron in the SNc is principally tightly bound to neuromelanin rather than to ferritin as in other brain regions and hence less chelateable in the SNc (Sian-Hulsmann *et al.* 2011, Zucca *et al.* 2014). Hence, a longer period of deferiprone therapy may be required before SNc iron levels decline. However, importantly this study demonstrates that deferiprone therapy does not result in a generalised removal of iron from the brain or its redistribution, which could have implications for other neurological functions. This was supported by the finding that 6 months deferiprone therapy did not adversely affect mood, cognitive function or general patient quality of life. However, some of these data differ from another clinical trial of deferiprone therapy in PD patients, the FAIRPARK clinical trial, where deferiprone at 30mg/kg/day was administered to PD subjects either at the initiation of the trial ('early start subjects'), or after 6 months of placebo therapy ('delayed start group') (Devos *et al.* 2014). Both 'early start' and 'delayed start' PD subjects showed a small but significant reduction in SNc iron after 6 months of therapy as assessed by MRI (Devos *et al.* 2014). This small effect may have achieved significance possibly due to the increased cohort size. Additionally, only one dose of deferiprone, 30mg/kg/day, was used in the 19 patients participating in the FAIRPARK study, compared to 11 subjects receiving 20 or 30mg/kg/day in this study, thus giving the FAIRPARK study greater power to detect significant small changes in MRI signal.

A further difference between the FAIRPARK study and this study was that the former observed a small but significant clinical improvement in the deferiprone-treated PD subjects compared to placebo-treated PD subjects (Devos *et al.* 2014). The delayed start PD subjects who received placebo over the first 6 months averaged a 1 point UPDRS-III decline in motor function, whilst the early start deferiprone group improved by an average of 2 UPDRS-III points over the same period, giving an overall UPDRS difference between the two groups of 3 points. In the present pilot a trend for an improvement in UPDRS-III motor scores was observed in the 30mg/kg/day deferiprone treated subjects compared to the placebo and 20mg/kg/day group although this was not significant. This may again relate to the FAIRPARK study utilising the more active dose

of 30mg/kg/day dose of deferiprone for the whole of their study and in a slightly greater number of subjects. In addition in the FAIRPARK study patients showed higher motor scores UPDRS =  $23.7 \pm 7$  by comparison to the PD patients in the present study,  $11 \pm 0.8$ , the former having been assessed in off medication state.

In the present chapter there was observed a reduction in serum ferritin concentrations in PD subjects treated with deferiprone compared to placebo that might reflect the mobilisation of iron stores particularly from the liver. With regards to brain iron mobilisation, deferiprone therapy was observed to be most efficient in those PD subjects starting with low serum ferritin content  $<100\text{ng/ml}$  at the start of the study. This might suggest that there is a need to lower body iron stores before iron can be mobilised from specific brain regions. Indeed, in animal studies where body iron stores were depleted, there was an increased release of iron from the brain (Taylor *et al.* 1991). There was a correlation between the plasma concentrations of ferritin and IL-6, suggesting that the iron status of PD patients with high plasma ferritin may reflect the anaemia of chronic disease. Furthermore, increased levels of pro-inflammatory cytokines, IL-1-alpha, IL-2, IL-6 and TNF-alpha are present in the brain, cerebrospinal fluid (Nagatsu and Sawada 2005) and plasma of PD patients (Stypula *et al.* 1996, Brodacki *et al.* 2008, Reale *et al.* 2009) such that changes in IL-6 observed in PD plasma might be indicative of inflammatory changes in the brain (Chen *et al.* 2008). It is unknown whether plasma hepcidin levels, the master regulator of iron metabolism, are altered in PD patients. Heparin binds to the sole known iron exporter, ferroportin, causing its internalisation and degradation, thereby inhibiting iron efflux (Nemeth *et al.* 2004). Heparin transcription can be stimulated by increases in serum iron as well as by inflammatory stimuli, most notably by IL-6, which signals via the IL6 receptor (IL-6Ra) and gp130 via the janus kinase (JAK) – STAT 3 pathway (Wrighting and Andrews 2006, Verga Falzacappa *et al.* 2007); such pathways are important since they can lead to the internalisation of iron in cells.

In conclusion, in the present chapter I demonstrated that short-term deferiprone therapy in PD subjects is safe and is associated with decreases of iron in specific brain regions. The administration of 30 mg/kg/day of deferiprone are associated with a trend in motor improvement. Further multi-centre studies with longer therapeutic window are required in order to elucidate the long-term and neuroprotective effect of deferiprone.

## 5.5.Limitations and future directions

There are some limitations in the present chapter that are worth to mention. First, the inclusion of a bigger sample size would enhance the clinical outcomes of chelation with deferiprone over time as well as iron removal in SNc as assessed with T2\* MRI. When the power calculation was performed, a number of 12 subjects were selected to be included in each therapeutic arm (placebo, 20 and 30 mg/kg/day of deferiprone), however, the final sample size was potentially reduced due to recruitment difficulties during the study.

Second, the clinical assessment of PD subjects in the “off-medication” state throughout the six months of study would potentially avoid the confounding factor of dopaminergic medication effect. In addition, the chelating effect of deferiprone in PD would be more evident after 6 months of treatment as indicated in FAIRPARK study (Devos *et al* 2014). Ideally, a 12 months duration would show more robust results in terms of therapeutic action of iron chelation in PD, especially when two therapeutic arms of deferiprone (20 and 30 mg/kg/day) and placebo were compared.

The current early stage PD cohort showed relatively low motor severity (UPDRS III on-medication state of 11) in contrast to comparative datasets of PD (Malek *et al* 2015). According to this, a potential limitation of this study inclusion of subjects with scans without evidence of dopaminergic deficits (SWEDD) and other non-PD subjects (i.e. dystonic tremor). The selection of PD subjects in the current study was performed by Movement Disorders Specialists, however, the assessment of nigrostriatal degeneration with DAT-scan was not carried out in all of the subjects included in the study.

The use of high-pass filtered phase imaging and quantitative susceptibility mapping may be of therapeutic interest in further chelation studies. Conventional relaxometry techniques (T2\*) estimate iron load in vivo using gradient echo sequences that are sensitive to variations in transverse relaxation times/rates between different tissues and that are affected by both macroscopic and microscopic magnetic field inhomogeneities (Haacke *et al* 2004). However, images produced using these methods can be affected by changes in tissue water content and other background field inhomogeneities such as those resulting from air-tissue interfaces (Haacke *et al* 2004). In contrast, SWI sequences, whilst also utilizing T2\* weighting, involve the collection of both magnitude and phase images using a three-dimensional velocity-compensated gradient echo sequence. In particular, phase images contain information on the

tissue magnetic susceptibility distribution. Since paramagnetic molecules such as ferritin and haemosiderin influence the local magnetic field and therefore the phase of the MR signal (Haacke *et al* 2004), phase offers greater specificity to quantifying brain iron load. Phase images are also more robust to the presence of noise (Haacke *et al* 2007), and the removal of low spatial frequency artefacts that could otherwise obscure inter-tissue phase differences (using a high-pass filter) (Haacke *et al* 2009) further improves its sensitivity to detecting abnormal iron levels. Thus, high-pass filtered phase images are a good alternative for monitoring the effects of iron chelators longitudinally

Finally, additional multi-center double-blinded randomized studies using two therapeutic arms of deferiprone (20 and 30 mg/kg/day) and placebo are needed to determine the neuroprotective effect of iron chelators in PD longitudinally (Moreau *et al.* 2018). Currently, the multi-center randomized and parallel-group FAIRPARK II is ongoing in different European centers using deferiprone 30 mg/kg/day during 9 months to assess the effect in motor and non-motor scores and the possible disease-modifying effect in the long term of treatment.

## 6. Multimodal imaging assessment of nigrostriatal pathways in Parkinson's disease using $^{11}\text{C}$ -PE2I PET and Neuromelanin-weighted MRI

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### 6.1. Introduction

Several studies have functionally and anatomically divided SNc in two tiers, a dorsal tier which extend to ventral tegmental area (VTA) and a ventral tier which spreads to laterally to the SN pars reticulata (SNr) (Lynd-Balta and Haber 1994, Haber *et al.* 2000). Topographical studies of the nigrostriatal projections in animal models reported that dorsal tiers of SNc projects to the ventral striatum, head of the caudate and anterior putamen whereas ventral tiers projects to the posterior putamen and caudate nuclei (Carpenter and Peter 1972, Szabo 1980, Haber *et al.* 2000).

The pattern of nigral neuronal loss in PD differs to normal ageing (Fearnley and Lees 1991). It has been demonstrated marked reduction of nigral pigmented in the ventral tier which spreads throughout the illness to the dorsal tiers in PD in comparison to the linear fallout of pigmented neurons of the ventral tier in normal ageing (Gibb and Lees 1991) It has been hypothesized that about 60% of nigral pigmented cellular loss is expected prior the appearance of classical motor symptoms (Fearnley and Lees 1991, Cheng *et al.* 2010). On the other hand the pronounced dopaminergic depletion in the putaminal areas is estimated with an order of 50-70%, with relatively indemnity of caudate nucleus (Kish *et al.* 1988, Cheng *et al.* 2010).

Pigmented neurons of SNc contain neuromelanin (NM) (see chapter 1) which in turn plays a toxic role in PD. Extracellular NM deposits release toxic metals and catecholaminergic products inducing oxidative stress, local microglial activation and further neurodegeneration (Zucca *et al.* 2015). NM acts as paramagnetic agent when

combining with metals such as iron and copper. Measurements of nigral depigmentation by using Neuromelanin-weighted MR imaging reported high sensitivity and specificity distinguishing between PD and healthy controls and is considered as a potential imaging tool for monitoring the disease severity (Sasaki *et al.* 2006, Kashiwara *et al.* 2011, Schwarz *et al.* 2011, Ogisu *et al.* 2013, Ohtsuka *et al.* 2013, Castellanos *et al.* 2015).

Functional imaging studies with positron emission tomography (PET) using  $^{18}\text{F}$ -DOPA have been traditionally used for measuring the integrity of dopaminergic nerve terminal in addition for assessing disease progression in PD patients (Morrish *et al.* 1996, Punal-Rioboo *et al.* 2009). However there is strong evidence of  $^{18}\text{F}$ -DOPA overestimates nerve terminal density in early PD regarding up-regulation of aromatic L-amino-acid decarboxylase (AADC) (Ribeiro *et al.* 2002). On the other hand the study of striatal dopamine capacity with dopamine transporters (DAT) PET ligands represent a more specific approach for studying the integrity of the dopaminergic innervation as are exclusively located on dopaminergic neurons (see review (Piccini 2003). In a recent work the highly specific DAT radioligand  $^{11}\text{C}$ -PE2I (Jucaite *et al.* 2006) reported greater sensitivity in detecting differences in motor severity and bradykinesia-rigidity symptoms than  $^{18}\text{F}$ -DOPA at cross-sectional and longitudinal settings (Li *et al.* 2017)

To date three studies have been undertaken for assessing the relationship between striatal dopamine transporter as measured with single-photon emission computed tomography and [ $^{123}\text{I}$ ] N- $\omega$ -fluoropropyl-2b-carbomethoxy-3b-(4-iodophenyl) tropane and post-mortem measurement of SNc pigmented neurons (Colloby *et al.* 2012, Kraemmer *et al.* 2014, Saari *et al.* 2017) with conflicting results. Post-mortem nigral cell counting was associated to striatal DAT binding in two studies (Colloby *et al.* 2012, Kraemmer *et al.* 2014), which included a heterogeneous small sample of non-parkinsonian disorders. This relationship was however not reported by Saari et al (Saari *et al.* 2017) who suggested that striatal DAT binding might not reflect the number of dopaminergic nigral neurons but axonal dysfunction or DAT expression. More recently Isaias et al (Isaias *et al.* 2016) conducted an in vivo multimodal assessment of nigrostriatal pathways measuring striatal dopamine transporter with DAT-SPECT and nigral pigmented load with Neuromelanin-weighted MRI. The authors reported positive correlations between both imaging markers, although the topographical patterns of nigrostriatal pathways were not described.

The objectives of the present chapter are:

- To assess the regional pattern of neuromelanin signal intensity loss within the SNc between PD and HC groups.
- To assess the correlation between regional subdivisions of nigral pigmented load (by using Neuromelanin-weighted MR imaging) and nigrostriatal terminal density (as measured with  $^{11}\text{C}$ -PE2I PET) in a moderate-stage PD cohort.

The hypotheses of the present chapter are:

- PD subjects will show reduced nigral pigmentation when compared with HC.
- Nigral depigmentation will be greater in ventral than dorsal tiers within the PD group.
- Loss of nigral-pigmented count in dorsal tiers will be associated with loss of dopaminergic nerve terminals in pre-commissural ventral striatum.
- Loss of nigral-pigmented count in ventral tiers will be associated with loss of dopaminergic nerve terminals in pre-commissural dorsal and post-commissural striatum.

## 6.2. Subjects and methods

### 6.2.1. Study subjects

Twenty-nine non-demented mild-moderate stage PD patients and twenty age and gender-matched healthy controls (HC) were included in this study (**Table 5.1**). Diagnosis of PD was performed by movement disorder specialists in accordance with the Parkinson's UK Brain Bank Criteria (Hughes *et al.* 1992), excluding atypical parkinsonism, concomitant vascular load, history of cognitive impairment, psychiatric disorders and any factors that would preclude MRI scanning.

The Movement Disorders Society Unified Parkinson's Disease Rating Scale Part III (UPDRS III) (Goetz *et al.* 2008) was used to calculate total motor severity in the practically-defined OFF medicated state and further subdivided into tremor (items 15-18) and bradykinesia-rigidity (items 2-9 and 14) subscores. A non-computerised motor tap

test was also administered in which patients were instructed to produce as many alternating button presses as possible within 30 seconds. The outcome measure was the average number of full button depressions achieved over two trials per side.

Patients were instructed to withdraw from standard release anti-PD medications 24 hours prior to motor and imaging assessments and 48 hours for prolonged release medications. Caffeine in any form was not permitted within 12 hours prior to scan. Levodopa equivalent daily dosage (LEDD) for each participant was calculated (Tomlison 2010) and shown in *Table 5.1*.

Data were collected under the TransEuro and PaMIR studies, funded by FP7 and Parkinson's UK respectively and carried out in accordance with the Declaration of Helsinki, after approval from the National Research Ethics Service.

### **6.2.2. Imaging acquisition**

#### **6.2.2.1. Magnetic Resonance Imaging**

MRI scans were acquired for both PD and HC participants on a 3T Siemens Magnetom Trio system with 32-channel head coil and consisted of neuromelanin-weighted T1-weighted turbo spin-echo (TSE: TR = 829ms; TE = 12ms; flip angle = 123°; echo train length = 4; echo spacing = 11.5ms; low specific absorption rate; FoV = 256\*256mm; matrix size = 320\*320) and high-resolution volumetric T1-weighted magnetization prepared rapid acquisition gradient echo (MPRAGE: TR = 2300ms; TE = 2.98ms; flip angle = 9°; time to inversion = 900ms; GRAPPA acceleration factor PE = 2; FoV = 240\*256mm; Matrix size = 240\*256) sequences.

For the MPRAGE, one whole brain volume was acquired consisting of 160 contiguous slices of 1mm thickness. For the TSE, 12 slices of 2.5mm thickness and slice gap of 0.25mm were acquired parallel to the AC-PC line with coverage of the whole midbrain and upper pons. Scans lasted 301 and 332 seconds respectively and patients were instructed to remain as still as possible for the duration.

The maximum delay between Neuromelanin-weighted MR imaging and  $^{11}\text{C}$ -PE2I PET scans was one month.

#### **6.2.2.2. Positron Emission Imaging**

PD participants underwent  $^{11}\text{C}$ -PE2I ( $[^{11}\text{C}]\text{N}$ -(3-iodopro-2*E*-enyl)-2 $\beta$ -carbomethoxy-3 $\beta$ -(4'-methylphenyl)nortropane) PET scans, acquired on a Siemens Biograph TruePoint HI-REZ 6 PET/CT system. Patients were positioned supine such that the transaxial plane was parallel to the AC-PC plane and movement minimised using memory foam padding and video monitoring to aid repositioning.  $^{11}\text{C}$ -PE2I tracer volumes (Injected dose =  $324.42 \pm 36.07$  MBq; Injected mass =  $3.72 \pm 1.31$   $\mu\text{g}$ ) were prepared to 10ml using saline solution and administered intravenously as a single bolus injection followed immediately by 10ml saline flush. Administration was at a rate of 1ml/s.

Dynamic emission data were acquired continuously while patients were at rest for 90 minutes post-injection. Data were binned into a dynamic series of 26 temporal frames (8 x 15s, 3 x 60s, 5 x 120s, 5 x 300s, 5 x 600s) and reconstructed with corrections for decay, scatter and attenuation using a filtered back-projection algorithm (direct inversion Fourier transform) with a matrix size of 128 x 128, zoom of 2.6 and 2mm isotropic pixel size and smoothed using a three-dimensional 5mm FWHM trans-axial Gaussian image filter. A low-dose CT transmission scan (0.36mSv) was acquired for attenuation and scatter correction

#### **6.2.3. Imaging procession and region of interest analysis.**

##### **6.2.3.1. Neuromelanin-weighted magnetic resonance imaging**

For the quantitative evaluation of NM-weighted MRI scans, a study-specific template was first created using grey and white matter MPAGE segmentations from both PD and HC participants and diffeomorphic anatomical registration through exponentiated Lie algebra (DARTEL) in SPM12. Software defaults were modified to enable template and MPAGE-to-template warp field generation in native voxel size (1mm<sup>3</sup>). NM-weighted T1 images were co-registered to the corresponding MPAGE and DARTEL warp fields

applied in one step to normalise all images to common space. The neuromelanin template could then be generated through voxel-wise averaging (mean) over the entire cohort (n=51).

Mesencephalic regions of interest (ROI) for the left/right substantia nigra pars compacta (SNc) were manually defined on five contiguous axial slices of the neuromelanin template on which the neuromelanin-related hyperintensity of the SNc was visible, at the level of the red nucleus and the inferior colliculus, in FSL (Jenkinson *et al.* 2012). The volume of the SNc on each axial slice was divided into two sections representing the dorsal and ventral tiers, in accordance with post-mortem histological investigations on the striatal afferences of the SNc reported by Haber *et al.* (Haber 2014). The decussation of the superior cerebral peduncle (CP) was also defined on the same axial slices as a reference region (*Figure 5.1 C*). Once all ROIs were delineated the inverse DARTEL warp fields for each participant were applied to generate individualised ROIs for sampling in native space. This was performed in order to reduce the effect of warping/normalisation on the absolute voxel intensities within the images. Registration was checked visually in FSL before extracting mean ROI intensity values using the `fslmeants` command. Contrast ratios (CR) were calculated in accordance with Sasaki *et al.* (Sasaki *et al.* 2006) using the formula  $(\text{SNc-CP})/\text{CP}$  for dorsal ( $\text{SNc-CR}_{\text{dor}}$ ) and ventral ( $\text{SNc-CR}_{\text{ven}}$ ) ROIs separately. Contrast ratios were first calculated for the most/least affected sides for each ROI, as indicated by the sum of the UPDRS III laterality items (items 3-8, 15-17) and motor tap test, before averaging across sides to yield bilateral CRs. Contrast ratios of the whole SNc ( $\text{SNc-CR}_{\text{total}}$ ) were calculated by averaging  $\text{SNc-CR}_{\text{ven}}$  and  $\text{SNc-CR}_{\text{dor}}$ .

#### **6.2.3.2. $^{11}\text{C}$ -PE2I PET**

Pre-processing and kinetic modelling for MPRAGE and  $^{11}\text{C}$ -PE2I PET was conducted using MIAKAT<sup>TM</sup> v3.4.2. (Molecular Imaging and Kinetic Analysis Toolbox, Imanova Imaging Centre, London, UK) (Gunn 2016), which utilises FSL (FMRIB Image Analysis Group, Oxford, UK) (Jenkinson *et al.* 2012), SPM (Statistical Parametric Mapping, Wellcome Trust Centre for Neuroimaging, London, UK) and in-house pre-processing and

kinetic modelling procedures within an integrated PET analytical framework and is implemented in MATLAB® (Mathworks, Natick, MA, USA).

First, MPAGE images were brain extracted, segmented and rigid-body registered to the Montreal Neurological Institute (MNI) template (Mazziotta *et al.* 1995). MPAGE images in “pseudo-MNI” space (MPAGE<sub>REG</sub>) were then used for manual subcortical ROI delineation in Analyze11.0, in accordance with the anatomical-landmark-based methods for striatal subdivision described in detail by Tziortzi *et al.* (Tziortzi *et al.* 2011). In brief, the ventral striatum (VSt) was defined on the coronal plane as the ventro-medial section of striatal gray matter anterior to the AC. The putamen was subdivided into three sections on the axial plane; posterior to the AC (post-commissural putamen), anterior to the AC and dorsal to the posterior commissure (PC) (pre-commissural dorsal putamen), anterior to the AC and ventral to the PC (pre-commissural ventral putamen). The caudate was subdivided into two sections on the axial plane; anterior to the AC (pre-commissural caudate) and posterior to the AC (post-commissural caudate). ROIs of the whole putamen and caudate nucleus were formed by merging pre-commissural and post-commissural subdivisions of the putamen and caudate respectively. Furthermore, the striatum was defined as the combination of the putamen, caudate nucleus and ventral striatum (*Figure 6.1A, 6.1B*). Cerebellar grey matter was automatically defined by applying the deformation fields estimated from the spatial normalisation (12-parameter affine transformation followed by non-linear warp) of the MNI template to the MPAGE<sub>REG</sub>, to an MNI-based regional atlas (CIC Atlas v1.2.). Grey matter segmentation maps were then applied to isolate cerebellar grey matter.

Dynamic PET data were corrected for intra-scan head movement using frame-to-frame rigid registration with the 16<sup>th</sup> frame as a reference. Registered frames between 10-90 minutes were subsequently summed to obtain signal-averaged (ADD) images that were co-registered to the subjects MPAGE<sub>REG</sub> using normalised mutual information as a cost function. Approximated co-registration matrices were then applied to both ADD images and realigned dynamic frames so that all images were in register with the subject MPAGE<sub>REG</sub> and the corresponding ROI map. Image processing was evaluated at each step before ROI maps were applied to the dynamic PET frames to generate regional time-activity curves (TACs) for all ROIs on the most and least affected sides separately, as indicated by the sum of the UPDRS-III laterality items (items 3-8, 15-17), as well as

bilaterally. The simplified reference tissue model (SRTM) (Lammertsma and Hume 1996) was used to calculate regional non-displaceable binding potential ( $BP_{ND}$ ). Cerebellar grey matter was used as a reference as previous studies have demonstrated negligible tracer uptake in this region (Hall *et al.* 1999, Halldin *et al.* 2003, Jucaite *et al.* 2006)

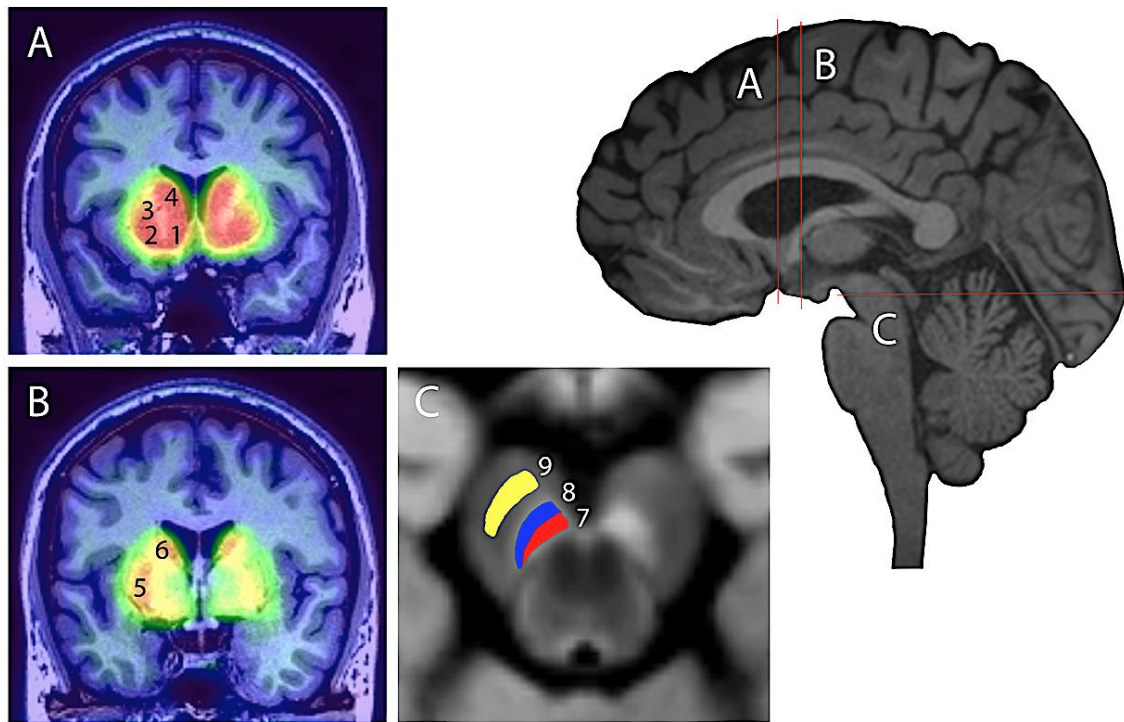


**Figure 6.1:** Sagittal midline view of a 3D T1-weighted MPRAGE illustrating coronal and axial planes of ROI delineation. **A and B** represent  $^{11}\text{C}$ -PE2I ADD images overlaid onto the structural  $\text{MPRAGE}_{\text{REG}}$  from two levels of the coronal plane either side of the anterior commissure along the y-axis. **A:** Pre-commissural striatum including ventral striatum (1), ventral putamen (2), dorsal putamen (3) and caudate (4). **B:** Post-commissural striatum including putamen (5) and caudate (6). **C** represents axial slices of neuromelanin-template at the level of the substantia nigra pars compacta, including delineation of the ventral (7) ( $\text{SNc-CR}_{\text{ven}}$ ) and dorsal (8) ( $\text{SNc-CR}_{\text{dor}}$ ) tiers as well as cerebral peduncles (9).  $\text{MPRAGE}$ = Three-dimensional T1 weighted gradient-echo sequence;  $\text{SNc-CR}_{\text{ven}}$ = Contrast ratios of ventral tiers of SNc;  $\text{SNc-CR}_{\text{dor}}$ = Contrast ratios of dorsal tiers.

#### 6.2.4. Statistical analyses

All statistical analyses were performed using SPSS statistical software (version 22.0 SPSS Inc., Chicago, Illinois).

Demographic data were compared between PD and HC groups using independent t-tests and Chi-square test for independence. Normal distribution of the data was assessed using Shapiro-Wilk tests ( $p < 0.05$ ) and evaluation of normal Q-Q plots and subsequent analyses were applied as appropriate.

To assess differences in SNc-CRs between groups and regions, a mixed analysis of variance (ANOVA) was conducted with group (PD and HC) as the between-subjects factor and region (bilateral SNc-CR<sub>dor</sub>, SNc-CR<sub>ven</sub>) as the within-subjects factor. The between-group difference in neuromelanin contrast ratios across the entire substantia nigra (SNc-CR<sub>total</sub>) was tested separately using an independent t-test. The asymmetry indices were calculated for the SNc-CR<sub>dor</sub> and SNc-CR<sub>ven</sub> according to clinical laterality in the PD group (most/least affected) and right/left in the HC group.

Pearson's product moment correlation coefficient was used to assess relationships between measures of disease severity (disease duration, UPDRS III total, tremor and bradykinesia/rigidity subscores), SNc-CRs for bilateral nigral regions (SNc-CR<sub>dor</sub>, SNc-CR<sub>ven</sub>) and <sup>11</sup>C-PE2I BP<sub>ND</sub> for bilateral striatal subregions (ventral striatum, pre-commissural dorsal putamen, pre-commissural ventral putamen, pre-commissural caudate, post-commissural putamen and post-commissural caudate).

Correlations between regional unilateral SNc-CRs and striatal <sup>11</sup>C-PE2I BP<sub>ND</sub> values according to clinical laterality as assessed by tap-test into most and least affected sides were performed using Pearson's product moment correlation coefficient. Not all regional combinations between SNc and striatum were assessed for a relationship. Instead, planned tests were performed based on previous anatomical work mapping connectivity between subregions of the substantia nigra and striatum (Tziortzi *et al.* 2011, Haber 2014). As such, correlations were conducted between SNc-CR<sub>dor</sub> and <sup>11</sup>C-PE2I BP<sub>ND</sub> in the pre-commissural ventral striatum, ventral putamen, dorsal putamen and caudate, not including post-commissural striatum (Figure 6.1, A1-4); SNc-CR<sub>ven</sub> and <sup>11</sup>C-PE2I BP<sub>ND</sub>

in the precommissural dorsal putamen and caudate not including ventral pre-commissural striatum (Figure 6.1, A1-2,4) as well as the post-commissural putamen and caudate (Figure 6.1, B5-6). Furthermore relationship between unilateral SNc-CR<sub>total</sub> and <sup>11</sup>C-PE2I BP<sub>ND</sub> from striatum, caudate and putamen nuclei was conducted for comparison with previous post-mortem (Colloby *et al.* 2012, Kraemmer *et al.* 2014, Saari *et al.* 2017) and imaging (Isaias *et al.* 2016) studies. Correlations were considered significant only if  $p < 0.05$  following Benjamini-Hochberg FDR adjustment for multiple comparisons.

## 6.3.Results

### 6.3.1. Demographics

Clinical and demographic characteristics for the PD and HC groups are summarized in Table 6.1. There were no significant differences in age ( $p=0.451$ ) or gender ( $p=0.054$ ) between groups that underwent the analysis of Neuromelanin-weighted MR imaging.

|                          | PD            | HC           | Statistic           | $p$   |
|--------------------------|---------------|--------------|---------------------|-------|
| Volunteers               | 29            | 20           |                     |       |
| Age (years)              | 56.44 ± 6.18  | 57.57 ± 9.83 | $t(47)= 0.49^a$     | 0.622 |
| Gender (m:f)             | 24:5          | 11:9         | $\chi^2(1)= 4.47^b$ | 0.054 |
| Disease duration (years) | 6.83 ± 1.88   | NA           | NA                  | NA    |
| UPDRS III                | 32.54 ± 10.99 | NA           | NA                  | NA    |
| Hoehn and Yahr scale     | 2.04 ± 0.18   | NA           | NA                  | NA    |
| LEDD                     | 744 ± 365     | NA           | NA                  | NA    |

**Table 6.1:** Demographic and clinical characteristics of PD and control subjects. Values represent the mean ± standard deviation (SD). PD= Parkinson's disease; HC= healthy controls; NA= not applicable; UPDRS-III=Unified Parkinson's Disease Rating Scale Part-III; LEDD= Levodopa equivalent daily dose; <sup>a</sup>= Indicates that differences were tested using independent  $t$ -tests; <sup>b</sup> = Indicates that differences were tested using Pearson's Chi-square test for independence; (df) = degrees of freedom.

### 6.3.2. Analysis of Neuromelanin Contrast Ratios

The difference in the mean bilateral SNc-CRs for each group was tested using a mixed ANOVA, with group (PD and HC) as the between-subjects factor and subregion of the substantia nigra (SNc-CR<sub>dor</sub>, SNc-CR<sub>ven</sub>) as the within-subjects factor.

Extreme values were screened using boxplots and studentized residuals. Data were normally distributed, as indicated by evaluation of normal Q-Q plots and non-significant Shapiro-Wilk tests, had homogenous variances, as revealed by Levene's test statistic and had homogeneity of covariance, as shown by Box's M. Mauchly's test revealed that the assumption of sphericity was violated, therefore Greenhouse-Geisser adjusted values were used.

The mixed ANOVA revealed significant interaction by region between groups ( $F(1, 47) = 19.570, p < 0.001$ ), indicating that the difference in SNc-CRs between each region of the SN depended on the grouping factor. Although ANOVA analysis did not show significant interactions within subjects group ( $F(1, 47) = 1.970, p > 0.05$ ), independent t-test displayed significant differences between SNc-CR<sub>dor</sub> and SNc-CR<sub>ven</sub> and in the PD group ( $t(54) = 2.741, p = 0.008$ ) but not in the HC group ( $t(36) = 1.995, p < 0.05$ ) (*Figure 6.2*).

Analysis of asymmetry indices showed significant differences between PD and HC groups in SNc-CR<sub>dor</sub> ( $t(45) = 1.679, p = 0.015$ ) and SNc-CR<sub>ven</sub> ( $t(45) = 1.679, p = 0.011$ ).

In terms of percentage of bilateral SNc-CR reduction, the PD group showed a ventro-medial pattern of neurodegeneration as compared to HC as follows SNc-CR<sub>ven</sub> (-34.73%) > SNc-CR<sub>dor</sub> (-23.56%). Additionally, an independent t-test for SNc-CR<sub>total</sub> showed a significant difference between groups ( $t(47) = 4.681, p < 0.001$ ) with a total nigral reduction of 28.83%,

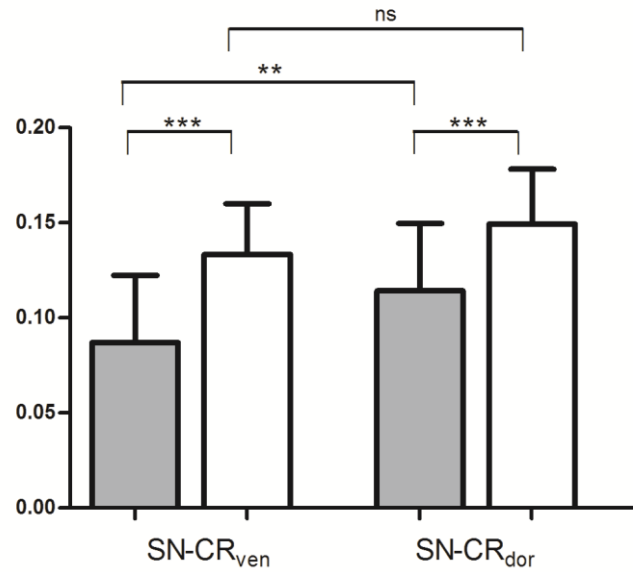


**Figure 6.2:** Bar chart showing results of the mixed ANOVA, including region (ventral and dorsal), as a within-subject factor and group (PD, HC) as between-subject factor. Grey bars represent the PD group and empty bars represent the HC group. Error bars represent 95% confidence intervals about the least-squares means of substantia nigra contrast ratios. Horizontal lines indicate planned post-hoc Bonferroni-adjusted pairwise comparisons. PD= Parkinson's disease; HC= Healthy control;  $SNc-CR_{ven}$ = Contrast ratios of ventral tiers of SNc;  $SNc-CR_{dor}$ = Contrast ratios of dorsal tiers; \*\*\*=  $p < 0.001$ ; \*\*=  $p < 0.01$ ; \*=  $p < 0.05$ ; ns = not significant.

### 6.3.3. Clinical correlations

Neuromelanin content in the ventral tiers of the SNc ( $SNc-CR_{ven}$ ) was negatively correlated with disease duration ( $r = -0.514$ ,  $p = 0.004$ ). No other associations between pigment load in ventral and dorsal tiers ( $SNc-CR_{ven}$ ,  $SNc-CR_{dor}$ ) with UPDRS III and subscores were found.

Nigrostriatal terminal density in the post-commissural putamen as measured with  $^{11}C$ -PE2I  $BP_{ND}$  was inversely correlated with UPDRS-III ( $r = -0.449$ ,  $p = 0.008$ ). Furthermore, bradykinesia-rigidity subscores were strongly associated with  $^{11}C$ -PE2I  $BP_{ND}$  in the pre-commissural dorsal putamen ( $r = -0.520$ ,  $p = 0.002$ ) and post-commissural caudate ( $r = -0.469$ ,  $p = 0.010$ ) and putamen ( $r = -0.556$ ,  $p = 0.001$ ). No significant correlations between

nigrostriatal terminal densities ( $^{11}\text{C}$ -PE2I BP<sub>ND</sub>) and tremor subscores were found. In additions correlation between  $^{11}\text{C}$ -PE2I BP<sub>ND</sub> in the pre-commissural dorsal putamen with disease duration ( $r = -0.377$ ,  $p = 0.044$ ) did not survive FDR corrections for multiple correlations test.

#### 6.3.4. Nigrostriatal correlations

In the PD group we assessed the correlations between the nigrostriatal terminal density in the striatal subdivisions ( $^{11}\text{C}$ -PE2I BP<sub>ND</sub>) and the Neuromelanin content of the SNc (SNc-CR<sub>ven</sub>, SNc-CR<sub>dor</sub>) in the contralateral to the least and most clinically affected sides.

When assessing the most affected side we found correlations between loss of neuromelanin load in the dorsal tiers (SNc-CR<sub>dor</sub>) and reduction of dopamine transporter bindings ( $^{11}\text{C}$ -PE2I BP<sub>ND</sub>) in the ventral striatum ( $r = 0.422$ ,  $p = 0.018$ ), in the Pre-Commissural caudate ( $r = 0.613$ ,  $p < 0.001$ ) and pre-commissural ventral ( $r = 0.463$ ,  $p = 0.008$ ) and dorsal putamen ( $r = 0.489$ ,  $p = 0.005$ ) (*Figure 6.3*). Furthermore depigmentation in nigral ventral tiers (SNc-CR<sub>ven</sub>) correlated with reduction of dopamine transporter bindings in the pre-commissural caudate ( $r = 0.542$ ,  $p = 0.002$ ) and dorsal putamen ( $r = 0.606$ ,  $p < 0.001$ ) in addition to the post-commissural putamen ( $r = 0.604$ ,  $p < 0.001$ ) and caudate ( $r = 0.438$ ,  $p = 0.014$ ) (*Figure 6.4*).

The least affected side only revealed significant associations between loss of neuromelanin load in the dorsal tiers (SNc-CR<sub>dor</sub>) and reduction of dopamine transporter binding in the pre-Commissural caudate ( $r = 0.358$ ,  $p = 0.028$ ) which did not survive the post-hoc FDR correction (*Figures 6.3 and 6.4*).

In addition the loss of neuromelanin load in the ventral and dorsal tiers (SN-CR<sub>total</sub>) showed significant correlations with loss of nigrostriatal density in the striatum ( $r = 0.549$ ,  $p = 0.001$ ), caudate ( $r = 0.568$ ,  $p < 0.001$ ) and putamen ( $r = 0.518$ ,  $p = 0.002$ ) in the most affected side, where as in the least affected side correlations in the caudate ( $r = 0.359$ ,  $p = 0.028$ ) did not survive the post-hoc FDR correction (*Figure 6.5*).

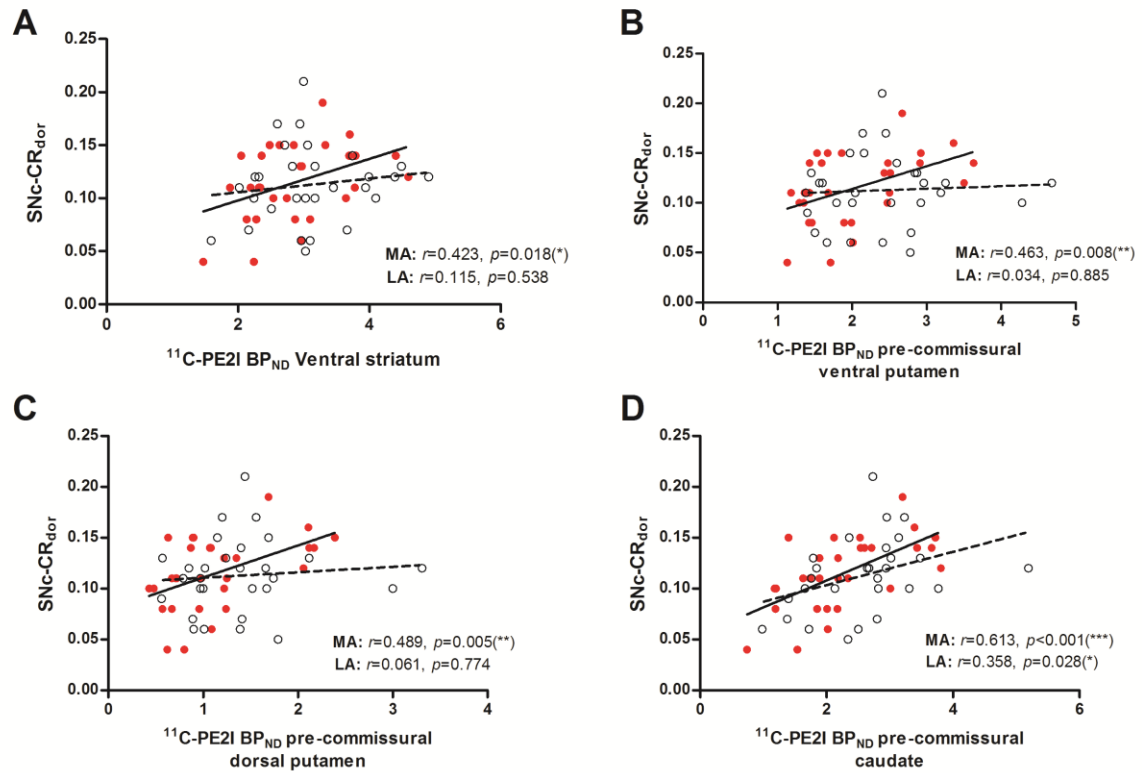


**Figure 6.3:** Scatterplots and Pearson's correlations showing positive relationships between SNc-CR<sub>dor</sub> and <sup>11</sup>C-PE2I BP<sub>ND</sub> in the ventral striatum (A; MA:  $*p<0.05$ , LA:  $p>0.05$ ), pre-commissural ventral putamen (B, MA:  $**p<0.01$ , LA:  $p>0.05$ ), pre-commissural dorsal putamen (C, MA:  $**p<0.01$ , LA:  $p>0.05$ ) and pre-commissural caudate (D, MA:  $***p<0.001$ , LA:  $p<0.05$ ) in the most affected (red circles, continuous line) and least affected sides (empty circles, dash line). MA= most affected; LA= least affected.

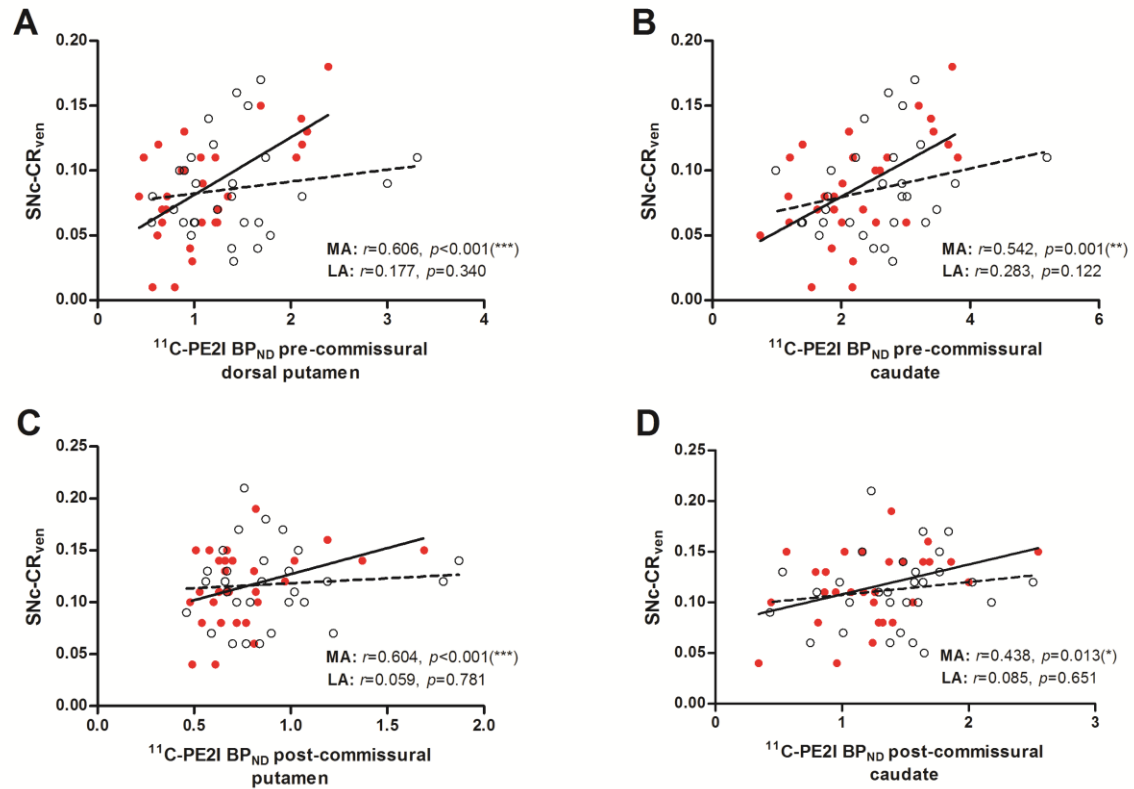


**Figure 6.4:** Scatterplots and Pearson's correlations showing positive relationships between Snc-CR<sub>ven</sub> and <sup>11</sup>C-PE2I BP<sub>ND</sub> in the pre-commissural dorsal putamen (A; MA: \*\*\* $p<0.001$ , LA:  $p>0.05$ ), pre-commissural caudate (B; MA: \*\* $p<0.01$ , LA:  $p>0.05$ ), post-commissural putamen (C; MA: \*\*\* $p<0.001$ , LA:  $p>0.05$ ) and post-commissural caudate (D; MA: \* $p<0.05$ , LA:  $p>0.05$ ) in the most affected (red circles, continuous line) and least affected sides (empty circles, dash line). MA= most affected; LA= least affected.

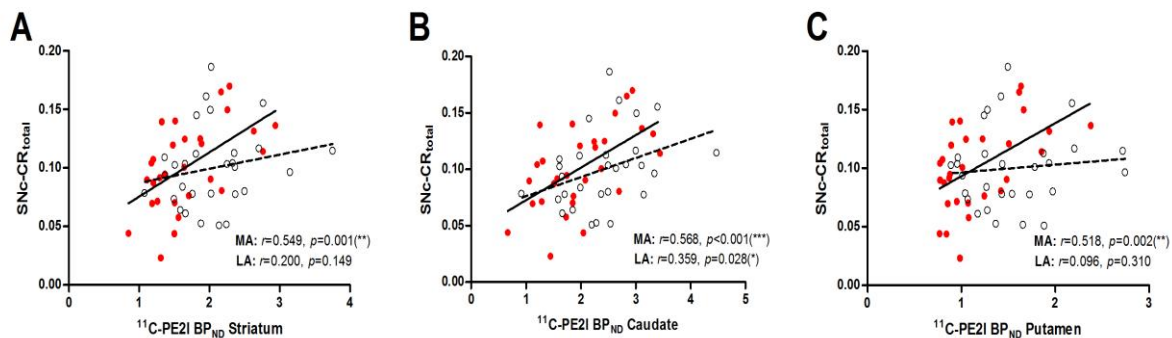


**Figure 6.5:** Scatterplots and Pearson's correlations showing positive relationships between Snc-CR<sub>total</sub> and <sup>11</sup>C-PE2I BP<sub>ND</sub> in the total striatum (A; MA: \*\* $p=0.01$ , LA:  $p>0.05$ ), total caudate (B; B: \*\*\* $p<0.01$ , LA: \* $p<0.05$ ), total putamen (C; MA: \*\* $p=0.01$  LA:  $p>0.05$ ) in the most affected (red circles, continuous line) and least affected sides (empty circles, dash line). MA= most affected; LA= least affected.

## 6.4. Discussion

The present chapter assessed the integrity of nigrostriatal pathways in a moderate-stage Parkinson's cohort by using NM-weighted MR imaging and <sup>11</sup>C-PE2I PET. PD subjects presented reduced nigral pigmentation load when compared to controls. Further ventral-dorsal pattern of nigral depigmentation was observed within the PD subjects. The associations between nigral depigmentation load and striatal dopamine transporter displayed inverse dorsal/ventral topography in the clinically most affected side, whereas were not present in the least affected side. Collectively these results imply that reduction of nigral pigmented neurons displays an uneven pattern of association with striatal dopaminergic loss according to clinical laterality in PD which in turn results in the presence of typical motor symptomology.

The degree of nigral pigmentation loss observed in the subdivisions of SNc into in the PD group displayed a remarkable dorsal-ventral pattern of neurodegeneration that goes in line with previous post-mortem studies (Gibb and Lees 1991) Dopaminergic neuronal loss first occurs in nigrosome 1 located the ventrolateral tiers on SNc (Damier *et al.* 1999) resulting in profound dopaminergic depletion in dorsal putamen (Kish *et al.* 1988). When

the disease progresses the dopaminergic loss extends to dorsal tiers of SNc and VTA which subsequent reduction of ventral striatal dopaminergic afferents (Fearnley and Lees 1991). The study of paramagnetic effects of neuromelanin with T1-weighted imaging has been recently shown as potential diagnostic and monitoring biomarker in PD (Sasaki *et al.* 2006, Kashihara *et al.* 2011, Schwarz *et al.* 2011, Matsuura *et al.* 2013, Ogisu *et al.* 2013, Ohtsuka *et al.* 2013, Castellanos *et al.* 2015, Reimao *et al.* 2015, Fabbri *et al.* 2017). Furthermore Neuromelanin-weighted MR imaging has been validated in post-mortem studies in PD (Kitao *et al.* 2013) demonstrating a direct association between the quantity of NM-containing dopaminergic neurons and the hyperintensity resulting from NM-weighted MR imaging in the SNc.

In the present chapter the associations between nigral pigmented markers and striatal dopamine transporter showed a topographical inverse ventro-dorsal pattern in the most affected side in PD. Ventral tiers of SNc containing the densocellular group and columnar cells (Haber 2014, Zhang *et al.* 2017) displayed significant associations with dopamine transporter in the post-commissural striatum. These findings are consistent with the previous neuropathological studies (Kish *et al.* 1988, Fearnley and Lees 1991) which result in the appearance of classical parkinsonian motor symptoms. Besides when assessing the dorsal striatal  $^{11}\text{C}$ -PE2I BP<sub>ND</sub> significant associations were observed with total motor severity as measured with UPDRS III and symptoms resulting to dopaminergic neurodegeneration such as bradykinesia and rigidity (Vingerhoets *et al.* 1997, Li *et al.* 2017). Furthermore associations between ventral tiers of SNc possibly corresponding to densocellular group and pre-commissural striatal dopamine transporter bindings consisting with central associative striatum are in line with the previous midbrain-striatum connectivity studies (Haber 2014, Zhang *et al.* 2017). Associative striatum receives dopaminergic inputs from the densocellular areas of the SNc overlapping with ventral-lateral nigral areas (Haber *et al.* 2000). Additionally the dorsal tier of SNc displayed significant associations with dopamine transporter bindings with of the ventral striatum and pre-commissural ventral putamen and caudate, which are functionally defined as limbic striatum. The latter associations occurred without including VTA in the present analysis. It is well known that dopaminergic neurons in VTA present a neuronal decline in later stages of PD (Uhl *et al.* 1985) however the interpretation of Neuromelanin-weighted MRI is still yet to be ascertained (Schwarz *et al.* 2017) probably due to its heterogeneous pigment distribution.

It is well known that nigral depigmentation occurs after the loss of dopaminergic markers in Parkinson's disease (Kordower *et al.* 2013) thus exhibiting a different pattern of degeneration especially at early stages. Although the rate of pigmented neurodegeneration lags behind the dopaminergic loss in all the time points, it appears to be closer and less variable in advanced stage of the disease. The uneven rate of degeneration of nigral pigmented load (see chapter 4.1 and 4.2) and dopaminergic nerve terminals (Ribeiro *et al.* 2002, Li *et al.* 2017) has been reported in the least and most clinically affected sides in PD, although their topographical *in vivo* associations were yet to be ascertained. In this chapter remarkable differences between nigrostriatal associations in the least and most affected sides regarding clinical laterality were reported in a moderate-stage PD cohort. I hypothesized that the asymmetrical pattern of nigrostriatal degeneration in both clinical side were reflected by unequal associations of nigral depigmentation measured with Neuromelanin-weighted MRI and dopamine transporter assessed with  $^{11}\text{C}$ -PE2I PET. Perhaps nigrostriatal associations would exhibit a parallel pattern in later stages of the disease, although further longitudinal studies are needed in order to determine temporal patterns of nigrostriatal relationships.

Previous studies assessing the relationship between striatal dopamine transporter using  $^{123}\text{I}$ -FP-CIT dopamine transporter single photon emission computed tomography (DAT-SPECT) and post-mortem nigral neuronal count (Kraemmer *et al.* 2014, Saari *et al.* 2017) showed heterogeneous results. While Kraemmer *et al.* (Kraemmer *et al.* 2014) and Colloby *et al.* (Colloby *et al.* 2012) reported reciprocal relationships between striatal dopamine transporter binding and post-mortem nigral measurements, Saari *et al.* (Saari *et al.* 2017) did not describe any associations between the latter measurements. Nevertheless these studies lack of *in vivo* validation of nigrostriatal impairment in PD as combined antero-mortem striatal dopamine transporter bindings and post-mortem neuronal nigral counts with variable time intervals. On the other hand the inclusion of non-parkinsonian disorders such as Alzheimer's disease and Creutzfeldt-Jakob disease cases as well as small sample size would result in difficult extrapolation of conclusions. In a recent *in vivo* multimodal study Isaias *et al.* (Isaias *et al.* 2016) evaluated the associations between nigral Neuromelanin-weighted MR imaging and dopaminergic striatal innervation with DAT-SPECT. The authors reported reduced nigral measures such as volumes and contrast-to-noise ratios correlated with total putamen and caudate

DAT bindings. Moreover in the present chapter significant associations between loss of total nigral depigmentation and loss of total dopaminergic nerve terminals of striatum, putamen and caudate nucleus in the most affected side are in line with results of Isaias *et al.* (Isaias *et al.* 2016). Besides in the current chapter evaluated the striatal dopamine transporters with  $^{11}\text{C}$ -PE2I which its specific binding is highest in striatum (Jucaite *et al.* 2006) when compared to  $^{123}\text{I}$ -FP-CIT which has also displays affinity for serotonin (SERT) and noradrenaline (NET) transporters (Abi-Dargham *et al.* 1996) hence providing a more specific in vivo measurement of striatal dopaminergic nerve terminals.

The present multimodal imaging protocol could potentially be considered as a promising *in vivo* indirect assessment of nigrostriatal pathways. To my knowledge previous imaging attempts assessing this neuronal circuitry with diffusional methods have showed conflicting results possibly due to methodological issues (Schwarz *et al.* 2013). Two recent studies reported significant diffusional tract alterations in nigrostriatal pathways by using deterministic tractography (Tan *et al.* 2015, Zhang *et al.* 2015) in PD. Authors reported systematic abnormalities in diffusion measures including reduction of fractional anisotropy as well as increases of mean diffusivity in the PD group when comparing with controls. Nevertheless diffusional measurements of nigrostriatal pathways imply several caveats that are worth to mention, such as difficulties distinguishing between functional directionality in fibre tracts not specifying the presence of striatonigral pathways or the potential inclusion of cortical-spinal crossing fibres.

In conclusion the present chapter provides evidence of the uneven topographical pattern of nigral neurodegeneration and striatal dopaminergic markers as assessed with a multimodal imaging protocol with Neuromelanin-weighted MR imaging and  $^{11}\text{C}$ -PE2I PET in a moderate-stage PD cohort. Although this multimodal in vivo imaging protocol could potentially be used for monitoring the disease progression and disease-modifying therapies in PD further longitudinal studies including cohorts with variable disease burden as well as healthy controls are needed.

## 6.5. Limitations and future directions

There are some limitations in the present chapter that are worth to mention. First, the nigrostriatal imaging assessment in normal ageing were not showed as the inclusion of a control group in the in the  $^{11}\text{C}$ -PE2I PET protocol was not done. Nigrostriatal circuitry has been studied in animal (Haber *et al.* 2000, Joel and Weiner 2000) and DTI studies (Zhang *et al.* 2017), although the lack of PET studies (Ito et al 2017) could be considered a major limitation to ascertain group differences.

Second, this chapter included a cross-sectional assessment of nigrostriatal pathways by using a multimodal imaging protocol in PD. In spite of the absence of the inclusion of healthy controls and analysis performed only in the cross-sectional setting, results provide evidence of dopaminergic nigrostriatal associations. Although this multimodal in vivo imaging protocol could potentially be used for monitoring the disease progression and disease-modifying therapies in PD further longitudinal studies including cohorts with variable disease burden as well as healthy controls are needed.

In addition, several disadvantages of PET studies should be mentioned such as the involvement of ionising radiation with the injection of radioactive tracers and the high cost of the procedure when compare to MR imaging.

To date, several studies combined functional and diffusional MRI modalities and dopaminergic PET studies in PD (Ekman *et al.* 2012, Lebedev *et al.* 2014, Son *et al.* 2016) thus examining the functional, structural and metabolic pathophysiological interplay of the disease. Perhaps the combination between resting-state functional MR and DTI with  $^{11}\text{C}$ -PE2I PET will provide promising results pertaining functional and structural connectivity of the nigrostriatal pathways.

# 7. Discussion

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## 7.1. Summary of key results

PD is the most common neurodegenerative cause of parkinsonism being also recognised as the second most prevalent neurodegenerative disorder after Alzheimer's disease. The neuropathological hallmark of PD consists in the presence of loss of dopaminergic neurons in the SNc and their following deafferentation of the striatum, which in turn result in the characteristic motor symptoms. Neuroimaging studies assessing structural such as iron deposition and neuromelanin load in SNc in PD have displayed consistent results when compared to normal ageing. Nevertheless the associations between motor severity and more specifically motor symptomatology with the previous nigral imaging measures presented inconsistent results. Furthermore the absence of longitudinal studies restricts the comprehensive validation of previous cross-sectional settings. Indeed the previous nigral imaging studies could be also applied in multimodal protocols with dopamine transporter PET studies to study the nigrostriatal pathways or as monitoring imaging tool of chelating therapies in PD.

The current thesis accomplished a multimodal nigral imaging assessment of iron deposition (chapter 3) and neuromelanin load (chapter 4) in an extensive and comprehensive cohort of PD subjects studied in two cross-sectional and longitudinal settings. Additional assessment of nigrostriatal pathways combining neuromelanin-weighted MR imaging and striatal dopamine transporter,  $^{11}\text{C}$ -PE2I PET, was showed in chapter 6. Finally a pilot study using the iron chelator deferiprone was included in order to evaluate relaxometry technique for monitoring the therapeutic effects.

### **7.1.1. Assessment of iron accumulation in deep grey matter nuclei of Parkinson's disease using high-pass filtered imaging.**

The current thesis presents two comprehensive cross-sectional and longitudinal analyses (subchapters 3.1 and 3.2) of an extensive cohort of early-moderate stage PD subjects using high-pass filtered imaging. Significant elevation of nigral iron levels were shown in both baseline and follow-up analyses in PD patients thus displaying an invariable finding when compared to increased iron accumulation in striatal nuclei such as putamen and globus pallidus in the cross-sectional cohort. Interestingly these results are in line with the recent meta-analysis of iron determination in PD with iron-sensitive imaging techniques and post-mortem quantification (Wang *et al.* 2016) which reports that in vivo iron imaging techniques may not authentically reflect iron changes in brain regions other than SN.

In the present thesis, nigral iron mineralisation presents significant relationship with motor severity as assessed with “off-medication” UPDRS-III scale in both baseline and follow-up studies. Furthermore, we demonstrated nigral iron mineralisation displays an ascendant stratified pattern of accumulation when PD patients were subdivided according to continuous groups of motor severity in both subchapters. The latter results took into account the age and original delineated area as study co-variants, thus offering a comprehensive understanding of nigral iron accumulation behaviour when assessed regarding to motor severity.

The associations between parkinsonian symptomatology and iron deposition in PD were also assessed in this chapter. Motor symptomatology resulting from dopaminergic neurodegeneration such as bradykinesia and rigidity was found to display significant associations with nigral iron deposition in both cross-sectional and longitudinal settings. Interestingly temporal changes of iron deposition in cerebellar dentate nucleus present significant relationships with changes in non-dopaminergic symptoms like tremor. According to the aforementioned results we hypothesise that associations of iron accumulation in SN and DN with bradykinesia-rigidity and tremor subscores respectively show two distinctive pathophysiological patterns of PD clinical progression.

### **7.1.2. Assessment of nigral depigmentation in Parkinson's disease using Neuromelanin in Parkinson's disease**

The current thesis presents two comprehensive cross-sectional and longitudinal analyses (subchapters 4.1 and 4.2; chapter 5) of an extensive cohort of early-moderate stage PD subjects using NM-weighted MR imaging. Significant reduction of nigral neuromelanin load was observed in both baseline and follow-up analyses in PD patients thus displaying an invariable finding in keeping with nigral elevation of iron. Topographical analysis of the NM deposition in the SNc showed greater reduction of pigment content in the ventral tiers when compared to dorsal areas.

Nigral depigmentation displayed significant relationship with disease duration and in lesser degree with motor severity as assessed with “off-medication” UPDRS-III scale at baseline setting. In addition associations of temporal changes of nigral depigmentation and changes in UPDRS III scale and bradykinesia was reported in the longitudinal study.

Furthermore, the degree of nigral depigmentation shows a descendant stratified pattern at baseline when PD patients were subdivided according to continuous groups of disease duration adjusted by original delineated area and age.

### **7.1.3. Iron chelation with deferiprone as therapeutic target in Parkinson's disease.**

The results presented in chapter 5 aimed to corroborate the importance of setting a double-blind randomized placebo-controlled studies with different therapeutic arms with deferiprone in the early stage of PD. Regardless of the small sample size, subjects treated with 30 mg/kg/day deferiprone display a trend of motor improvement after 6 month of treatment unlike patients with placebo or 20 mg/kg/day deferiprone. However the assessment of regional brain iron removal showed significant reduction in extranigral grey matter areas such as dentate and caudate nuclei, iron chelation in SN may be the result of longer period of treatment. The present results were in part in line with the

FAIRPARK clinical trial, reporting a small but significant reduction of motor severity after 6 months of treatment with deferiprone with subsequent reduction of nigral iron accumulation in a crossed double-blind placebo-control study. The latter study however include increased cohort size as well as 30 mg/kg/day deferiprone, conferring though great statistical power than the present study.

The use of high-pass filtered phase imaging in PD as described in chapter 3 may be of therapeutic interest. MR relaxometry data were not consistent between both studies, detecting different degrees of regional chelation in SN and extranigral deep grey matter nuclei. Conventional relaxometry techniques (T2\* and R2\*) estimate iron load *in vivo* using gradient-echo (GE) sequences that are sensitive to variations in transverse relaxation times/rates between different tissues and which are affected by both macroscopic and microscopic magnetic field inhomogeneities (Haacke *et al.* 2004). In contrast phase images contain information on the tissue magnetic susceptibility distribution and are not affected by total content of tissue water, thus offering greater specificity to quantifying brain iron load. In addition phase images are also more robust to the presence of noise (Haacke *et al.* 2007) and the removal of low spatial frequency artefacts that could otherwise obscure inter-tissue phase differences (using a high-pass filter) further improve its sensitivity to detecting abnormal iron levels.

#### **7.1.4. Assessing nigrostriatal pathways with multimodal imaging assessment using NM-weighted MR imaging and <sup>11</sup>C-PE2I PET in Parkinson's disease.**

In the chapter 6 the relationship between anatomical and functional subdivisions of SNc and striatum was assessed with Neuromelanin-weighted MR imaging and <sup>11</sup>C-PE2I PET in order to ascertain the nigrostriatal circuitry in a cohort of moderate-stage PD subjects.

The contralateral to the most affected clinical side displayed significant associations between the ventral tiers of SNc with motor and associative striatal areas, i.e. post-commissural and pre-commissural central striatum, whereas the dorsal tiers with the limbic pre-commissural ventral striatum. The latter results did not reach significant

associations when assessing the ipsilateral to the most affected clinical side. The present asymmetrical pattern of nigrostriatal associations might reflect a different rate of neurodegeneration between most and least affected side, which could follow a parallel pattern in advanced stage of the disease.

## 8. References

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# Appendix A: Scientific papers published in peer-reviewed journals arising from this thesis

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- **Martin-Bastida A**, Lao-Kaim NP, Loane C, Politis M, Roussakis AA, Valle-Guzman N, Kefalopoulou Z, Paul-Visse G, Widner H, Xing Y, Schwarz ST, Auer DP, Foltynie T, Barker RA, Piccini P. Motor associations of iron accumulation in deep grey matter nuclei in Parkinson's disease: a cross-sectional study of iron-related magnetic resonance imaging susceptibility. *European Journal of Neurology*. 2017 Feb; 24(2):357-365.
- **Martin-Bastida A**, Pietracupa S, Piccini P. Neuromelanin in parkinsonian disorders: a review. *International Journal of Neuroscience*. 2017 Dec; 127(12):1116-1123.
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- Pietracupa S, **Martin-Bastida A**, Piccini P. Iron metabolism and its detection through MRI in parkinsonian disorders: a systematic review. *Neurological Sciences*. 2017 Dec; 38(12):2095-2101.

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# Motor associations of iron accumulation in deep grey matter nuclei in Parkinson's disease: a cross-sectional study of iron-related magnetic resonance imaging susceptibility

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**Background and purpose:** To determine whether iron deposition in deep brain nuclei assessed using high-pass filtered phase imaging plays a role in motor disease severity in Parkinson's disease (PD).

**Methods:** Seventy patients with mild to moderate PD and 20 age- and gender-matched healthy volunteers (HVs) underwent susceptibility-weighted imaging on a 3 T magnetic resonance imaging scanner. Phase shifts (radians) in deep brain nuclei were derived from high-pass filtered phase images and compared between groups. Analysis of clinical laterality and correlations with motor severity (Unified Parkinson's Disease Rating Scale, Part III, UPDRS-III) were performed. Phase shifts (in radians) were compared between HVs and three PD subgroups divided according to UPDRS-III scores using analysis of covariance, adjusting for age and regional area.

**Results:** Parkinson's disease patients had significantly ( $P < 0.001$ ) higher radians than HVs bilaterally in the putamen, globus pallidus and substantia nigra (SN). The SN contralateral to the most affected side showed higher radians ( $P < 0.001$ ) compared to the less affected side. SN radians positively correlated with UPDRS-III and bradykinesia–rigidity subscores, but not with tremor subscores. ANCOVA followed by *post hoc* Bonferroni-adjusted pairwise comparisons revealed that SN radians were significantly greater in the PD subgroup with higher UPDRS-III scores compared to both lowest UPDRS-III PD and HV groups ( $P < 0.001$ ).

**Conclusions:** Increased nigral iron accumulation in PD appears to be stratified according to disease motor severity and correlates with symptoms related to dopaminergic neurodegeneration. This semi-quantitative *in vivo* iron assessment could prove useful for objectively monitoring PD progression, especially in clinical trials concerning iron chelation therapies.

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## Introduction

Iron plays an important role in Parkinson's disease (PD) neurodegeneration. Postmortem and *in vitro* studies have demonstrated links between iron accumulation and the cardinal pathological features of PD, the loss of dopamine neurons in the substantia nigra (SN) pars compacta [1] and the presence of  $\alpha$ -synuclein-rich Lewy bodies [2]. Free ferrous iron ( $\text{Fe}^{2+}$ ) acts as a catalyst in a reaction with hydrogen peroxide (Fenton reaction), producing highly toxic reactive oxygen species and ferric iron ( $\text{Fe}^{3+}$ ) that lead to oxidative-stress-related damage to cellular components including proteins, lipids and DNA [3]. To relieve potential toxicity, excess ferrous iron within deep brain nuclei is accumulated and transported into the core of apoferritin molecules where it is oxidized and safely stored in high concentrations in the ferric state [4]. Since ferritin (iron-containing apoferritin) is highly paramagnetic and indeed one of the only non-haem iron molecules present in sufficient quantity to cause local field inhomogeneities [4,5], magnetic resonance imaging (MRI) can be used to measure ferritin-bound ferric iron *in vivo*. Given that the biosynthetic rate of apoferritin is modulated by the availability of free iron [6,7], changes in ferritin-related MR signal are reflective of alterations in iron load.

*In vivo* iron quantification using MRI relaxometry ( $T2^*$ ,  $R2^*$ ) or susceptibility-weighted imaging (SWI) consistently demonstrates increased SN iron levels in patients with PD relative to healthy volunteers (HVs) [8–18], whilst results pertaining to striatal regions such as the caudate nucleus, putamen and globus pallidus appear highly heterogeneous [8–16]. Several studies have observed relationships between increased nigral iron accumulation, disease duration and motor severity [12–14,18] although to our knowledge there are currently no cross-sectional studies that have evaluated whether significant changes in iron accumulation exist between different stages of disease burden in mesencephalic or striatal nuclei. In addition, it remains unclear as to the effect of iron accumulation on differing motor symptoms, with the two studies investigating tremor and akinetic/rigid predominance yielding conflicting results [16,17].

Consequently the goal of this study was (i) to evaluate differences in iron accumulation in deep brain nuclei between PD groups of varying motor severity and (ii) to investigate the association between motor symptomatology and iron load. Phase images obtained as part of an SWI protocol were used because they contain information on the tissue magnetic susceptibility distribution, a property shown to be influenced by ferritin and haemosiderin

(breakdown product of ferritin) and which correlates with iron concentration postmortem [14,19]. Conventional relaxometry techniques are also affected by background field inhomogeneities such as tissue water content and air–tissue interfaces; thus phase images represent a more direct means to estimate iron [4]. This work demonstrates the value of iron quantification as an objective means by which patients can be monitored and stratified, particularly from the viewpoint of developing iron modifying therapies.

## Methods

### Subjects

Seventy non-demented mild to moderate stage PD patients and 20 age- and gender-matched HVs were included in this study (see Table S1 for recruitment information). The diagnosis of PD was performed by movement disorder specialists according to the Parkinson's UK Brain Bank criteria [20], excluding atypical parkinsonism, concomitant vascular load, history of cognitive impairment and neurodegenerative disorders other than PD.

Data were collected under the TransEuro and PaMIR studies, funded by FP7 and Parkinson's UK respectively, and carried out in accordance with the Declaration of Helsinki after approval from the National Research Ethics Service Committee. All participants gave written informed consent before participation.

### Motor assessment

Motor symptomatology was measured using the Unified Parkinson's Disease Rating Scale, Part III (UPDRS-III). Patients were instructed to withdraw from medication 24 h prior to assessment. Off-medication UPDRS-III scores were then subdivided into tremor (sum of items 15–18) and bradykinesia–rigidity subscores (sum of items 2–9 and 14). Levodopa-equivalent daily dose (LEDD) was calculated.

### Susceptibility-weighted imaging

Susceptibility-weighted images were acquired on a 3 T Siemens Magnetom Trio system with a 32-channel phased-array head coil running a T2-weighted three-dimensional gradient-echo sequence (SWI: TR = 28 ms, TE = 20 ms, flip angle  $15^\circ$ , bandwidth 120 Hz per pixel, matrix size  $294 \times 320$ , FoV =  $230 \times 230$  mm, GRAPPA acceleration factor 2). Seventy-two slices of 1.9 mm thickness and slice gap 20% were obtained in an interleaved order

parallel to the anterior–posterior commissural line in the left-hand reference system. A small number of participants (four PD, seven HVs) underwent a modified SWI protocol in which 88 slices of 0.9 mm thickness with a 20% slice gap were acquired. Phase images were reconstructed automatically on the Siemens workstation (Syngo MR B17 software, SWI version 1, Siemens, Erlangen, Germany), which included application of a  $64 \times 64$  high-pass filter to remove low spatial frequency effects.

### Regions of interest analysis

Manual region of interest (ROI) delineation was performed by a trained investigator (AMB) on both PD and HV high-pass filtered phase images using SPIN (Signal Processing in Nuclear Magnetic Resonance; MRI Institute, Detroit, MI, USA). All study subjects were anonymized prior to delineation, thus blinding

group and disease severity. ROIs were hand drawn on a single axial slice and included head of the caudate nucleus, putamen and globus pallidus (Fig. 1). The SN was delineated on the third axial slice ventral to the most dorsal aspect of the red nucleus. For images acquired under the modified SWI protocol, the fifth axial slice ventral to the most dorsal aspect of the red nucleus was used (Fig. S1). Independent sample *t* tests showed no significant differences in SN radians between the two SWI protocols for either group ( $P > 0.05$ ; Table S2). Subjects with microvascular lesions or physiological calcifications in the globus pallidus were not included in the analysis.

Mean phase-shift values, number of voxels, standard deviations and regional areas were extracted for each ROI unilaterally before averaging to obtain bilateral data. Phase-shift values were converted to radians according to the formula provided for the Siemens left-handed system: radians =  $(\phi - 2048)\pi/2048$  [21].

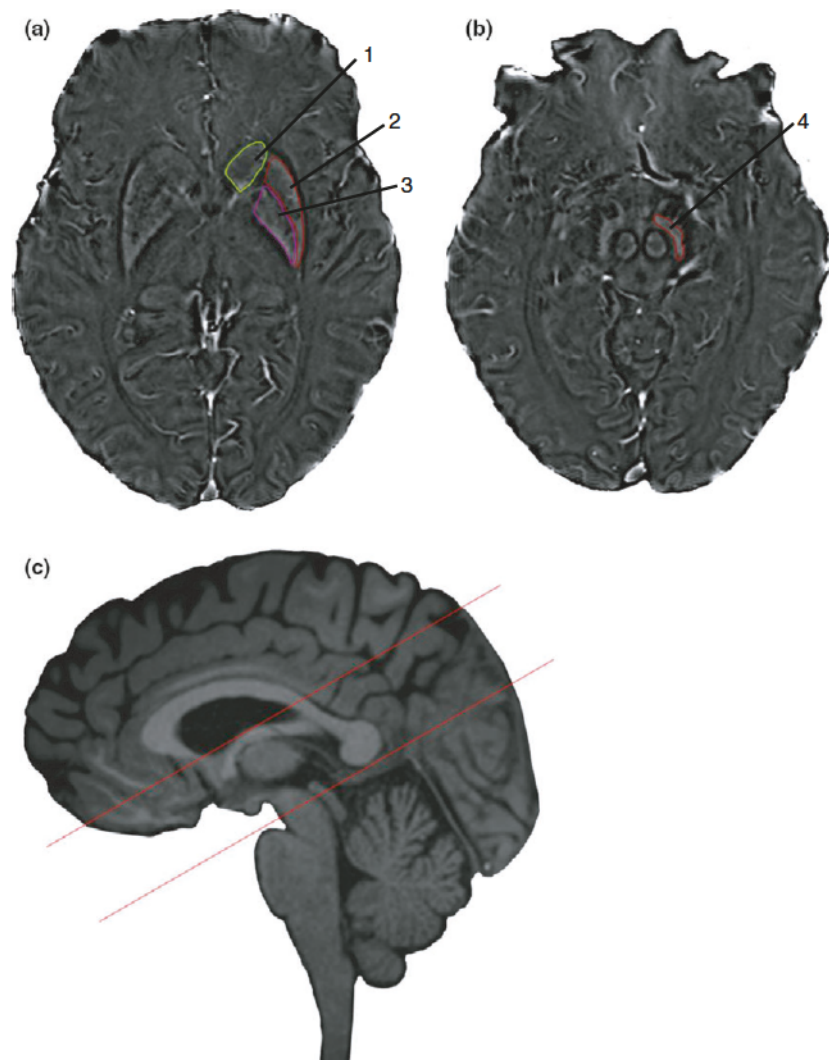


**Figure 1** Regions of interest (ROIs) delineated: (a) head of caudate nucleus (area 1), putamen (area 2), globus pallidus (area 3) and (b) substantia nigra (area 4); (c) sagittal three-dimensional MPRAGE T1-weighted image indicating level of ROI delineation. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

### Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 22.0 SPSS Inc., Chicago, IL, USA).

Group demographics and bilateral radian for each region were compared between PD and HVs using independent *t* tests. ROIs showing a significant difference were considered for analyses of covariance (ANCOVA) in which PD patients, subdivided into three disease severity groups using UPDRS-III tertile calculations (Table 1), were compared to the HV group, adjusting for age and regional area and followed with *post hoc* Bonferroni-adjusted pairwise comparisons.

To assess hemispheric iron load differences in the PD group, radian for clinically most/least affected sides, as indicated by UPDRS-III laterality items, were compared for each region using independent *t* tests.

Pearson's correlation coefficient was used to assess relationships between each ROI and measures of disease severity (disease duration, UPDRS-III total, tremor and bradykinesia/rigidity). Correlations were considered significant only for  $P < 0.05$  following Benjamini–Hochberg false discovery rate (FDR) adjustment.

## Results

### Participants

Clinical and demographic data are summarized in Table 1. There were no significant differences in age ( $P = 0.347$ ) between PD and HVs. Although the male:female ratio was higher in the PD group ( $P = 0.039$ ), no significant gender differences in iron accumulation were found.

As expected, PD subgroups exhibited significant differences in motor severity, disease duration, LEDD and Hoehn and Yahr scores (Table 1). There were no significant differences between subgroups for age or gender.

### Phase-shift analysis

Shapiro–Wilk's test revealed that putaminal and pallidal radian were not normally distributed ( $P < 0.05$ ). Square root transformations were therefore applied to the putamen and logarithmic transformation to the globus pallidus. The PD group showed significantly more radian in the putamen, globus pallidus and SN ( $P < 0.001$ ) compared to HVs (Fig. 2). No significant difference was found in the caudate nucleus.

Table 1 Demographic and clinical characteristics of HV and PD groups

|                      | HV group     | PD group      | Statistics          | <i>P</i> | PD group 1   | PD group 2   | PD group 3   | Statistics          | <i>P</i> |
|----------------------|--------------|---------------|---------------------|----------|--------------|--------------|--------------|---------------------|----------|
| Volunteers           | 20           | 70            |                     |          | 23           | 22           | 25           |                     |          |
| Age (years)          | 53.11 ± 11.7 | 55.78 ± 7.22  | $t = 3.38$ (23,18)  | 0.347    | 55.78 ± 7.76 | 54.66 ± 5.08 | 57.26 ± 8.51 | $F = 0.694$ (2, 67) | 0.558    |
| Gender (m:f)         | 10:10        | 52:18         | $\chi^2 = 4.28$ [1] | 0.039    | 15:8         | 15:7         | 22:3         | $\chi^2 = 7.74$ [2] | 0.052    |
| Disease duration     | NA           | 5.39 ± 2.45   |                     | –        | 4.33 ± 1.97  | 6.19 ± 2.29  | 5.62 ± 2.71  | $F = 3.77$ (2, 67)  | 0.028    |
| UPDRS-III            | NA           | 31.47 ± 11.56 |                     | –        | 19.3 ± 3.11  | 29.27 ± 3.13 | 44.60 ± 6.42 | $F = 213.9$ (2, 67) | 0.0001   |
| Hoehn and Yahr scale | NA           | 1.87 ± 0.47   |                     | –        | 1.63 ± 0.48  | 1.86 ± 0.46  | 2.12 ± 0.33  | $F = 7.07$ (2, 67)  | 0.01     |
| LEDD                 | NA           | 563 ± 344     |                     | –        | 411 ± 244    | 647 ± 431    | 640 ± 313    | $F = 3.11$ (2, 67)  | 0.03     |

HV, healthy volunteer; LEDD, levodopa-equivalent daily dose; NA, not applicable; PD, Parkinson's disease; UPDRS-III, Unified Parkinson's Disease Rating Scale, Part III. PD patients were subdivided into three disease severity groups using UPDRS-III tertile calculations. Values represent the mean ± SD. [1] and [2] indicate degrees of freedom obtained by Chi Square analysis.

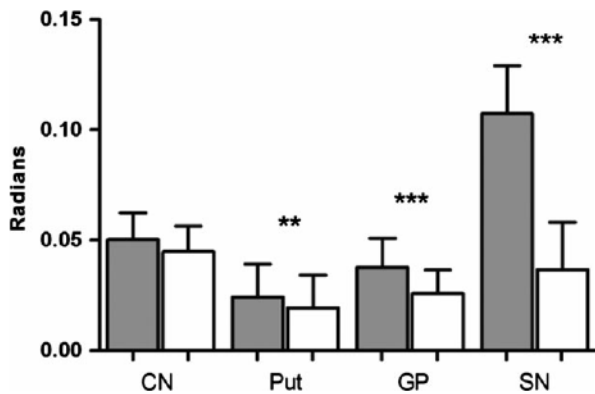


Figure 2 Comparison of radians between PD (grey bars) and HVs (white bars). CN, caudate nucleus; Put, putamen; GP, globus pallidus; SN, substantia nigra. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ .

ANCOVA including HV and PD subgroups indicated main effects of motor severity in the putamen ( $F = 6.346$ ,  $P < 0.001$ ), globus pallidus ( $F = 7.998$ ,  $P < 0.001$ ) and SN ( $F = 65.008$ ,  $P < 0.001$ ; Table 2). *Post hoc* Bonferroni-adjusted pairwise comparisons showed that the radians in all three PD subgroups were significantly higher than HVs in the globus pallidus ( $P = 0.025$ ,  $P < 0.001$  and  $P = 0.001$  respectively) and SN ( $P < 0.001$ ,  $P < 0.001$  and  $P < 0.001$  respectively; Table 2). Putaminal radians were significantly higher in PD subgroups 1 and 2 ( $P = 0.006$ ,  $P < 0.001$ ) compared to HVs. Pairwise comparisons between the PD subgroups revealed significance only in the SN, i.e. PD group 1 showed significantly lower radians compared to PD groups 2 ( $P = 0.049$ ) and 3 ( $P = 0.001$ ). Although PD group 3 showed the most radians in the SN, no statistically significant difference was found compared with PD group 2 ( $P = 0.236$ ; Fig. 3).

#### Analysis of clinical laterality

The SN contralateral to the clinically most affected side showed more radians ( $P < 0.001$ ) compared to the least affected side. No other significant results were found.

#### Correlations with disease duration and motor severity

Pearson's correlation analysis revealed significant positive correlations between SN radians and total UPDRS-III ( $r = 0.420$ ,  $P < 0.001$ ) and bradykinesia–rigidity subscores ( $r = 0.407$ ,  $P = 0.001$ ) but not with tremor ( $r = 0.219$ ,  $P = 0.071$ ; Fig. 4). Correlations remained significant after Benjamini–Hochberg FDR correction for multiple comparisons.

Within the HV group, SN radians positively correlated with age ( $r = 0.533$ ,  $P = 0.016$ ).

#### Validation of phase-shift data

To validate whether phase-shift data provide a quantitative measure of iron, Pearson's correlations were conducted between deep brain grey matter nuclei radians from the HV group and the distribution of non-haem iron at autopsy, as reported by Hallgren and Sourander [22]. A positive correlation was found after exclusion of the caudate nucleus due to spatial discordance with regard to regional iron measurements ( $r = 0.874$ ,  $P = 0.01$ ; Fig. S2).

## Discussion

This cross-sectional MRI study evaluated regional iron deposition in deep brain nuclei, using high-pass filtered phase images to quantify average phase shift (radians) in a large cohort of PD patients as well as age-matched HVs.

Higher levels of iron, as measured using radians, were found in the SN of PD patients relative to the HV group. Further, when PD groups were subdivided

Table 2 Analysis of covariance matrices adjusting for age and regional area

|                              |            | Mean $\pm$ SD      | Controls<br>( $N = 20$ ) | PD group 1<br>( $N = 23$ ) | PD group 2<br>( $N = 22$ ) | PD group 3<br>( $N = 25$ ) | ANCOVA                              |
|------------------------------|------------|--------------------|--------------------------|----------------------------|----------------------------|----------------------------|-------------------------------------|
| Putamen <sup>a</sup>         | Controls   | 0.130 $\pm$ 0.052  |                          |                            |                            |                            | $F = 6.346$ (3, 82)<br>$P = 0.001$  |
|                              | PD group 1 | 0.183 $\pm$ 0.035  |                          |                            |                            |                            |                                     |
|                              | PD group 2 | 0.195 $\pm$ 0.036  |                          |                            |                            |                            |                                     |
|                              | PD group 3 | 0.172 $\pm$ 0.054  |                          |                            |                            |                            |                                     |
| Globus pallidus <sup>b</sup> | Controls   | −1.621 $\pm$ 0.176 |                          |                            |                            |                            | $F = 7.998$ (3, 82)<br>$P < 0.001$  |
|                              | PD group 1 | −1.488 $\pm$ 0.153 |                          |                            |                            |                            |                                     |
|                              | PD group 2 | −1.413 $\pm$ 0.123 |                          |                            |                            |                            |                                     |
|                              | PD group 3 | −1.441 $\pm$ 0.143 |                          |                            |                            |                            |                                     |
| Substantia nigra             | Controls   | 0.037 $\pm$ 0.021  |                          |                            |                            |                            | $F = 65.008$ (3, 82)<br>$P < 0.001$ |
|                              | PD group 1 | 0.094 $\pm$ 0.017  |                          |                            |                            |                            |                                     |
|                              | PD group 2 | 0.111 $\pm$ 0.021  |                          |                            |                            |                            |                                     |
|                              | PD group 3 | 0.117 $\pm$ 0.023  |                          |                            |                            |                            |                                     |

Coloured boxes indicate the significance level of *post hoc* Bonferroni-corrected pairwise comparisons:  $P > 0.05$  (blue),  $P < 0.05$  (yellow),  $P < 0.01$  (orange),  $P < 0.001$  (red). <sup>a</sup>Square-root transformation; <sup>b</sup>Logarithmic transformation. [Colour table can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

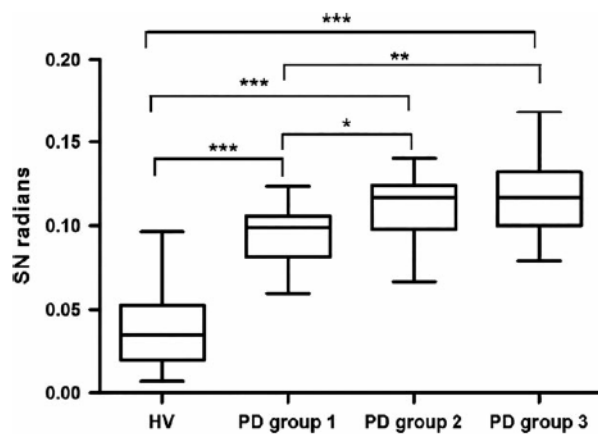


Figure 3 Box and whisker plot displaying ANCOVA results for the SN. PD group 1, UPDRS-III =  $19.3 \pm 3.11$ ; PD group 2, UPDRS-III =  $29.27 \pm 3.13$ ; PD group 3, UPDRS-III =  $44.60 \pm 6.42$ . *Ad hoc* Bonferroni-adjusted pairwise comparison significance levels indicated as follows: \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ .

according to UPDRS-III score, the SN showed a stratified distribution in which iron accumulation increased with motor severity. The SN is particularly vulnerable to abnormal increases in iron. Neuromelanin (NM), which under normal conditions acts as a neuroprotective iron chelator, is released into the extracellular space as a result of nigral cell death. Extracellular NM then releases ferric iron, which in turn promotes oxidative stress, microglial activation and further neuronal death [23]. Indeed, increased nigral iron has been demonstrated in imaging studies using both SWI and relaxometry techniques [8–18]. Recent longitudinal studies have also shown that changes in SN iron accumulation over time correlate with changes in motor severity [24,25]. Our design provides further evidence that this effect is not limited to increases within individuals but that significant changes can be seen between patients at different stages of disease. It is noted that, although there was an increase in iron from moderate to high PD groups,

this was not significant, suggesting that the rate of accumulation may decrease as the disease progresses. These findings cannot be explained by age differences, which are well known to correlate with nigral iron levels [26], nor between-patient nigral size, since both were added as covariates in our model.

Increased iron deposition was also found in the putamen and globus pallidus, although no difference was found in the caudate nucleus. Results relating to striatal mineralization have been highly variable, with several studies reporting increases [8,11,13,15], reductions [9,10] or normal levels [12,14,16] in PD. One possible explanation for this heterogeneity could be the presence of calcifications in the striatum, which may cause SWI and relaxometry techniques to underestimate iron levels. Recently, however, Ulla *et al.* [24] demonstrated in an early PD cohort that, whilst there was a significant increase in  $R2^*$  signal in the caudal putamen between baseline and 3-year follow-up compared to HVs, the rostral putamen remained at normal levels. Thus, the heterogeneity of results within the literature may be attributable to a rostro-caudal gradient of mineralization, which in turn may be dependent on disease duration. In addition, our data, although non-significant, show a trend for an initial increase of iron accumulation from low to moderate PD motor severity followed by a decrease from moderate to high motor severity in both the putamen and globus pallidus. Interestingly, this trend is in accordance with Ryvlin *et al.* [9] who suggested that mineralization in these regions inversely correlates with disease duration when the duration of illness is above 10 years. In contrast to the SN, it is clear that the mechanism behind striatal iron accumulation is not yet understood. Further longitudinal studies with patients followed up to the point of moderate to high motor severity are required to characterize the trajectory of striatal mineralization in PD more accurately.

Substantia nigra iron has previously been shown to correlate strongly with disease duration and motor severity [12–14,18]. Here, it is found that this

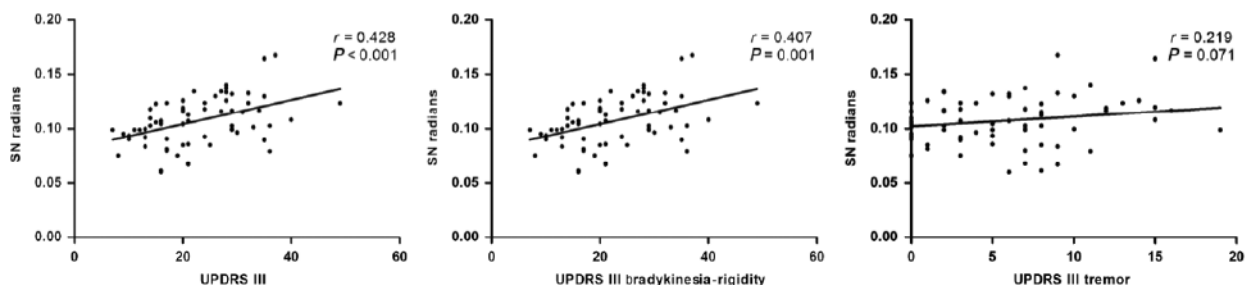


Figure 4 Pearson's correlations of SN radians with UPDRS-III, bradykinesia–rigidity and tremor scores.

relationship may be driven by bradykinesia/rigidity symptomatology, since although a strong relationship was found between these measures no association with tremor was identified. Bradykinesia and rigidity symptoms result as a direct consequence of dopaminergic nigral neurodegeneration [27], which is associated with regional iron accumulation [1]. In contrast, tremor not only reflects dopaminergic nigro-striatal degeneration but may also be associated with serotonergic and noradrenergic pathology [28]. Indeed, it was recently found that dopaminergic presynaptic terminal availability as well as storage capacity in the putamen and caudate nucleus were negatively associated with bradykinesia and rigidity scores, but not with tremor (Li W, Lao-Kaim NP, Roussakis AA, Martin-Bastida A, Loane C, Valle-Guzman N, Kefalopoulou Z, Politis M, Foltynie T, Barker RA, Piccini P, unpublished observations). Bunzeck *et al.* found a significant association between striatal iron accumulation and tremor symptomatology, suggesting that putaminal iron may be used as a predictor for tremor dominance in early PD [17]. However, their measures were collected in the 'on' medication state whilst our assessments were conducted in the 'off' medication state in order to avoid the confounding effect of variable clinical benefit with respect to tremor. Thus our data suggest that iron levels in the SN may be a feasible biomarker of bradykinesia and rigidity progression in PD.

The use of high-pass filtered phase imaging may be of therapeutic interest. Iron chelators that cross the blood–brain barrier, such as deferiprone, used mainly for peripheral haematological disorders, show a disease-modifying effect in animal models of PD [29]. So far, two randomized clinical trials with this drug have been conducted in PD patients, showing small reductions in motor severity after 6–12 months of treatment without major medication-related side effects [30] (Martin-Bastida A, Ward RJ, Newbould R, Piccini P, Sharp D, Kabba C, Patel MC, Spino M, Connelly J, Fernando T, Crichton RR, Dexter DT unpublished observations). However, MR relaxometry data were not consistent between these studies, detecting different degrees of regional chelation. Conventional relaxometry techniques ( $T2^*$  and  $R2^*$ ) estimate iron load *in vivo* using gradient echo sequences that are sensitive to variations in transverse relaxation times/rates between different tissues and that are affected by both macroscopic and microscopic magnetic field inhomogeneities [31]. However, images produced using these methods can be affected by changes in tissue water content and other background field inhomogeneities such as those resulting from air–tissue interfaces [4,31]. In contrast, SWI sequences, whilst also utilizing  $T2^*$  weighting, involve the collection of both magnitude and phase images

using a three-dimensional velocity-compensated gradient echo sequence. In particular, phase images contain information on the tissue magnetic susceptibility distribution. Since paramagnetic molecules such as ferritin and haemosiderin influence the local magnetic field and therefore the phase of the MR signal [31], phase offers greater specificity to quantifying brain iron load. Phase images are also more robust to the presence of noise [32], and the removal of low spatial frequency artefacts that could otherwise obscure inter-tissue phase differences (using a high-pass filter) [33] further improves its sensitivity to detecting abnormal iron levels. Thus, high-pass filtered phase images are a good alternative for monitoring the effects of iron chelators longitudinally.

There are some limitations with our study. First, the slice with the highest iron content was analysed in order to avoid background artefacts. Although no ROI size differences were found between PD and HVs, this method may not reflect the iron content of the entirety of each nucleus.

Secondly, although it is well documented that the pars compacta and pars reticulata present differential levels of mineralization, the SN was analysed without further segmentation. Postmortem studies including those immunostaining for calbindin  $D_{28K}$  [34] have provided valuable information on the definition of nigral compartments based on dopamine-cell-independent anatomical landmarks. However, translation to MRI segmentation is not straightforward owing to technical considerations (acquisition volume, slice orientation and thickness) and imperfect correspondence between anatomically defined nigral distribution and that observed on T2-weighted images [35,36]. Nevertheless, attempts have been made to manually separate the nigra albeit with significant methodological variation [12,24], highlighting disagreement on best localization procedures. More sophisticated techniques have recently been developed involving multimodal analysis of both SWI and NM-sensitive T1-weighted MRI (magnetization transfer) with which reduced NM pigmentation has been demonstrated in the SN pars compacta in PD with very high sensitivity and specificity [37,38]. However, the authors note a spatial overlap of  $12.6\% \pm 4.6\%$  between the two SN volumes further reflecting the difficulty of accurately segmenting the SN [39]. Given the inability of phase images to resolve intra-nigral boundaries, the SN was delineated as a whole in order to maintain the accuracy of our measurements, although it is acknowledged that this was at the cost of specificity and conclusiveness with regard to the level of mineralization for each sub-region and their relationship to PD pathology. Higher MR field strength and the use of quantitative susceptibility

mapping [40] which can further reduce phase image artefacts may provide greater spatial validity with respect to iron and improve the accuracy with which nigral compartments can be interrogated.

In summary, our results demonstrate that the degree of iron accumulation in PD is stratified according to disease severity in the SN but not in striatal sub-regions and that nigral iron is associated with the severity of bradykinesia and rigidity rather than tremor. Phase imaging techniques could be an important tool for monitoring clinical progression and establishing the efficacy and neuroprotective effects of iron chelation therapies in PD patients of variable clinical phenotype.

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### Disclosure of conflicts of interest

The authors declare no financial or other conflicts of interest.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Axial slices of two different subjects comparing SN delineation between the main (slice thickness 1.9, no. of slices 72) and modified (slice thickness 0.9, no. of slices 88) SWI protocols.

**Figure S2.** Pearson's correlations between phase-shift radians of deep brain grey matter nuclei from the healthy volunteer group ( $n = 20$ ) with the distribution of non-haem iron at autopsy (mg iron/100 g fresh tissue), as reported by Hallgren and Sourander (1958).

**Table S1.** Study volunteer recruitment from TransEuro and PaMIR studies.

**Table S2.** Independent sample  $t$  tests comparing SN radians between the main SWI protocol 1 (slice thickness 1.9, no. of slices 72) and the modified SWI protocol 2 (slice thickness 0.9, no. of slices 88) for the HV and PD groups. Values represent the mean  $\pm$  SD.

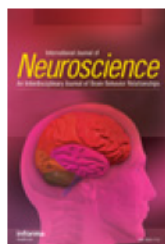
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REVIEW ARTICLE



## Neuromelanin in parkinsonian disorders: an update

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### ABSTRACT

Neuromelanin (NM) is a dark pigment that accumulates linearly with aging in substantia nigra (SN) and locus coeruleus (LC). The dual protective and toxic role of NM has been hypothesized according to its intraneuronal or extraneuronal deposition. The melanized dopaminergic neurons in SN and LC seem to have special vulnerability to neurodegeneration in Parkinson's disease (PD). The paramagnetic properties of NM due to its association to metals like iron induce T1 prolongation; hence the measurement of SN-sensitive contrast could be a useful diagnostic biomarker in neurodegenerative disease like PD and other atypical parkinsonisms. This paper will review NM histopathology and neurochemistry studies in health and diseases and the role of imaging targeting NM load in parkinsonian disorders.

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### Introduction

Neuromelanin (NM) is a complex, dark and insoluble pigment present in several neurons, specifically concentrated in the dopaminergic neurons of substantia nigra (SN), ventral tegmental area, the noradrenergic neurons of locus coeruleus (LC) and in the ventrolateral reticular formation and nucleus of the solitary tract in the medulla oblongata [1,2]. Furthermore, it was clearly demonstrated with electron microscopy and neurochemical investigations that NM could be also found in the putamen, premotor cortex and cerebellum [3,4].

NM is dark brown to black under visible light pigment and it is similar to other human melanin. At electron microscopy level, NM is distributed on irregularly shaped granules of varying electron density and size (0.5–2.5 nm). This pigment is located at the opposing end of the nucleus in the neuronal soma surrounded by a double membrane. The NM-pigmented neurons of SN and LC appear as macroscopically dark areas [5].

The exact structure of NM is still unknown. However, it seems to have a 3D structure composed of melanin groups bound to dolichoic chains of beta-sheet peptides [3,4,6]. In the melanic component, there are dihydroxyindole and benzothiazine rings and dopamine (DA)- and cysteinildopamine-derived units of proteins that are involved in the melanin synthesis [7]. These components are sequestered in autophagic organelles and processed to form the final NM [7].

It is still not clear whether NM synthesis is due to the autoxidation process or it is enzymatically controlled. NM derives from the metabolism of catecholamines, DA in the SN and norepinephrine (NE) in the LC. NM synthesis appears to be activated by an excess of cytosolic catecholamines that are not accumulated into synaptic vesicles by the synaptic vesicular monoamine transporter 2 (VMAT2). In addition, DA seems to be oxidized via iron-mediated oxidation catalysis that leads to reaction with beta-sheet proteins and formation of melanin-protein complex [8].

The initiation of pigmentation in the human brain starts at approximately three years of age and in adulthood, NM fills almost half of the cytoplasm [5,9]. NM continues to accumulate linearly in the SN during aging with a peak NM concentration between 50 and 90 years [1]. Fedorow and colleagues [10] suggested three phases in the production of NM consisting of the following: first, the initiation of production or synthesis which begins around the age of three; second, the growth with maturation, which implies a certain regulation of NM; and finally, sustained maturation without excessive volumetric growth.

### The role of neuromelanin in normal conditions

NM has a strong affinity for transition metals including iron, copper, lead and zinc [3,11,12]. Iron is sequestered by the melanic portion of NM and maintained in a redox

inactive form [13,14]; hence, NM could act as a “black hole” chelating redox-active metal.

NM binds iron in two different high- and low-affinity sites. Most of the iron is bound to the high-affinity sites, but under iron overload conditions, the high-affinity sites of NM are saturated, and iron binds to the low-affinity sites [3,15]. In the latter sites, iron is sequestered in a reactive form and could play a deleterious role by promoting redox reactions. Therefore, the binding of iron could have a protective role under physiological conditions, but a toxic gain of function may manifest when iron overload is present in the brain [1].

NM appears to have antioxidant properties contributing to regulate the cellular oxidative stress [1]. Furthermore, NM synthesis removes excess of catecholamines from the cytosol [9].

NM is able to bind a variety of organic molecules including lipids, pesticides and toxic compounds [3,11,12,16]. The herbicide paraquat, which has a similar structure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has a strong affinity with NM [17]. Antipsychotic drugs such as chlorpromazine and haloperidol, the antidepressant imipramine and the antimalarial chloroquine have a strong affinity for NM [18]. In addition, NM may also be associated with lipids like cholesterol and other high molecular polyunsaturated complex, as lipids are part of more than 20% of its structure [4,7].

### Neuromelanin in Parkinson's disease

In Parkinson's disease (PD), the brain's extracellular NM granules are preceded by nigral neurodegeneration; hence, NM released from the dying neurons is phagocytized

by the activated microglia. Microglia activation releases pro-inflammatory factors initiating an adaptive autoimmune response that leads to a vicious cycle of neurodegeneration and neuroinflammation [3,19–21]. Extracellular NM also releases metals and toxic compounds stored in normal conditions intraneuronally, potentially resulting in increased oxidative stress. NM has also been reported to inhibit the 26S proteasome [22], which may obstruct protein degradation causing accumulation of abnormal proteins such as alpha-synuclein aggregates (Figure 1).

Hirsch and colleagues [23], by using a quantitative analysis of NM-pigmented neurons and tyrosine hydroxylase (TH) immunohistochemistry, were among the first authors to demonstrate a link between the pigmentation of SN and the vulnerability of nigral neurons to neurodegeneration in parkinsonism. They found that healthy subjects showed a dense TH neuronal distribution in SN and tegmental extranigral A8 and A10, while PD cases showed a marked reduction of TH neurons in substantia nigra pars compacta (SNc) (77%) with moderate reduction of extranigral tegmental nucleus (A8 and A10: 43% and 48%, respectively) and a strong correlation with the number of NM-pigmented neurons. In progressive supranuclear palsy (PSP) cases, in addition to a marked reduction of TH cells in A10, there was a reduction of TH- and NM-pigmented cells in SN, but less marked than in the PD group. These findings suggest a direct correlation between PD cell loss and the percentage of NM-pigmented neurons, thus showing a selective vulnerability of pigmented subpopulation of DA-containing neurons.

Yamada and colleagues [24] studied the distribution of calbindin-D28K (CaBP), a calcium-binding protein, which may have a protective function in the pathological

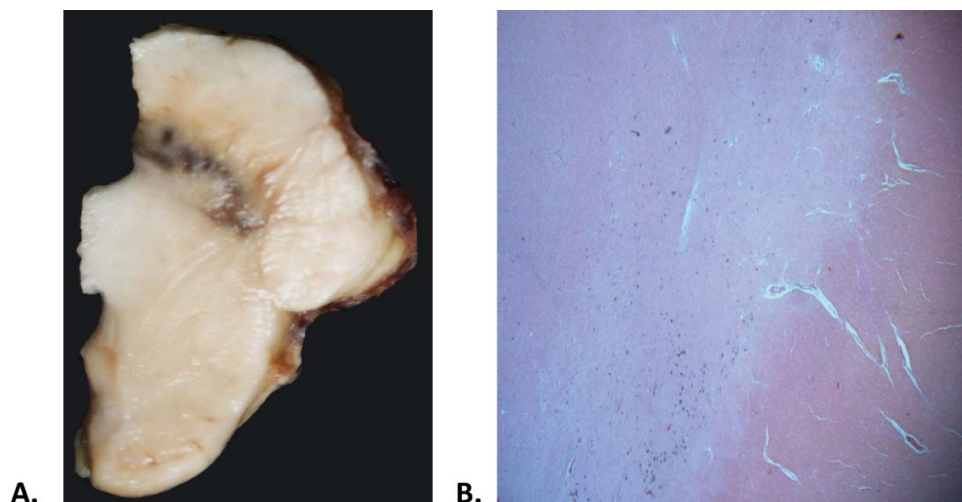


**Figure 1.** Macroscopic and microscopic appearances of midbrain in PD (A and B, respectively). (A) Transverse section of the midbrain shows pigment loss of SN. (B) Hematoxylin- and eosin-staining show neuronal loss, extraneuronal neuromelanin pigment and gliosis in the ventral-lateral tier of the SNc. Both images are courtesy of Parkinson's Disease Brain Bank, Imperial College London.

process of PD, in healthy volunteers, PD patients and one multiple system atrophy (MSA) case. The CaBP-positive SN regions were found preserved when compared with controls, but a substantial reduction of NM-pigmented neurons was found in the PD group and the MSA case (70% and 65%, respectively) with many extraneuronal deposits of NM due to neurodegeneration.

Gibb and Lees [25] examined 6 healthy controls, 6 PD cases and 13 subjects without PD, but with nigral Lewy bodies and cell loss to study cell distribution of the SNc. In healthy controls, the heavily and lightly pigmented neurons were located in the dorsal and the ventrolateral tiers, respectively. In PD and incidental cases, there was a complete loss of pigmented neurons in the ventrolateral SN group, which reflects greater DA depletion in the dorsal putamen compared to the caudate nucleus.

The micro-architecture of SN in PD, the parkinsonian variant of MSA (MSA-P) and PSP patients were studied by Fearnley and Lees [26]. In healthy controls, a linear decrease of NM-pigmented neurons in SNc was associated with age, showing a reduction of 4.7% per decade with regional selectivity in the dorsal and medial tiers. In the PD cases, there was an exponential decrease of NM cells with 45% of loss in the first decade of the disease and a significant regional selectivity in the lateral ventral tier, thus showing an opposite pattern to that found in aging processes. MSA cases showed similar neuronal loss distribution to PD, but the reduction in the dorsal tier was greater than in PD. Interestingly, PSP cases showed also a reduction in the medial tier of SN when compared with PD.

A morphometric approach of SNc was used by Rudow and colleagues [27] by measuring the total number and body volume of TH-positive nigral and pigmented neurons in young, middle-aged and older healthy control groups along with a PD cohort. Significant loss of pigmented and TH-positive neurons were found in middle-aged and older healthy control groups (28% and 36%, respectively), but in these cohorts, there was also present a significant cellular hypertrophy when compared to the younger controls. In contrast, the PD group showed up to 80% loss of pigmented cells and significant cellular atrophy when compared to all control groups. These findings suggest that cellular hypertrophy could represent a compensatory mechanism occurring with age in healthy controls; however, these mechanisms may be lost in PD.

Damier et al. [28] used compartmental patterns of calbindin immunostaining to subdivide the pattern of loss of DA-containing neurons in PD. The authors identified within the SNc, DA-containing neurons in the calbindin-rich regions named matrix and in five calbindin-poor neuronal subgroups which were labeled as “nigrosomes”

and identified as 1, 2, 3, 4 and 5 according to their caudo-rostral localization. The degree of neuronal loss was significantly higher in the nigrosomes than in the matrix. Depletion was maximum (98%) in the main subgroup (nigrosome 1), located in the caudal and mediolateral part of the SNc. Neuronal loss was also detectable in more medial and rostral nigrosomes, with nigrosome 1 > nigrosome 2 > nigrosome 4 > nigrosome 3 > nigrosome 5, therefore following a caudo-rostral gradient.

More recently, Kordower and colleagues [29] studied the extent of nigrostriatal degeneration in 28 PD cases with different disease duration. A progressive reduction of 50%–90% of TH dopaminergic cells in SN was found from the first year from diagnosis to the more advanced disease. In addition, 30%–60% variable NM-pigmented cell loss was observed, with greater NM /TH cell ratios at all the time points. This finding suggests that the loss of DA markers precedes the loss of pigmented cells implicating that there may be two different neuronal subpopulations in SNc with different neurodegenerative vulnerability.

### Imaging of neuromelanin in Parkinson's disease

Enochs and colleagues [30] were the first to quantitate the binding of metals to synthetic melanin *in vitro*, and to test whether such binding by natural melanin can be detected in cultured melanoma cells *in vivo* with MR imaging. They indeed proved that melanotic melanomas are hyperintense on T1-weighted (T1w) images because of paramagnetic metal scavenging.

Many of the recent imaging studies have used high-resolution T1w with fast spin echo (FSE) on 3-Tesla MR to visualize NM-sensitive contrast in the midbrain and pons. T1 prolongation with a strong magnetic field can suppress the signal of the surrounding brain tissue of the NM-pigmented nuclei. The contrast obtained in NM-sensitive MRI seems to be directly related to the reduction of T1 from the melanin compounds. In addition, magnetization transfer (MT) also helps to distinguish NM-rich areas from the surrounding brain tissue; however, it is not directly affected by the presence of NM (Figure 2) [31].

Several neuroimaging studies have demonstrated specific reduction of NM contrast using NM-sensitive T1w in SNc and LC (Table 1) [32–39].

The first approach to measuring the NM-sensitive T1w FSE contrast was performed by Sasaki and colleagues [32]. In this study, PD patients and age-matched healthy volunteers were scanned with T1w FSE, and then the imaging compared with macroscopic specimens at the level of the midbrain and upper pons from two cadavers, one with pathologically diagnosed

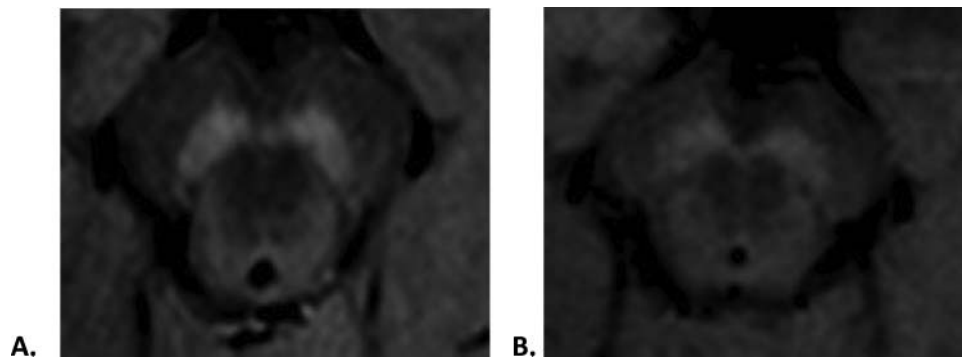


**Figure 2.** Neuromelanin-sensitive magnetic resonance imaging of SNc. (A) NM-sensitive MRI of the midbrain of a 50-year-old healthy control woman showing increased bilateral hyperintensity areas corresponding to SNc. (B) NM-sensitive MRI of a 53-year-old woman with five years history of PD showing bilateral decrease of contrast in SNc suggesting nigral dopaminergic neurodegeneration. Both images are taken from PaMIR (Parkinson's Disease Magnetic Imaging Repository) study, University of Nottingham and Imperial College London, funded by Parkinson's UK.

PD and one without any neurological disease. Measurement of high T1 signal intensity was performed in SNc and LC with circular regions of interest (ROIs) along with the measurement of the surrounding pontine tegmentum and decussation of the superior cerebellar peduncles. Contrast ratios of the LC/pontine tegmentum and SNc/superior cerebellar peduncles were significantly reduced in PD groups when compared to healthy volunteers. T1w FSE findings in the midbrain showed NM distribution in SNc in a band-like high signal similar to the postmortem specimen. In addition, radiological findings at the pons level of LC showed high signal “spots” due to NM deposition in LC, identical to the gross specimen.

Kashikara and colleagues [33] also used T1w FSE to study a cohort of PD patients and a group of healthy controls that had suffered from minor ischemic stroke. The PD subjects were subclassified according to the Hoehn and Yahr staging (HY), with 20 PD patients corresponding to each HY stage. The NM-sensitive areas of SNc were bilaterally manually traced and the volume was calculated by multiplying the area by slice thickness. The mean SNc volumes were significantly reduced in patients with PD compared to the controls. Furthermore, the volumes in PD patients were significantly decreased at each HY stage and correlated with PD disease duration.

More recently, Schwarz and colleagues [34] analyzed 10 PD patients classified as early stage ( $N = 6$ ) and late

**Table 1.** Overview of studies using NM-sensitive T1-weighted sequence in Parkinson's disease and other parkinsonisms.

|                           | MR sequence | Controls | PD                               | Parkinsonisms                       | SNc PD vs. controls                                                           | LC PD vs. controls |
|---------------------------|-------------|----------|----------------------------------|-------------------------------------|-------------------------------------------------------------------------------|--------------------|
| Sasaki et al. (2006)      | T1w FSE     | 22       | 17                               |                                     | Reduced                                                                       | Reduced            |
| Kashikara et al. (2011)   | T1w FSE     | 54       | 80                               |                                     | Reduced                                                                       | NA                 |
|                           |             |          |                                  |                                     | Correlation disease duration                                                  |                    |
| Kashikara et al. (2011)   | T1w FSE     | 54       | 80                               | 28 MSA<br>11 PSP<br>10 CBD<br>8 SCA | Reduced<br>No differences between PD and parkinsonisms                        | NA                 |
| Schwarz et al. (2011)     | T1w FSE, MT | 12       | 10                               |                                     | Reduced                                                                       | Similar            |
| Otsuka et al. (2013)      | T1w 3D FSE  | 22       | 37                               |                                     | Reduced in lateral/central SN<br>No differences early/advanced PD             | NA                 |
| Ohtsuka et al. (2014)     | T1w 3D FSE  | 22       | 37                               | 11 MSA<br>10 PSP                    | Reduced<br>lateral/central in PD and MSA<br>No differences between MSA and PD | Reduced<br>MSA     |
| Matsuura et al. (2014)    | T1w FSE     | 23       | 32                               | 9 MSA                               | Reduced in PD and MSA<br>No differences between MSA and PD                    | Reduced<br>MSA     |
| Reimão et al. (2015)      | T1w FSE     | 10       | 12 <i>de novo</i><br>10 early PD |                                     | Reduced<br>No differences between PD groups                                   | NA                 |
| Reimão et al. (2015)      | T1w FSE     | 10       | 12                               | 15 ET                               | Reduced<br>No differences with ET group                                       | NA                 |
| Castellanos et al. (2015) | T1w FSE     | 23       | 23 iPD<br>8 LRRK2<br>5 Parkin    |                                     | Reduced<br>No differences between PD groups                                   | Reduced            |

SNc, substantia nigra pars compacta; LC, locus coeruleus; PD, Parkinson's disease; MSA, multiple system atrophy; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; SCA, spinocerebellar ataxia; ET, essential tremor; iPD, idiopathic Parkinson's disease; LRRK2, leucine-rich repeat kinase 2, PARK 8 gene; Parkin, PARK 2 gene; T1w, T1-weighted; FSE, fast spin echo; MT, magnetization transfer.

stage ( $N = 4$ ) and 12 age-matched healthy volunteers. Interestingly, they added for the first time a spectral pre-saturation inversion-recovery pulse to the T1w FSE, which confers an MT effect and adds a better resolution contrast between the NM-rich areas and the surrounding areas. The size and signal intensity of SNc and LC were normalized to the surrounding background mesencephalic and pontine regions. The T1 hyperintense area in the SN was significantly smaller in patients compared with controls ( $-60\%$ ). In addition, size reduction was more pronounced in more advanced disease ( $-78\%$ ) than in early-stage disease ( $-47\%$ ). In spite of the small PD sample, they suggest that reduction in the size of NM-rich SN correlates well with the postmortem observations of dopaminergic neuron loss, and that this new optimized NM-sensitive T1 scan technique could be used to assess SN signal reduction in PD as a putative marker of NM loss.

Regional differences of NM contrast within the SNc according to the rostral-caudal gradient of neuronal loss in PD were studied by Ohtsuka and colleagues [35]. PD patients were subclassified in the early and late stages (1.5 and 12 years of disease duration) in addition to the healthy controls. Three subdivisions were traced along the SNc (lateral, central and medial). Significant reduction in the lateral and central SNc contrast ratios were found in the PD patients group when compared to the healthy controls. These results support the neuropathological finding that neurodegeneration in SNc in PD progresses from lateral to central to medial regions.

Ogisu and colleagues [36] used a novel 3D turbo field-echo (TFE) sequence with off-resonance MT pulse for NM-sensitive MRI. The SNc volume was measured semi-automatically by applying a region-growing technique at various thresholds measuring the signals with the superior cerebellar peduncle. The average SNc volume in the PD group was significantly smaller than that in the control group at all the thresholds. At higher thresholds, the area under the curve and likelihood ratios, based on receiver operative characteristic (ROC) analysis, increased with balanced sensitivity and specificity. However, at lower thresholds, the sensitivity tended to increase, but the specificity decreased. The authors claimed that their method could help to distinguish PD from controls with high sensitivity and specificity, particularly at an early stage of PD, therefore, helping in the early diagnosis of PD.

In two recent studies, Reimão and colleagues [37,38] performed a semi-automated MRI analysis of T1w in SNc. In the first study [37], 12 *de novo* PD patients and 10 treated PD with 2–5-year disease duration were analyzed along with healthy controls. SNc-related hyperintensity and SN NM /midbrain ratio were significantly reduced in

both PD groups when compared to healthy controls, with a substantial overlap between the two PD groups. They suggested that PD NM changes remain stable during the course of the disease. However, given the rather limited number of patients and range of disease severity, it seems difficult to sustain this conclusion. Their second study [38] aimed to evaluate the diagnostic accuracy of NM-contrast MR technique in the differential diagnosis of PD and essential tremor (ET). They found that T1 high signal in the SN region was markedly decreased in the PD group compared to the ET and age-matched subjects, with the ventrolateral segment most affected. No significant differences were found in the ET group when compared with the healthy controls. As a high specificity in discriminating ET from early-stage PD was found (93%), the authors suggest that this technique could be useful for differential diagnosis.

Castellanos et al. [39] analyzed the diagnostic accuracy of automated segmentation and quantification method of the SNc and LC volumes based on the NM-sensitive MRI in 23 patients with idiopathic PD (iPD) and 13 with monogenic PD genotyped with Parkin (PARK2) and LRRK2 (leucine-rich repeat kinase 2, PARK8) mutations. Significant differences between ipsilateral and contralateral SNc volumes, in relation to the more clinically affected side, were found in iPD. In addition, contralateral atrophy in the SNc showed the highest power to discriminate PD subjects from controls. The authors suggested that this automatic technique could be an unbiased PD biomarker.

The relationship between NM distribution and iron accumulation in SN is yet to be ascertained. Reimão et al. [40] studied 12 *de novo* PD patients, 10 early-stage PD and 10 control subjects with NM-sensitive T1w and T2\* sequence to study the regional correlation between NM and iron accumulation. Marked reduction in NM load was found along the SN in both PD subgroups with no significant differences in the nigral mineralization when assessed with T2\*. The authors confirm the lack of correlation between iron and NM in SNc, suggesting that both the pathophysiological pathways present in neurodegeneration might not be parallel in PD.

Nigral NM load might be useful to explore the nigrostriatal pathway integrity in PD in combination with striatal DA transporter (DAT) single-photon emission computed tomography (SPECT) imaging. Recently, Isaias et al. [41] assessed the relationship between NM load and iron accumulation in SN with dopaminergic striatal markers in equally sized PD and healthy volunteers groups. Nigral contrast ratios and NM volumes positively correlated with striatal DAT uptake values; however, no other associations with nigral-iron accumulation were demonstrated. These results suggest that the NM-

sensitive T1w could be potentially used in combination with positron emission imaging to further study nigrostriatal pathway abnormalities in PD.

Premotor symptoms like rapid eye movement (REM) sleep behavior disorder (RBD) are considered as the highest PD risk as well as hyposmia. It has been hypothesized that the LC/subcoeruleus (LCS) is directly implicated in muscle tone during REM sleep in animal models. Garcia-Lorenzo et al. [42] studied a large cohort of PD with and without RBD using video-polysomnography and multimodal MRI including the NM-sensitive T1w. Significant reduction of NM signal in the LCS was found in PD with RBD group. In addition, the percentage of abnormally increased muscle tone in REM sleep negatively correlated with the reduction of LCS NM signal. The authors suggest that this technique could be used to identify prodromal premotor symptoms of PD in longitudinal studies.

### Imaging of neuromelanin in parkinsonian syndromes

Differential diagnosis at early stages among PD and other hypokinetic parkinsonian syndromes like MSA, PSP and corticobasal degeneration (CBD) is usually difficult, due to the similarity of neurological symptoms, hence the need of a new early-stage radiological biomarker. To date, only a few NM-sensitive T1-contrast studies have attempted to demonstrate the validity of this approach for differential clinical diagnosis of parkinsonisms (Table 1).

Kashikara and colleagues [43] analyzed a large cohort of 80 PD volunteers subclassified according to HY staging, 28 MSA patients, 11 PSP patients, 10 CBD volunteers, 9 patients with sporadic cerebrosplinal ataxias (SCA) and 54 healthy volunteers who suffered from minor acute ischemic stroke. The SNc volumes were significantly reduced in early PD, MSA, PSP and CBD when compared to healthy volunteers and SCA group. Nigral volume loss was more marked in PD and PSP patients followed by MSA and CBD cases, although differences were not statistically significant within the parkinsonian syndromes group.

Ohtsuka [44] studied the signal attenuation in both the SNc and LC in a cohort of 30 patients with early PD, 10 MSA-P patients, 13 PSP and 22 healthy controls. Marked reduction in SNc and LC contrast ratios were found in PD and MSA groups when compared with the healthy controls. In addition, the PSP group showed no differences when compared with the PD and MSA groups. Interestingly, the MSA-P seems to have similar topographic distribution of NM-sensitive reduction within the SNc as PD.

Matsuura analyzed the SNc and LC contrast ratios of 9 MSA patients (5 MSA parkinsonian variant and 4 MSA cerebellar variant) and 32 PD patients [45]. They found a marked reduction of LC and SNc contrast ratios in both MSA and PD when compared to healthy volunteers. Furthermore, the MSA group showed the most prominent reduction in LC contrast ratios than the PD group. The authors claimed that these results were attributed to the fact that the cerebellar subtype of MSA was included in the study.

Mitochondrial neurological cytopathies like progressive external ophthalmoplegia (PEO) and parkinsonism due to mutations in POLG-1 can be assessed with NM-sensitive T1w sequence. Mukai et al. [46] studied two familiar PEO and parkinsonism patients in comparison to iPD patients. The NM-related signal intensity of SNc was significantly reduced in the PD, PEO and parkinsonism groups when compared with healthy controls. Nevertheless, no significant LC involvement was found in the PEO and parkinsonism patients confirming topographical differences in neuronal depletion due to mitochondrial mutations. The clinical features of parkinsonism related to POLG-1 mutation were indistinguishable from iPD; however, the NM MRI showed a distinct pattern in this subject compared to the iPD. The NM imaging results were consistent with the neuropathological findings reported in cases of POLG-1 mutations, in which neurons of the SN were profoundly affected while those in the LC were preserved. The authors concluded that the 3-T NM MRI may be useful for differentiating POLG-1 mutation-associated parkinsonism from iPD, and those POLG-1 mutations may cause selective neuronal loss in the SN via a mechanism different from that of iPD.

### Conclusions

NM plays a toxic role in neurodegenerative conditions like PD. Extracellular NM deposits release toxic metals inducing oxidative stress, microglial activation and chronic local neuroinflammation. The NM-pigmented neurons of the SNc are particularly vulnerable to neurodegeneration in PD where the neuronal loss is earliest and greatest in the ventral-lateral tier of the SN, resulting in the well-documented rostro-caudal gradient of reduction of nigrostriatal projections.

T1w FSE sequence is a feasible technique for visualizing NM-containing regions within the SN and LC. Manual and semi-automated analysis of contrast ratios and SNc areas in PD have consistently demonstrated significant reduction when compared to the healthy controls. Manual analysis of SNc has also been able to show the topographical caudal-rostral gradient of SN depigmentation consistent with the neuropathological studies. In spite of

the promising results of both analyses, manual approaches seem to assess more accurately the nature of NM depigmentation in SNc than the semi-automated analysis.

In conclusion, NM imaging in SNc is a relatively simple, non-invasive technique that allows distinguishing PD and healthy controls with high accuracy, and it could therefore be considered as a potential diagnostic biomarker. A limitation so far is that almost all the studies have included only a limited number of subjects. Larger cross-sectional and longitudinal studies are necessary to confirm the value of this technique as a diagnostic biomarker, and also to explore whether the SN depigmentation is associated with the PD severity and the progression of the disease.

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## Disclosure statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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# SCIENTIFIC REPORTS

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## Brain iron chelation by deferiprone in a phase 2 randomised double-blinded placebo controlled clinical trial in Parkinson's disease

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Parkinson's disease (PD) is associated with increased iron levels in the substantia nigra (SNc). This study evaluated whether the iron chelator, deferiprone, is well tolerated, able to chelate iron from various brain regions and improve PD symptomology. In a randomised double-blind, placebo controlled trial, 22 early onset PD patients, were administered deferiprone, 10 or 15 mg/kg BID or placebo, for 6 months. Patients were evaluated for PD severity, cognitive function, depression rating and quality of life. Iron concentrations were assessed in the substantia nigra (SNc), dentate and caudate nucleus, red nucleus, putamen and globus pallidus by T2\* MRI at baseline and after 3 and 6 months of treatment. Deferiprone therapy was well tolerated and was associated with a reduced dentate and caudate nucleus iron content compared to placebo. Reductions in iron content of the SNc occurred in only 3 patients, with no changes being detected in the putamen or globus pallidus. Although 30 mg/kg deferiprone treated patients showed a trend for improvement in motor-UPDRS scores and quality of life, this did not reach significance. Cognitive function and mood were not adversely affected by deferiprone therapy. Such data supports more extensive clinical trials into the potential benefits of iron chelation in PD.

Parkinson's disease (PD) is the most common motor disorder, affecting approximately 1–2% of the population. Clinical diagnosis of PD is based on the presence of unilateral onset of bradykinesia, rigidity, resting tremor and postural instability. The main neuropathological hallmarks of PD are the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), the presence of intracellular Lewy bodies, deposition of  $\alpha$ -synuclein<sup>1</sup> and high iron deposition in the SNc<sup>2,3</sup>. The latter has been confirmed from post mortem studies, magnetic resonance imaging (MRI), and transcranial ultrasound studies<sup>4–6</sup>. Additionally, electron probe x-ray microanalysis has confirmed that individual SNc dopaminergic neurons in PD have raised iron levels<sup>7</sup> as well as an increased labile iron pool<sup>8</sup>. Such low molecular weight iron can cause oxidative damage to proteins, lipids and DNA by generating reactive oxygen species (ROS)<sup>9</sup>, processes for which there is evidence in the PD brain<sup>9–11</sup>. ROS activity will be exacerbated by decreased anti-oxidant levels, e.g. reduced glutathione, in the PD brain<sup>12</sup> while  $\alpha$ -synuclein aggregation is promoted by the excess iron<sup>13,14</sup>. Therefore the removal of such iron from the SN may be beneficial in retarding the progression of PD.

Animal studies have shown that iron chelators are able to cross the blood brain barrier and are able to remove excess iron from various brain regions in models of brain iron overload<sup>15–17</sup>. In early studies, we demonstrated that iron chelators, desferrioxamine, deferiasirox and deferiprone, currently in clinical use for treating iron overload in thalassemia major, significantly attenuated SNc dopaminergic neuronal and striatal dopamine loss in the 6-hydroxydopamine PD model, whilst reducing hydroxyl radical formation<sup>17</sup>. The potential benefit of using an

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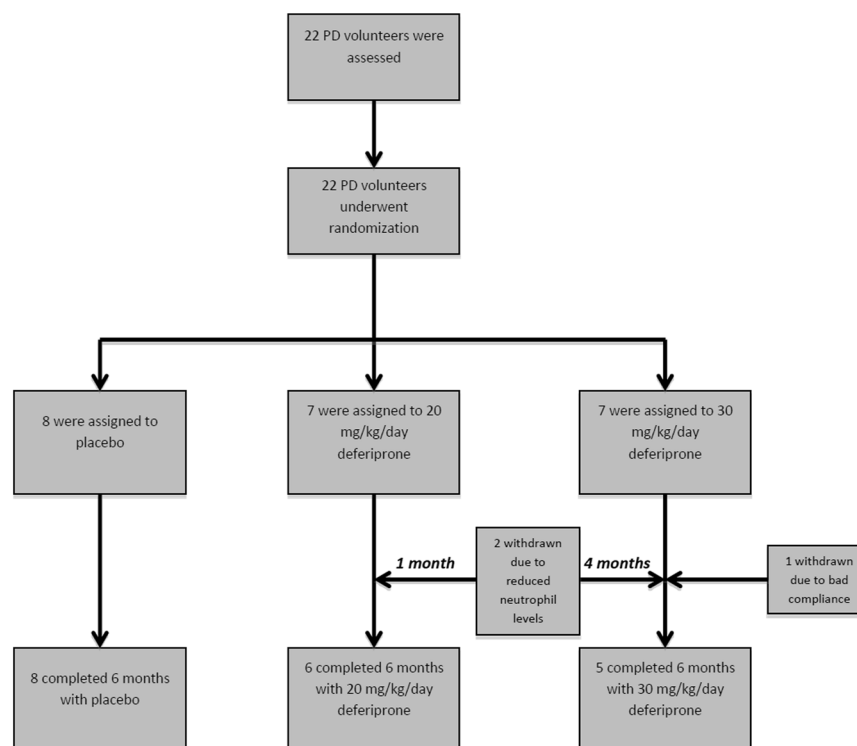


**Figure 1.** Flow diagram showing the random assignments of the two doses of deferiprone, 20 or 30 mg/kg and placebo to the 22 Parkinson's disease patients.

iron chelator in a neurodegenerative condition, in the absence of systemic iron overload, was exemplified in a clinical trial utilizing deferiprone in Friedreich Ataxia (FA), where excessive concentrations of iron are present in mitochondria, caused by mutations in the frataxin gene. Cardiomyopathy, as well as neurodegeneration in the dorsal root ganglia and the dentate nucleus are prominent features in Friedreich Ataxia, and may be related to the dysregulation of iron and the resultant excess in regionalized intracellular labile iron. Deferiprone treatment, with 20 to 30 mg/kg/day, divided into 2 daily oral doses, for up to 6 months, in 9 FA patients, reduced dentate nucleus iron content, as assessed by MRI, whilst concomitantly reducing neuropathy and ataxic gait in some patients<sup>18</sup>.

Since iron has been implicated in the pathology of PD, its removal by iron chelators, administered at low doses, might be of therapeutic benefit in PD. The main objectives of our double-blinded, randomized, placebo-controlled, pilot clinical trial with deferiprone in PD were firstly to evaluate changes in brain iron content by T2\* MRI, secondly to assess drug safety, and thirdly to assess subjects' clinical status as characterised by PD disability utilising the Movement Disorders Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS), mood, cognitive function and quality of life. Lastly, markers of iron homeostasis and peripheral inflammation were investigated to ascertain whether inflammation might be a contributing factor in the iron accumulation in the brain and also may hinder removal of iron from the brain. Additionally, pro-inflammatory cytokines, particularly IL-6, have been shown to play a pivotal role in peripheral and CNS iron metabolism since it stimulates the release of hepcidin, the master regulator of iron metabolism, for the liver and chorio plexus leading to decreased cellular iron export and macrophage iron retention<sup>19, 20</sup>.

## Patients and Methods

We prospectively studied 22 patients (12 males and 10 females; aged 50–75 years) with early stage PD, disease duration of less than 5 years (Queen Square Brain Bank criteria at Hoehn and Yahr stage 1 to 2 in the “on-medication” state) who were receiving stable PD medication regimes. Participants were excluded if they had another suspected cause for their parkinsonism, other neurological or psychiatric disorders such as history of major depression, insulin-dependent diabetes mellitus, renal or liver disease, or were immunocompromised as revealed by neutropenia, HIV, etc. Written informed consent was obtained from each patient on their initial screening visit before enrolment. The protocol was approved by Imperial College London (Reference crol1715; Date 11/05/11), the Oxford Institutional Ethical Committee (REC reference 11/SC/0101; Date 26/05/2011), MHRA (EudraCT 2011-001148-31; Date 29/12/2011) and registered at the National Health Authority. The clinical trial was registered on the publicly accessible database [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01539837; Date: 22/02/12). Methods were carried out in “accordance” with the approved guidelines.

The PD patients,  $n = 22$ , were recruited by AM (between 18/04/2012 – 27/03/2013) and randomly selected to receive placebo or 20 or 30 mg/kg/day deferiprone (80 mg/ml deferiprone solution or excipient matched placebo provided by ApoPharma Inc., Toronto, ON, Canada) which was divided into 2 daily oral doses, morning and evening, and administered for 6 months (Fig. 1). Randomisation was carried out by the Hammersmith Hospital

|                             | Placebo          | 20 mg/kg/day<br>Deferiprone | 30 mg/kg/day<br>Deferiprone | Stats            | p value |
|-----------------------------|------------------|-----------------------------|-----------------------------|------------------|---------|
| Number                      | 8                | 7                           | 7                           |                  |         |
| Gender (m:f)                | 3:5              | 4:3                         | 5:2                         | $\chi^2 = 1.721$ | 0.414   |
| Age (years)                 | $64.38 \pm 3.23$ | $68.57 \pm 2.17$            | $62.85 \pm 2.74$            | $F = 1.008$      | 0.384   |
| Disease duration<br>(years) | $3.54 \pm 0.34$  | $2.82 \pm 0.69$             | $3.02 \pm 2.69$             | $F = 0.675$      | 0.521   |
| LEDD                        | $284 \pm 55$     | $395 \pm 57$                | $432 \pm 118$               | $F = 0.964$      | 0.399   |
| UPDRS III                   | $11.38 \pm 1.29$ | $11.43 \pm 1.31$            | $10.57 \pm 1.34$            | $F = 0.129$      | 0.879   |
| MADRS                       | $8.25 \pm 1.78$  | $7.14 \pm 1.49$             | $5.14 \pm 1.37$             | $F = 0.995$      | 0.388   |
| MMSE                        | $29.25 \pm 0.49$ | $29.42 \pm 0.29$            | $28.57 \pm 0.61$            | $F = 0.833$      | 0.450   |
| PDQ 39                      | $23 \pm 4.03$    | $31.42 \pm 10.35$           | $24.28 \pm 6.29$            | $F = 0.381$      | 0.689   |

**Table 1.** Demographic data of Parkinson's disease patients. Values are represented as mean  $\pm$  SEM.

Pharmacy and the randomization scheme was generated using the web site [Randomization.com](http://www.randomization.com) (<http://www.randomization.com>). The code for the randomisation was only disclosed at the conclusion of the trial or if the patient experienced unexpected adverse side effects. Patients, care providers and those assessing clinical outcomes were blinded to the intervention given. Of the original group of 22 patients, 19 completed the 6-month course of deferiprone, with three brain MRI scans, at 0, 3 and 6 months, repeated neurological examinations, as well as a variety of blood investigations. At this stage the trial was concluded. The analysis of the study was performed on the subjects that remained in.

The primary outcome of this double-blinded placebo-controlled study is to assess whether deferiprone is able to modify regional brain mineralization as assessed with T2\* MR in PD patients. The secondary outcome is based in the drug safety of deferiprone in PD.

**Neurological measures.** All PD patients were clinically assessed at the Hammersmith Hospital Campus, Imperial College London at baseline, 2 months, 4 months and 6 months for PD severity using MDS-UPDRS part 3 whilst on-medication. Anti-Parkinsonian medication dosages were maintained at a constant level for each individual throughout the study. Calculation of levodopa equivalent dosage (LED) was performed using published formulae<sup>21</sup>, Table 1. In addition, each subject was assessed by the Minimental State of Folstein (cognitive function), the Montgomery Asberg DRS (depression rating) and the PDQ39 (quality of life) questionnaires, Table 1.

**Blood and urine analysis.** Blood specimens were provided by each PD patient weekly for haematological assessment to ensure that there were no adverse effects, particularly neutropenia. Agranulocytosis occurs in 1–2% of beta-thalassemia patients, who receive much higher doses of deferiprone, 75–100 mg/kg/day. Biochemical assessments were made on a monthly basis to assess various markers of iron metabolism, immunoglobulin status, hepatic and renal function, as well as levels of deferiprone and deferiprone 3-O-glucuronide to ensure patient compliance. Spot urine samples were collected weekly to assess iron excretion.

Inductively coupled plasma mass spectrometry (Thermo XII, Massachusetts, USA), was used for the analysis of urinary iron. Urine samples were diluted in 1% nitric acid to which the internal standard <sup>69</sup>gallium had been added. A calibration curve was prepared in a similar fashion and internal quality controls were included in all analytical runs. Creatinine was analysed by a standard biochemical method. The results were expressed as nmol Fe/mmol creatinine.

**Quantification of deferiprone and deferiprone glucuronide.** Deferiprone and its main metabolite, deferiprone glucuronide, were assessed in the plasma to monitor patient compliance. Perchloric acid (200  $\mu$ L, 0.4 M) was added to plasma samples (200  $\mu$ L) to precipitate plasma proteins. After brief vortexing, followed by 10 min incubation on ice, the samples were centrifuged (35,000  $\times$  g) for 15 min at 4 °C. The supernatant (10  $\mu$ L) was injected onto the HPLC column and analysed by UV detection.

**Magnetic Resonance Imaging, MRI.** MRI measurements were acquired at 0, 3 and 6 months using a 3T Siemens Verio (Siemens Healthcare, Erlangen, Germany) equipped with a 32-channel phased array head coil. A 3D T1-weighted spoiled gradient echo volume of isotropic 1  $\times$  1  $\times$  1 mm resolution was acquired at six echo times: 6.29, 13, 21, 29, 37, and 49 ms in each TR of 56 ms. A 240  $\times$  256  $\times$  160 mm sagittal FOV and flip angle of 35° was used, with a parallel imaging factor of 2, requiring 10 m:14 s. A double-echo inversion recovery sequence (MP2RAGE)<sup>22</sup> was also acquired with identical resolution and coverage at inversion times of 409 ms and 1100 ms in a 5 s TR with a parallel imaging factor of 2, requiring 10 m:57 s. This sequence gives two co-registered volumes: one with fluid nulled similar to the ADNI MPRAGE<sup>23</sup> and one with white matter nulled for subcortical visualization. The signal decay in each voxel across the six echo acquisition was fit to the non-linear signal equation:

$$S(TE) = S_0 \cdot e^{-\left(\frac{TE}{T2^*}\right)} + C$$

where  $S_0$  represents the effective proton density, including partial T1 recovery, and the  $C$  term captures a rectified noise floor from magnitude imaging with possible low SNR in the final echo volume. The first echo volume of the six echo acquisition was co-registered to the fluid-nulled volume from the first imaging visit using the FMRIB

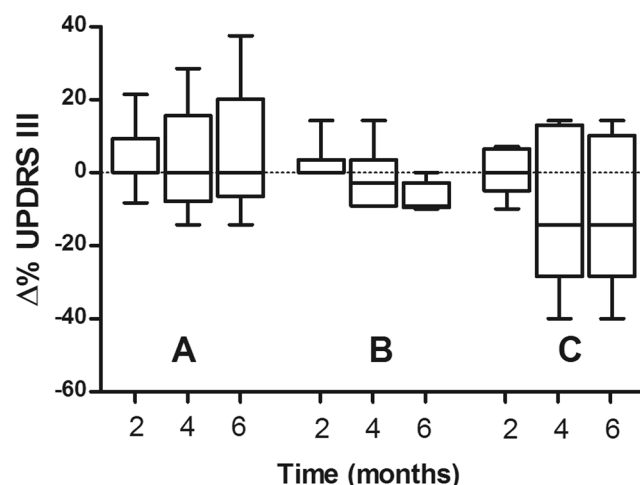


**Figure 2.** Percentage change in the median UPDRS III motor scale of PD patients receiving placebo (A,  $n = 8$ ), 20 (B,  $n = 6$ ) or 30 (C,  $n = 5$ ) mg/kg/deferiprone for 2, 4 and 6 months of treatment. Values are presented in box plots with whiskers representing the minimum and maximum values.

software library (FSL) FLIRT tool (FSL version 4.1.9 <http://fsl.fmrib.ox.ac.uk/fsl>). The resulting linear transformation matrix was then applied to the T2\* volume for that visit. The first echo volume was also used for automatic segmentation using the FSL FIRST tool (FSL version 4.1.9 <http://fsl.fmrib.ox.ac.uk/fsl>) for automated delineation of the caudate, putamen, and globus pallidus.

All six echoes were automatically summed by the custom sequence software to result in a high SNR<sup>24</sup>. This volume was used for the manual delineation of the substantia nigra (SN), red nucleus (RN), and the dentate nucleus (DN).

**Statistical Analysis.** All statistical analyses were performed using SPSS statistical software (version 21 SPSS Inc., Chicago, Illinois) and SAS 9.3.

Demographic data were compared between PD and HV groups using independent t-tests.

The statistical methodology used in the analysis was Mixed Models applied to longitudinal data. This method takes into account the correlation between the time points (screening visit, third and sixth months) within the different brain regions of interest. Estimated values from T2\* values means were analysed using Pairwise comparisons with post-hoc Bonferroni correction using T2\* as dependent variable. The threshold for statistical significance was set to  $p < 0.05$ .

Correlation analysis between IL-6, TNF-alpha and ferritin with T2\* regional values were performed with non parametric Spearman tests with post hoc Benjamini-Hochberg corrections for multiple correlations.

## Results

**Demographics.** Demographics of enrolled patients are summarized in Table 1.

**Clinical Parameters.** *Side effects of deferiprone therapy.* Deferiprone therapy was essentially well tolerated by PD subjects with only minor side effects being reported. Seven PD subjects reported an exacerbation of pre-existing muscular/joint pain, while another 3 PD patients experienced mild gastrointestinal upset during the first months of the study, all of which resolved without any clinical intervention. In one PD patient we detected an increase of liver enzymes after 4 weeks of deferiprone administration which returned to normal values after a 7-day break from deferiprone therapy. In two PD subjects administered deferiprone, a reduction in neutrophil cell numbers was observed warranting drug withdrawal. In the first subject the neutrophil decline was very rapid and severe, with zero cells being detected. In the second subject there was a slower decline in neutrophil numbers and the subject was withdrawn from the drug prior to the neutrophil numbers reaching critical levels. These subjects were monitored subsequently and their neutrophil numbers returned quickly to within normal values after deferiprone withdrawal.

*Neurological assessment.* In the placebo-administered and 20 mg/kg/day deferiprone PD groups the motor-UPDRS scores increased over the 6 months' treatment period which indicated a slight worsening of motor disability (Fig. 2A), however this did not reach significance. Conversely, there was a trend for a decrease in motor-UPDRS scores over the 6-month treatment period in the 30 mg/kg/day deferiprone treatment groups compared to their baseline value prior to drug treatment, indicative of improvement, but this did not reach significance (Fig. 2A). A similar but more pronounced trend was observed with the PDQ-39 scores, with the placebo and 20 mg/kg/day PD groups showing strong trends for worsening quality of life whilst the 30 mg/kg/day deferiprone PD group showed a slight improvement over 6 months but this did not reach statistical significance (Fig. 2B). There were no changes in the clinical rating obtained from the minimal State of Folstein or the Montgomery-Asberg depression scale within the placebo or deferiprone drug treated groups throughout the study (data not shown).

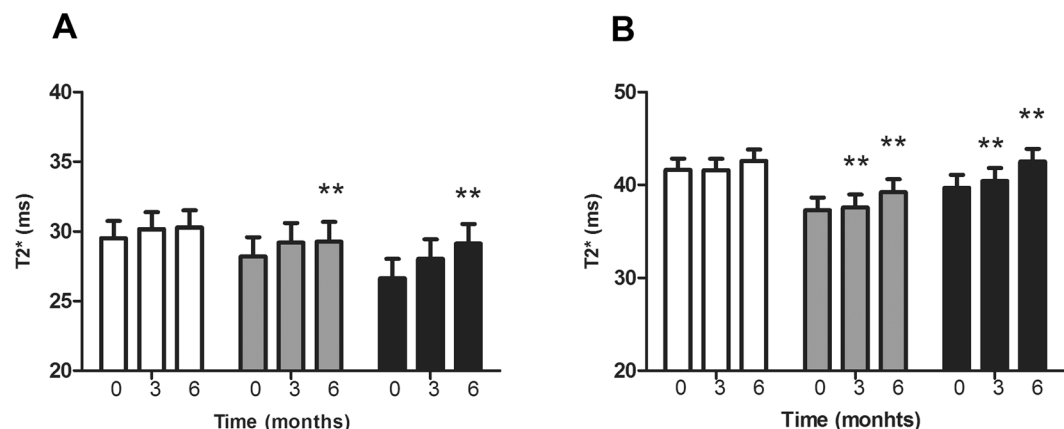


**Figure 3.** Mean T2\* MRI values in the dentate nucleus (A) and caudate nucleus (B) of Parkinson's disease subjects receiving 20 mg/kg/day, (grey bars, n = 6) or 30 mg/kg/day (black bars, n = 5) mg/kg/day deferiprone or placebo (open bars, n = 8) at 3 months and 6 months. Values are represented as mean  $\pm$  SEM. T2\* value is prior to the commencement of treatment, and then after 3 and 6 months of drug treatment. \*\*p < 0.01 as assessed by Pairwise comparison.

|              | Months | Substantia Nigra | Red Nucleus      | Dentate               | Caudate               | Putamen          | Pallidum         |
|--------------|--------|------------------|------------------|-----------------------|-----------------------|------------------|------------------|
| Placebo      | 0      | 28.09 $\pm$ 1.52 | 27.37 $\pm$ 0.82 | 30.51 $\pm$ 0.59      | 43.92 $\pm$ 1.22      | 37.26 $\pm$ 1.18 | 27.41 $\pm$ 1.39 |
|              | 3      | 29.36 $\pm$ 1.84 | 28.76 $\pm$ 1.06 | 30.54 $\pm$ 0.86      | 43.65 $\pm$ 1.62      | 35.76 $\pm$ 0.90 | 26.25 $\pm$ 0.58 |
|              | 6      | 28.22 $\pm$ 1.81 | 28.58 $\pm$ 1.23 | 30.74 $\pm$ 0.65      | 45.98 $\pm$ 2.08      | 36.55 $\pm$ 0.86 | 26.43 $\pm$ 0.87 |
| 20 mg/kg/day | 0      | 26.41 $\pm$ 1.25 | 28.10 $\pm$ 1.19 | 29.14 $\pm$ 0.84      | 38.86 $\pm$ 1.53      | 33.99 $\pm$ 1.80 | 25.55 $\pm$ 1.23 |
|              | 3      | 27.05 $\pm$ 1.23 | 26.86 $\pm$ 1.56 | 30.15 $\pm$ 0.49      | 40.29 $\pm$ 1.74 (**) | 33.54 $\pm$ 1.27 | 25.43 $\pm$ 1.08 |
|              | 6      | 27.68 $\pm$ 1.06 | 27.78 $\pm$ 1.05 | 30.59 $\pm$ 0.87 (**) | 41.05 $\pm$ 3.43 (**) | 33.94 $\pm$ 1.75 | 25.89 $\pm$ 0.96 |
| 30 mg/kg/day | 0      | 23.89 $\pm$ 2.24 | 27.21 $\pm$ 1.56 | 27.16 $\pm$ 1.56      | 40.75 $\pm$ 1.68      | 33.67 $\pm$ 2.14 | 25.72 $\pm$ 1.76 |
|              | 3      | 23.74 $\pm$ 1.84 | 26.99 $\pm$ 1.04 | 29.04 $\pm$ 1.82      | 44.69 $\pm$ 1.93 (**) | 37.24 $\pm$ 2.31 | 24.97 $\pm$ 1.14 |
|              | 6      | 24.57 $\pm$ 1.69 | 27.55 $\pm$ 1.12 | 29.86 $\pm$ 1.10 (**) | 44.55 $\pm$ 1.57 (**) | 38.19 $\pm$ 2.04 | 26.26 $\pm$ 1.33 |

**Table 2.** Mean T2\* values of six brain region in PD patients analysed at 0, 3 and 6 months after administration of either 20 mg/kg/day or 30 mg/kg/day deferiprone or receiving placebo. Results are presented as mean  $\pm$  standard error. \*\*p < 0.01.

**MRI.** Administration of 20 or 30 mg/kg/day deferiprone resulted in a significant time-dependent decrease in iron concentrations in both the dentate and caudate nuclei, (Fig. 3). An increase in the mean T2\* MRI values was evident in the dentate nucleus: the values increased significantly from  $29.2 \pm 0.8$  to  $30.6 \pm 0.9$  and from  $27.2 \pm 1.6$  to  $29.9 \pm 1.1$  (p < 0.001) after six months of 20 mg/kg and 30 mg/kg/day deferiprone administration, respectively, while no changes were evident in the placebo group (Fig. 3A). Similarly, in the caudate nucleus: T2\* values increased significantly in both treatment groups from  $38.9 \pm 1.5$  to  $41.1 \pm 3.4$  (p = 0.007) in the 20 mg/kg/day group and from  $40.8 \pm 1.7$  to  $44.6 \pm 1.6$  (p = 0.0002) in the 30 mg/kg/day group after 6 months of therapy (Fig. 3B). Across each treatment group there were no significant changes in T2\* mean values for SNc, red nucleus, putamen or globus pallidus in PD patients receiving either 20 mg/kg/day or 30 mg/kg/day over the period of the study (Table 2). However, we did observe a decrease in SNc iron concentration with deferiprone therapy in three patients, two of whom were hypoferritinemic by the end of the study.

No significant correlations regional iron accumulation measured with T2\* were found with UPDRS-III scores or disease duration.

**Blood parameters.** *Plasma levels of deferiprone and deferiprone glucuronide.* PD patients who received the higher dose of the chelator, 30 mg/kg/day, showed a significantly higher concentration of both deferiprone and deferiprone glucuronide than those administered 20 mg/kg/day, confirming that the patients had conformed to the instructions of the clinical trial (data not shown).

**Haematological and biochemical parameters.** Haemoglobin, transferrin and serum iron levels, transferrin saturation, and blood cell counts did not differ between placebo administered and deferiprone-treated patient groups during the 6-month period of the clinical study (Table 3). Ferritin concentrations in the PD patient groups administered either 20 or 30 mg/kg/day deferiprone gradually declined over the 6-month period, compared to the PD placebo group, which remained comparable to baseline values (Fig. 4). In two PD patients where the initial ferritin value was <100 ng/ml, it was noteworthy that there was a greater decrease in brain iron in the

|                                     | Months | Haemoglobin<br>( $10^9/L$ ) | WBC (g/L)       | Neutrophils<br>( $\times 10^9/L$ ) | Platelets<br>( $\times 10^9/L$ ) | Ferritin<br>(ng/ml) | Serum Iron<br>(nmol/L) | Transferrin<br>Saturation<br>(%) |
|-------------------------------------|--------|-----------------------------|-----------------|------------------------------------|----------------------------------|---------------------|------------------------|----------------------------------|
| <i>Placebo</i>                      | 0      | $141 \pm 0.59$              | $6.23 \pm 0.41$ | $3.95 \pm 0.33$                    | $223 \pm 18$                     | $100 \pm 27$        | $14 \pm 1.2$           | $25 \pm 2.7$                     |
|                                     | 2      | $137 \pm 0.61$              | $5.58 \pm 0.33$ | $3.33 \pm 0.20$                    | $222 \pm 15$                     | $77 \pm 19$         | $16 \pm 1.4$           | $25 \pm 2.6$                     |
|                                     | 4      | $137 \pm 0.63$              | $5.98 \pm 0.41$ | $3.36 \pm 0.31$                    | $219 \pm 15$                     | $82 \pm 21$         | $15 \pm 1.8$           | $27 \pm 4.1$                     |
|                                     | 6      | $137 \pm 0.55$              | $5.78 \pm 0.29$ | $3.37 \pm 0.13$                    | $220 \pm 12$                     | $88 \pm 21$         | $17 \pm 1.4$           | $25 \pm 2.9$                     |
| <i>20 mg/kg/day<br/>Deferiprone</i> | 0      | $136 \pm 0.67$              | $6.70 \pm 0.81$ | $4.08 \pm 0.66$                    | $222 \pm 20$                     | $189 \pm 62$        | $21 \pm 2.1$           | $32 \pm 5.5$                     |
|                                     | 2      | $132 \pm 0.81$              | $6.78 \pm 0.62$ | $4.30 \pm 0.59$                    | $237 \pm 22$                     | $187 \pm 62$        | $22 \pm 1.9$           | $36 \pm 5.4$                     |
|                                     | 4      | $134 \pm 0.63$              | $6.93 \pm 0.97$ | $4.48 \pm 0.78$                    | $215 \pm 21$                     | $165 \pm 59$        | $18 \pm 2.2$           | $30 \pm 2.4$                     |
|                                     | 6      | $135 \pm 0.63$              | $6.61 \pm 0.62$ | $4.30 \pm 0.56$                    | $224 \pm 23$                     | $141 \pm 47$        | $18 \pm 1.1$           | $33 \pm 6.6$                     |
| <i>30 mg/kg/day<br/>Deferiprone</i> | 0      | $141 \pm 0.48$              | $6.73 \pm 0.66$ | $4.38 \pm 0.38$                    | $249 \pm 17$                     | $132 \pm 37$        | $17 \pm 1.9$           | $24 \pm 2.2$                     |
|                                     | 2      | $134 \pm 0.41$              | $6.38 \pm 0.42$ | $4.26 \pm 0.20$                    | $229 \pm 14$                     | $136 \pm 50$        | $21 \pm 2.5$           | $28 \pm 1.9$                     |
|                                     | 4      | $140 \pm 0.27$              | $5.63 \pm 0.33$ | $3.70 \pm 0.23$                    | $217 \pm 14$                     | $115 \pm 41$        | $20 \pm 1.9$           | $30 \pm 1.7$                     |
|                                     | 6      | $138 \pm 0.32$              | $6.05 \pm 0.64$ | $4.20 \pm 0.53$                    | $223 \pm 17$                     | $107 \pm 38$        | $19 \pm 1.2$           | $28 \pm 2.9$                     |

**Table 3.** Haematological and iron status measurements expressed as medians  $\pm$  SEM of Parkinson's disease patients receiving 20 mg/kg/day ( $n = 6$ ) or 30 ( $n = 5$ ) mg/kg/day deferiprone or placebo ( $n = 8$ ) for 6 months. WBC - white blood cells.

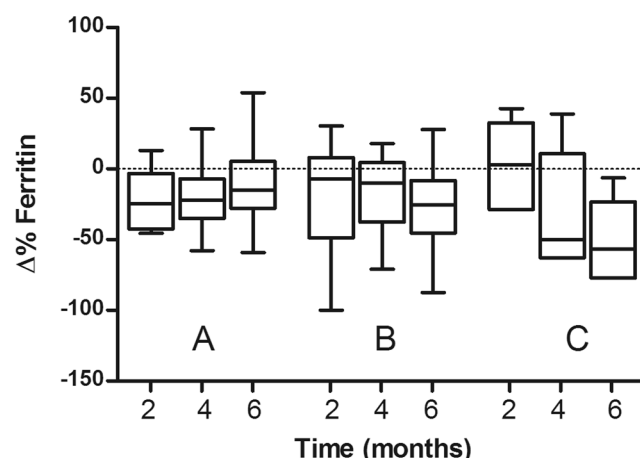


**Figure 4.** Percentage change in the median plasma ferritin values of PD patients receiving placebo (A,  $n = 8$ ), 20 (B,  $n = 6$ ) or 30 (C,  $n = 5$ ) mg/kg/deferiprone for 2, 4 and 6 months of treatment. Values are presented in box plots with whiskers representing the minimum and maximum values.

dentate and caudate nuclei, as well as the SNc, as assessed by T2\* MRI, after iron chelation (Fig. 5A) when compared to other PD patients where the plasma ferritin level commenced at values  $>100$  ng/ml (Fig. 5B).

**Urinary iron content.** The iron content of spot urinary samples was assessed at monthly intervals over the 6-month trial period. There was an increase in the median value of urinary iron values in PD patients over the six month period who received deferiprone therapy, 20 mg/kg/day and 30 mg/kg/day;  $172 \pm 160$  and  $132 \pm 104$  nmol Fe/mmol creatinine respectively, compared to  $23.1 \pm 4.8$  nmol Fe/mmol creatinine in the placebo group.

**Plasma Inflammatory markers.** Plasma concentrations of IL-6 and TNF $\alpha$  were analysed at monthly intervals during the 6-month clinical trial period. Whilst there were no changes in IL-6 and TNF $\alpha$  concentration with time in either the placebo or deferiprone treated PD groups, there was a positive correlation between the plasma levels of IL-6 and ferritin at Time 0 (Fig. 6). No further correlations were found between IL-6, TNF alpha, ferritin and regional T2\* values at baseline and follow up visits.

## Discussion

This pilot clinical trial with the iron chelator deferiprone clearly demonstrated that over the 6 months' treatment period there was gradual iron removal from specific brain regions, namely the caudate nucleus and dentate nucleus, as assessed by T2\* MRI, in the majority of the PD patients while only three subjects showed alterations in the T2\* MRI values for SNc. Deferiprone therapy was well tolerated by the PD patients, with a few patients experiencing mild side effects, while a decline in white cell counts warranting drug withdrawal was evident in 2 PD subjects. Deferiprone administration did not adversely affect PD severity, mood, cognitive function or quality of life or peripheral iron status. Furthermore, serum ferritin levels appear to be a surrogate marker to identify

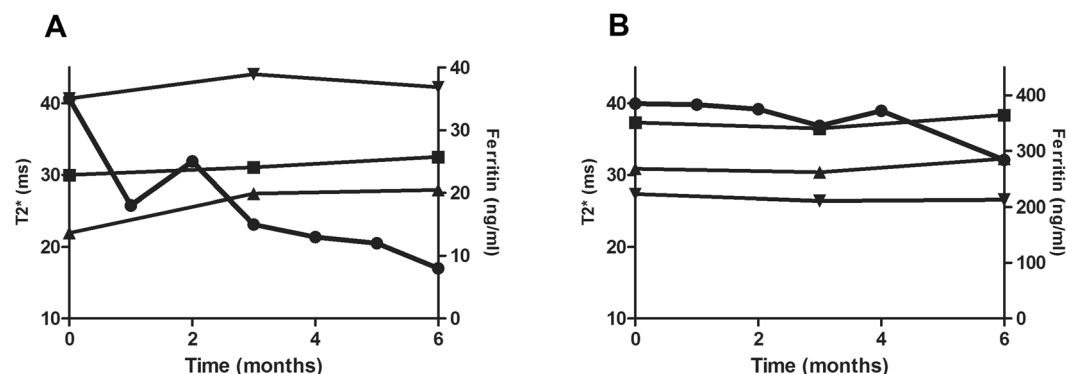


**Figure 5.** Examples of changes in ferritin levels and T2\* values in two PD patients receiving chelation therapy for 6 months. (A) Plasma ferritin level <100 ng/ml at the beginning of the study (B) plasma ferritin level >300 ng/ml. Ferritin values are denoted by circles, caudate nucleus T2\* values by upright triangles, dentate nucleus values by downward triangles and substantia nigra by squares.

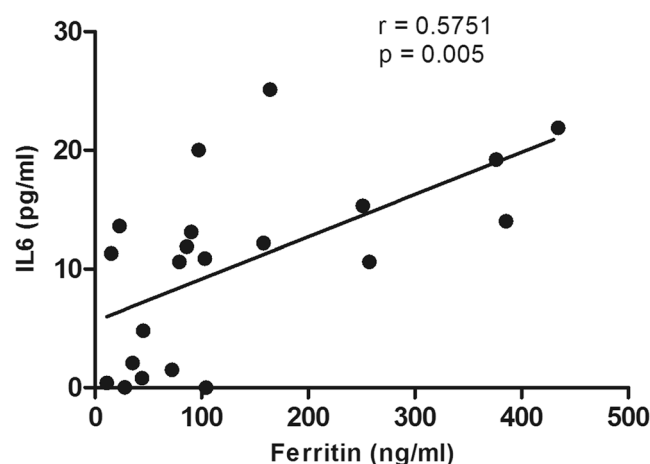


**Figure 6.** Correlation between IL-6 (pg/ml) and ferritin (ng/ml) baseline values from all clinical trial PD subjects. Statistical evaluation is by non-parametric correlation of Spearman ( $r = 0.5751$ ,  $p = 0.005$ ).

which patients might respond optimally to iron chelation by deferiprone. Additionally, plasma ferritin levels correlated with IL-6 concentrations, implicating a link between peripheral inflammation and iron metabolism in PD.

Our MRI findings of a decrease in the iron content of the dentate nucleus correlate with the clinical trial in FA patients carried by Boddaert and colleagues<sup>18</sup>, who also utilised 20 or 30 mg/kg/day doses of deferiprone over 6 months. Furthermore, Velasco-Sanchez *et al.*<sup>25</sup> administered 20 mg/kg/day deferiprone in addition to the antioxidant idebenone, to FA patients and showed an increase in T2\* values in dentate nucleus after 11 months. Nevertheless we did not find any association between tremor sub-scores and dentate iron accumulation.

In this present study, short-term deferiprone treatment was only observed to remove iron from the SNc in three patients receiving deferiprone. This could possibly be explained by the fact that iron in the SNc is principally tightly bound to neuromelanin rather than to ferritin as in other brain regions and hence less chelateable in the SNc<sup>26,27</sup>. Hence, a longer period of deferiprone therapy may be required before SNc iron levels decline. However, importantly this study demonstrates that deferiprone therapy does not result in a generalised removal of iron from the brain or its redistribution which could have implications for other neurological functions. This was supported by the finding that 6 months deferiprone therapy did not adversely affect mood, cognitive function or general patient quality of life. However, some of these data differ from another clinical trial of deferiprone therapy in PD patients, the FAIRPARK clinical trial, where deferiprone at 30 mg/kg/day was administered to PD subjects either at the initiation of the trial ('early start subjects'), or after 6 months of placebo therapy ('delayed start group')<sup>28</sup>. Both 'early start' and 'delayed start' PD subjects showed a small but significant reduction in SNc iron after 6 months of therapy as assessed by MRI<sup>28</sup>. This small effect may have achieved significance possibly due to the increased cohort size. Additionally, only one dose of deferiprone, 30 mg/kg/day, was used in the 19 patients participating in the FAIRPARK study, compared to 11 subjects receiving either 20 or 30 mg/kg/day in this study, thus giving the FAIRPARK study greater power to detect significant small changes in MRI signal.

A further difference between the FAIRPARK study and this study was that the former observed a small but significant clinical improvement in the deferiprone-treated PD subjects compared to placebo-treated PD subjects<sup>28</sup>.

The delayed start PD subjects who received placebo over the first 6 months averaged a 1 point UPDRS decline in motor function, whilst the early start deferiprone group improved by an average of 2 UPDRS points over the same period, giving an overall UPDRS difference between the two groups of 3 points. In our pilot study we observed a trend for an improvement in UPDRS motor scores in the 30 mg/kg/day deferiprone treated subjects compared to the placebo and 20 mg/kg/day group although this was not significant. This may again relate to the FAIRPARK study utilising the more active dose of 30 mg/kg/day dose of deferiprone for the whole of their study and in a slightly greater number of subjects. In addition in the FAIRPARK study, PD patients ( $n = 19$ ) who were recruited, had a clinical diagnosis of PD made less than 3 years before enrolment, whereas subjects recruited for this present study had a disease duration of less than 5 years. However the FAIRPARK patients showed higher motor scores  $UPDRS = 23.7 \pm 7$  by comparison to the PD patients in the present study,  $11 \pm 0.8$ , the former having been assessed in off medication state.

As in the FAIRPARK study we also observed a reduction in serum ferritin concentrations in PD subjects treated with deferiprone compared to placebo. This would reflect the mobilisation of iron stores particularly from the liver. With regards to brain iron mobilisation, deferiprone therapy was observed to be most efficient in those PD subjects starting with low serum ferritin content  $< 100$  ng/ml at the start of the study. This might suggest that there is a need to lower body iron stores before iron can be mobilised from specific brain regions. Indeed, in animal studies where body iron stores were depleted, there was an increased release of iron from the brain<sup>29</sup>. There was a correlation between the plasma concentrations of ferritin and IL-6, suggesting that the iron status of PD patients with high plasma ferritin may reflect the anaemia of chronic disease. Indeed, increased levels of pro-inflammatory cytokines, IL-1 $\beta$ , IL-2, IL-6 and TNF $\alpha$  are present in the brain, cerebrospinal fluid<sup>30</sup> and plasma of PD patients<sup>31–33</sup> such that changes in IL-6 observed in PD plasma might be indicative of inflammatory changes in the brain<sup>34</sup>. It is unknown whether plasma hepcidin levels, the master regulator of iron metabolism, are altered in PD patients. Heparin binds to the sole known iron exporter, ferroportin, causing its internalisation and degradation, thereby inhibiting iron efflux<sup>35</sup>. Heparin transcription can be stimulated by increases in serum iron as well as by inflammatory stimuli, most notably by IL-6, which signals via the IL6 receptor (IL-6Ra) and gp130 via the janus kinase (JAK) – STAT 3 pathway<sup>36, 37</sup>; such pathways are important since they can lead to the internalisation of iron in cells.

In conclusion, we have demonstrated that short term deferiprone therapy in PD subjects is safe and is associated with decreases of iron in specific brain regions. These studies support future longer-term clinical trials in PD where the neuroprotective effects of deferiprone can be fully assessed.

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## Author Contributions

A.M.B., R.J.W., R.N., D.T.D. wrote the main manuscript text. R.J.W., R.N., P.P., D.S., M.S., J.C., F.T. and D.T.D. contributed to the design of the studies. A.M.B. prepared the figures and tables. All authors reviewed the manuscript.

## Additional Information

**Competing Interests:** Whilst Michael Spino, John Connelly and Fernando Tricta are employees of ApoPharma Inc., the drug company did not financially sponsor the clinical trial; however they did generously donate free of charge deferiprone and matched placebo and helped with the critique of the manuscript.

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# Iron metabolism and its detection through MRI in parkinsonian disorders: a systematic review

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**Abstract** Iron deposition in the brain normally increase with age, but its accumulation in certain regions is observed in a number of neurodegenerative diseases including Parkinson's disease (PD) and other parkinsonisms. Whether iron overload leads to dopaminergic neuronal death in the SN of PD patients or is instead simply a by-product of the neurodegenerative progression is still yet to be ascertained. Magnetic resonance imaging (MRI) is a non-invasive method to assess brain iron content in PD patients. In PD, accurate radiologic visualization of basal ganglia is required. Deep gray matter nuclei are well presented in T2- and T2\*-weighted images. T2\*-weighted gradient-echo (GRE) is widely used to assess calcifications and also for iron detection. On the other hand, new methods specifically designed for detecting iron-induced susceptibility differences can be further improved by sequences like susceptibility-weighted imaging (SWI). In the present review, we aim to summarize the available data on brain iron deposition in PD.

**Keywords** Parkinsonian disorders · Parkinson's disease · Iron · Magnetic resonance imaging

## Introduction

Iron accumulates in the brain in healthy aging; however, its accumulation in specific regions is observed in neurodegenerative diseases including Parkinson's disease (PD) [1]. Specific areas of the brain, such as globus pallidus (GB), substantia nigra (SN), dentate nucleus (DN), and motor cortex (MC), show increased mineralization in healthy brain. Neurons store iron in the form of ferritin, which exists as light (L) and heavy (H) chains. The iron redox couple mediates the transfer of single electrons through the reversible oxidation/reduction reactions of Fe(II) and Fe(III). The biological redox potential, electronic spin state as well as the reactivity of iron, is determined by the nature of the ligand to which the species are bound. Iron configuration dictates whether an iron-based biomolecule is responsible for reactions involving oxygen transport and storage, electron transfer, or oxidation/reduction of other molecules [2]. Reactions involving iron in the body are predominately redox-based, hydrolytic, or involve polynuclear complex formation [3]. This is especially important for the brain, where some of the highest concentrations of iron in the body are maintained [4].

It has been hypothesized by a number of studies that nigral mineralization may be a surrogate biomarker of PD. Mutations in proteins involved in neuronal iron homeostasis have been linked with PD, including transferrin (Tf) [5], iron regulatory protein 2 (IRP2) [6], ferritin (Ft) [7], and divalent metal transporter 1 (DMT1) [8]. In experimental animal models, direct injection of iron in SN causes dopaminergic neurodegeneration [9]. In addition, feeding neonatal mice with iron can trigger parkinsonism and nigral degeneration [10].

Neurodegenerative disorders characterized by brain iron accumulation, like aceruloplasminemia [11, 12], neuroferritinopathy [13, 14], and neurodegeneration with brain iron accumulation (NBIA) [15] present parkinsonian symptoms.

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Loss-of-function mutations of IRPs show similar iron accumulation observed in idiopathic PD. Conversely, modulation of iron content shows beneficial effects on PD animal models. PD toxin model, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) cause SN iron accumulation in mice. These PD models show reduction of dopaminergic degeneration in SN and increased striatal dopamine turnover after administration of iron chelators [16–18]. Iron-mediated toxicity in these models can also be ameliorated by genetic or pharmacologically restoring Ft [16] and Cp [19].

The reason why iron accumulates in PD could be explained by a number of iron-related proteins that are altered in PD. Ft levels have been found to be decreased in postmortem PD brains [20]; moreover, loss of iron storage capacity leads to increased free unbound iron being more available for oxidative reactions. Iron accumulation in PD might be caused by increased neuronal iron import. DMT1 has been shown to be elevated in the SN of postmortem PD brains [21]. This could promote iron import, but the levels of transferrin receptor 1 (TfR1), which is required for DMT1-mediated iron import were found unchanged when corrected for neuronal loss [22–24]. On the other hand, increased iron deposition might also be attributed to reduced iron export. Although ceruloplasmin (Cp) levels have been shown to be unaltered in PD brains, however its activity is reduced in SN, which could narrow iron efflux [19].

Furthermore, tau protein can play a role in iron accumulation in PD [25]. A selective reduction of tau found in SN of PD patients may also contribute to iron accumulation by preventing APP-mediated iron export [26].

The role of neuromelanin (NM) in iron chelation has been recently proposed, NM binds iron in two different high and low affinity sites. Most of the iron is bound to high affinity sites, but under iron overload conditions, the high affinity sites of NM are saturated, and iron binds to the low affinity sites [27, 28]. In the latter sites, iron is sequestered in the reactive form and could play a deleterious effect by promoting redox reactions when NM is released to extraneuronal matrix. Therefore, the binding of iron could have a protective role under physiological conditions, but a toxic gain of function may manifest when iron overload is present in the brain [29]. Neurotoxic interactions between iron and dopamine have been recently proposed to explain degeneration of the SN in PD [30]. Moreover, specific T1-weighted magnetic resonance imaging (MRI) sequence has been demonstrated to detect NM signal changes to accurately discriminate PD patients from controls. Nevertheless, the relationship between iron accumulation and NM using multimodal imaging techniques has not found clear results [31].

Recent developments in MRI make it now possible to examine brain iron content in PD patients. Imaging studies have

shown increased iron accumulation at early stages of PD even before symptom onset [32–34]. The SN iron accumulation in PD patients, shown by MRI, correlates with disease severity [35, 36] and duration. In this review, we will focus on the MRI methods used for iron detection in PD. The Medline database on PubMed was searched for relevant papers (last accessed on the March 2017) using the following queries: “Brain iron deposition” and “Parkinson’s disease” or “Parkinsonism.” We only considered in this review MRI studies, excluding metabolic studies.

## MRI methods to study iron accumulation

T2-weighted spin echo (SE) and fluid attenuation inversion recovery (FLAIR) are the standard MRI protocol in neurodegenerative diseases showing hyperintensity in areas of white matter (WM) lesions [37]. FLAIR is used to maximize the detectability of lesions attenuating the signal generated by cerebrospinal fluid (CSF) [38]. Furthermore, SE-based sequences have recently been modified with sampling perfection with application optimized contrasts (SPACE) using different flip-angle evolution [39, 40]. This technique is a T2-weighted three-dimensional turbo SE sequence with long echo time (TE) that enables fast high-resolution three-dimensional images [39, 40]. T2\*-weighted-GRE is also useful for detecting microbleeds and calcifications [38]. Deep gray matter nuclei are well presented in T2- and T2\*-weighted images; however, methods specifically designed for detecting iron-induced susceptibility differences can be further improved by sequences like susceptibility-weighted imaging (SWI).

SWI is a novel high-resolution MRI modality which [41–43] requires a different from traditional spin-density, T1, or T2 imaging recalled echo pulse sequence to acquire images. The use of long TE allows for phase development between the excitation pulse and data acquisition that is sufficient for the differentiation of susceptibility variations [41]. Due to its sensitivity, this method exploits the susceptibility differences between tissues and uses the phase image to detect these [42–44]. The magnitude and phase data are combined to produce an enhanced contrast magnitude image that is exquisitely sensitive to venous blood, hemorrhage, and iron deposition. Furthermore, susceptibility-weighted-angiography (SWAN) is useful to precisely delineate small blood vessels, microbleeds, iron, and calcium deposits. SWAN is innately less affected by the chemical shift compared to SWI with significantly enhanced susceptibility information. Finally, quantitative susceptibility mapping (QSM) provides a novel contrast mechanism in MRI [45] converting the phase shifts to localize magnetic susceptibility.

## Brain iron deposition in PD

In 1986, Drayer et al. [46] assessed for the first time the differences in T2 relaxation times in basal ganglia and SN in six patients diagnosed with multi system atrophy (MSA) and progressive supranuclear palsy (PSP) and 14 healthy controls (HC). Authors found decreased T2 relaxation times in the putamen (PUT), and less prominent decreased in the caudate nucleus (CN) and lateral pars compacta of the SN in the MSA and PSP population compared with HC. Conversely, other authors [47], studied postmortem brains of five subjects: four dying of non-neurological conditions and one subject with PD by means of T2-weighted images, showing no differences in iron distribution in the basal ganglia between both study groups. Antonini et al. [48] evaluated T2 relaxation times in 30 PD patients and 33 HC using a 1.5 T MRI. When compared to HC, PD patients showed decreased T2 values in CN, putamen (PUT), and SN; however, no clinical correlations were found with the aforementioned regional increased iron accumulations.

In the last decade, several studies have focused their interest in evaluating iron overload in PD patients (Table 1).

**Table 1** Overview of studies using T2, T2\*, SWI, and QSM sequence in Parkinson's disease and other parkinsonisms

|                            | MR sequence | HC | PD             |
|----------------------------|-------------|----|----------------|
| Drayer et al., [46]        | T2 GE       | 14 | 6 MSA<br>6 PSP |
| Brooks et al., [47]        | T2          | 6  | 1 PD           |
| Antonini, [48]             | T2 GE       | 33 | 30             |
| Zhang et al. [49]          | T2 GE       | 30 | 42             |
| Grabner et al. [50]        | SWI         | 5  | 25             |
|                            | T1          |    |                |
| Zhang et al. [51]          | SWI         | 26 | 40             |
|                            | T1          |    |                |
| Jin et al. [52]            | SWI         | 45 | 45             |
|                            | T1          |    |                |
| Wang et al. [53]           | SWI         | 14 | 20             |
|                            | T1 IR       |    |                |
|                            | T1 FSE      |    |                |
|                            | T2 FLAIR    |    |                |
| Ulla et al. [54]           | T2*ESWAN    | 26 | 27             |
|                            | T1          |    |                |
| Wu et al. [55]             | GRE         | 54 | 40             |
|                            | T1          |    |                |
|                            | T2          |    |                |
| Barbosa et al. [56]        | SWI         | 30 | 20             |
|                            | T1          |    |                |
| Schwarz et al. [57]        | R2 and R2 * | 90 | 19             |
|                            | SWI         |    |                |
| He et al. [58]             | QSM         | 35 | 44             |
| Guan et al. [59]           | QSM         | 40 | 60             |
| Hopes et al. [60]          | QSM         | 20 | 70             |
|                            | T1          |    |                |
| Peckham et al. [61]        | T2*         | 16 | 18             |
|                            | SWI         |    |                |
| Martin Bastida et al. [62] | SWI         | 20 | 70             |

Increased evidence using SWI techniques confirm that parkinsonian patients show increased iron accumulation in deep brain gray nuclei when compared to HC [49–52, 55, 59, 60]. Several works [57, 62] also demonstrated increased phase values in the SN of PD positively correlating with UPDRS motor scores (UPDRS III) [49] and Hoehn and Yahr (H&Y) stage [55] as well as a different iron accumulation in clinical laterality [53]. In particular, Zhang et al. [49] investigated a population of 42 PD patients and 30 HC analyzing SN and basal ganglia. PD patients showed an increased deposition in SN, PUT, and red nucleus (RN) bilaterally when compared to HC. In addition, SN phase radians correlated with UPDRS III of the most affected side. On the other hand, Wu and colleagues [55] demonstrated elevated iron deposition in PUT, CN, RN, GP, and SN of 54 PD patients (18 PD patients with HY < 1.5 and 36 PS patients with HY > 1.5) compared to 40 HC. The phase values of the GB and the SN showed negative correlations with the HY stage. According to other data [50], the regional phase values of 25 PD patients can reveal the differences between PD patients with symmetric and lateralized PD, even though the study included only a small number of PD patients and HC. By contrast, other authors [59, 61] found increased nigral mineralization in PD patients when compared to HC; however, no correlations with motor severity as assessed with UPDRS III and disease stage were found. In the study by Pechkam et al. [61], the authors addressed the lack of significant correlations to the small number of PD patients studied (18 patients). Previous results also suggest a positive correlation between phase shifts values in SN and serum ceruloplasmin levels in PD patients [52] in 45 PD patients (25 with decreased ceruloplasmin levels and 20 with normal serum ceruloplasmin levels and 45 HC). The original finding in this study that PD patients with reduced ceruloplasmin levels showed decreased nigral phase values when compared to HC whilst PD patients with normal ceruloplasmin levels demonstrated no changes in phase values when compared to HC, suggests a role in iron deposition mediated by ceruloplasmin levels. In a recent cross-sectional study [62], of 70 non-demented PD using high-pass filtered phase imaging, PD patients showed higher levels of iron in SN, GP, and PUT in PD patients compared to HC. Further correlations with nigral mineralization were found with motor severity as assessed with UPDRS III in off-medicated state and bradykinesia-rigidity subscores. Moreover, when PD groups were subdivided according to UPDRS-III score, iron mineralization appears to be stratified according to disease motor severity, confirming the idea that patients with a more severe disease show a greater iron accumulation.

Other authors, Schwarz et al. (2014) [57], investigated the usefulness of nigrosome-1 detection using SWI. Their sample was divided as follows: a prospective case control study in 19 subjects (10 PD, 9 HC) and a retrospective cross-sectional study in 95 consecutive patients (9 PD, 81 non-PD, 5 non-

diagnostic studies exclude). Two independent raters classified subjects into PD and non-PD assessing the presence or absence of nigrosome-1. Almost 90% of participants were correctly classified, demonstrating a high sensitivity of the current imaging sequence.

ESWAN sequences are also useful in detecting iron accumulation. Wang et al. (2013) [53] demonstrated the presence of smaller main MPV in the SN of PD patients as well as negative correlation between the extent of reduction and severity of motor symptoms, in a population of 20 PD patients with different disease stages and 14 HC.

Recent works have been focused on evaluating the utility of quantitative magnetic resonance (QMR) [56, 58 and 59]. In a recent study (60) including 60 PD patients and 30 HC and comparing R2, R2\* with quantitative susceptibility mapping (QSM), authors found QSM more accurate in detecting iron accumulation. Further increased iron accumulation measured with QSM in SN of PD patients but also a spread in other structures such as RN and GP in patients with a more advanced disease were found [59]. In addition, authors demonstrated positive correlation between QSM values and disease duration and UPDRS III in a large number of subjects including 60 PD patients (45 with HY > 2.5 and 15 with HY > 3) and 40 HC [58].

Finally, two longitudinal studies evaluated so far brain iron overload in PD patients by means of R2\* over a period of 3 and 2 years, respectively [54, 60]. In the first longitudinal study [54], authors evaluated 26 PD patients and 26 HC by a first MRI whereas 14 PD patients and 18 HC 3 years after by a second MRI, whilst the second longitudinal study included 70 PD patients in the cross-sectional arm and 35 PD patients and 20 HC in the follow-up [60]. In both studies, authors found significant changes in nigral R2\* values in comparison with HC which correlated with changes in disease severity in the SN.

## Discussion

In the last decade, a great number of studies evaluated iron overload in parkinsonian disorders and other neurodegenerative diseases. So far, there is accordance between authors that iron is a promising biomarker for diagnosis in PD and other parkinsonisms. However, the present studies present some limitations. First of all, most of them are retrospective studies, to our knowledge, only two longitudinal studies confirmed so far, that iron overload increases as the disease progresses [54, 60]. Secondly, there is still lack of agreement between authors on which is the most accurate sequence to study iron deposition. Indeed, a recent meta-analysis which compared postmortem results with iron deposition detected by using MRI [63] demonstrated that both R2\* and SWI have some limitations in evaluating iron deposition. This meta-analysis confirmed an

increased iron accumulation in SN and PUT as assessed by R2\*, SWI, and postmortem studies. On the other hand, whilst both SWI and R2\* indicated an increase in iron levels in the RN, postmortem samples failed to find a correlation between disease severity and iron deposition in RN. A number of confounding factors can influence imaging results. Calcifications, microbleeds, and myelinated fibers can disrupt the iron signals in both R\* and SWI. These difficulties may be overcome applying QSM sequences as previous studies already confirmed [53, 56, 57].

NM loss may be another confounding factor able to reduce iron signals since in normal conditions it stores big quantity of iron in the brain. A recent study [31] investigated the relationship between iron content in the SN and neuromelanin signal changes combining T1 neuromelanin-sensitive MRI sequence and T2\* relaxometry. In this study, iron content in the SN of PD patients did not show any significant correlation with neuromelanin MRI signal changes, which may indicate that neuromelanin reduction and iron accumulation derive from different pathogenetic mechanisms. MRI techniques sensitive to both NM changes and iron accumulation have been hypothesized to be potential surrogate biomarkers of PD [54, 60, 62, 64–69]. In a recent study [69], 43 PD patients (13 late stage PD, 12 de novo PD patients, 10 PD patients with a 2–5-year disease duration, and 10 HS) were studied with NM MRI-sensitive. Authors were able to identify a significant NM depletion parallel with disease progression between PD patients in an early stage of disease and groups of PD patients belonging to more advanced disease stages. Moreover, other authors already found significant correlations between NM loss and disease stage and severity [66]. On the other hand, previous studies confirmed the role of iron as a promising diagnostic biomarker in PD, finding an increased iron accumulation in the SN correlating with disease duration and motor impairment [62] as well as a reliable marker of disease progression [54, 60].

The use of iron sensitive MRI sequences may also be of therapeutic interest. Iron chelators, such as deferiprone, showed a disease-modifying effect in animal models of PD [70]. So far, two randomized clinical trial with this drug have been conducted in PD patients, showing mild reductions in motor severity after 6–12 months of treatment associated with iron removal in SN, CN, and DN [71]. In the future, action of iron chelation and other drugs can be followed with MRI as well as iron overload over disease progression.

## Conclusions

A number of studies have demonstrated that iron plays a role in PD. Moreover, topographic distribution of iron could help

in some cases of parkinsonism in early stage differential diagnosis, even though it is not clear if the increase in iron content in PD is a pathogenetic or epiphenomenic event related to dopaminergic neurodegeneration. So far a limitation of previous studies is that almost all of them have included only a relatively small number of subjects. Larger studies are necessary to confirm that iron can be considered as a novel biomarker in PD and a new therapeutic target.

#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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